Thesis for doctoral degree (Ph.D.) 2007

Karolinska Institutet

Thesis for doctoral degree (Ph.D.) 2007

STUDIES OF CELL SIGNALLING USING BACTERIAL TOXINS AND ORGANIC ELECTRONIC DEVICES

Peter Kjäll

Thesis for doctoral degree (Ph.D.)
2007

STUDIES OF CELL SIGNALLING USING BACTERIAL TOXINS AND ORGANIC ELECTRONIC DEVICES

Peter Kjäll

Karolinska Institutet

From DEPARTMENT OF MICROBIOLOGY, TUMOR AND CELL BIOLOGY
Karolinska Institutet, Stockholm, Sweden

STUDIES OF CELL SIGNALLING USING BACTERIAL TOXINS AND ORGANIC ELECTRONIC DEVICES

Peter Kjäll

Karolinska Institutet

Stockholm 2007

Published by Karolinska Institutet.

ISBN 978-91-7357-364-1

Printed by

www.reproprint.se
Gårdsvägen 4, 169 70 Solna

I am very curious of things……..

ABSTRACT

One of the most versatile biological signalling entities is the calcium ion, Ca^{2+}. With a characteristic spatial and temporal signalling pattern, this universal second-messenger directs diverse cellular functions, e.g. secretion of neurotransmitters at the synaptic cleft to regulation of transcription and fertilization. The temporal feature of Ca^{2+}signal can be described as oscillations occurring with periodicities ranging from μs to h.

The bacterial exotoxin α-haemolysin (Hly) is a pathophysiologically relevant protein known to induce Ca^{2+} oscillations that leads to production of pro-inflammatory cytokines in target cells. A role for this uropathogenic *E.coli*-encoded virulence factor was recently identified, as Hly was shown to modulate inflammatory responses *in vivo*. In this thesis, we investigate the molecular details of signalling pathways activated by Hly. All functional domains of Hly are required for cell signalling, and we suggest that the Ca^{2+} binding domain interacts with the membrane-bound receptor glycophorin. Components of the LPS-recognition system are essential to recruit Hly to the cell membrane, where signalling is initiated in specialized microdomains termed lipid rafts. We show that the signalling pathway involves the small GTPase RhoA, the linker protein ezrin, and the cytoskeleton. Ca^{2+} oscillations are generated by concerted actions of Hly-activated voltage-operated Ca^{2+} channels in the plasma membrane and generation of the second-messenger IP_3, which causes release of Ca^{2+} from the ER via the IP_3-receptor.

The "tool-kit" available to study the dynamic range of Ca^{2+} signals is limited. We envisaged that a powerful tool for such studies would be a device that is able to induce ion signalling with specified temporal and spatial resolution. To this end, we developed an organic electronic ion pump as a bio-interface to electronically induce Ca^{2+} signalling in neuronal cells. We show that the conjugated polymer PEDOT:PSS is bio-compatible. A device is designed that is able to transport ions, e.g. H^+, K^+ and Ca^{2+}, with high degree of electronic control and ON/OFF ratios exceeding 300. By integrating this device with cells, electronic control of cellular Ca^{2+} influx is achieved, showing that an electronically controlled ion conductor circuit can trigger a biological output. A prototype is manufactured demonstrating that the ion pump can be used to achieve controlled oscillations of an ion concentration. We believe that these novel devices have great potential as tools to study Ca^{2+} signalling patterns with specific frequencies in biological specimens. But most importantly, this work shows a successful integration of two research fields, which has paved the path for the novel research area termed "Organic bioelectronics".

LIST OF PUBLICATIONS

I. Role of the lipopolysaccharide-CD14 complex for the activity of hemolysin from uropathogenic Escherichia coli.
Månsson LE, **Kjäll P**, Pellett S, Nagy G, Welch RA, Bäckhed F, Frisan T, Richter-Dahlfors A. Infection and Immunity 2007, 997-1004.

II. Molecular characterization of α-hemolysin-induced Ca^{2+} signalling in renal epithelial cells.
Kjäll P, Oxhamre C, Söderblom T, Mikoshiba K, and Richter-Dahlfors A.
Submitted manuscript.

III. Electronic control of Ca^{2+} signalling in neuronal cells using an organic electronic ion pump.
Isaksson J*****, **Kjäll P***, Nilsson D, Robinson N. D, Berggren M and Richter-Dahlfors A. Nature Materials 2007, 673 - 679.
*****contributed equally.

IV. Electronically controlled pH gradients and proton oscillations.
Isaksson J, Nilsson D, **Kjäll P**, Robinson N. D, Richter-Dahlfors A and Berggren M.
Submitted manuscript.

CONTENTS

LIST OF ABBREVIATIONS

a.u	Arbitrary units
$[Ca^{2+}]$	Calcium concentration
CaM	Calmodulin
CCD	Charge-coupled device e.g cooled CCD cameras
CCE	Capacitative calcium entry
CICR	Calcium induced calcium release
ClyA	Cytolysin A
CNF-1	Cytotoxic necrotizing factor 1
DAG	Diacylglycerol
e^-	Electron
E. coli	*Escherichia coli*
EGFP	Enhanced green fluorescent protein
FET	Field-effect transistor
Hly	α-Hemolysin (*E. coli*)
IL	Interleukin
IP_3	Inositol-1, 4, 5-trisphosphate
IP_3R	IP_3 Receptor
$[K^+]$	Potassium concentration
LBP	LPS binding protein
LED	Light-emitting diod
LLO	Listeriolysin O (*L. monocytogenes*)
LPS	Lipopolysaccharide
OFET	Organic field-effect transistor
PANI	Polyanilline
PEDOT	poly(3,4-ethylene dioxythiopene)
PIP_2	Phosphatidylinositol 4, 5-bisphosphate
PKC	Protein kinase C
PLC	Phospho lipase C
Ply	Pneumolysin (*S. pneumoniae*)
PPy	Poly-pyrrole
PSS	poly(styrene sulphonate)
PTC	Proximal tubule cell (Kidney epithelium)
ROCC´s	Receptor operated calcium channels
RTX	Repeats in toxin
S/cm	Siemen per cm
Sat	Secreted autotransporter toxin
SOCC´s	Store operated calcium channels
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
UPEC	Uro-pathogenic *E. coli*
UTI	Urinary tract infection
V	Potential
VOCC´s	Voltage operated calcium channels

1 INTRODUCTION – INFECTION BIOLOGY

1.1 HISTORICAL PERSPECTIVES OF MICROBIAL PATHOGENESIS

The world of microbial pathogenesis begun in 1674 when the Dutch biologist and father of the modern microscope, Antonie van Leeuenhoek decided to analyze a drop of drinking-water with one of his own manufactured microscopes. Before his eyes he saw millions tiny vibrating specimens he later called "animal-molecules" or "animalcules". More than 50 years later German zoologist Christian Gottfried Ehrenberg designated them bacteria after the Greek word *bacterion* meaning "small staff". In the end of the 18th century, the Danish biologist Otto Müller extended the knowledge of van Leuenhoek´s first observation by organizing bacteria into genera and species according to the classification methods of Carl von Linnè. During the 19th century pioneering German pathologist Friedrich Henle proposed the "Germ Theory of disease", a criteria for proving that microorganisms were responsible for causing human disease. Contemporary scientists Robert Koch and Louis Pasteur confirmed this theory by a series of elegant experiments that proved that microorganisms were responsible for causing tuberculosis, cholera, plague, rabies and anthrax. By 1880s, bacteriology was formed as an official scientific discipline, much through the work of Robert Koch, who also discovered that bacteria could be grown in laboratory conditions on nutrition containing agar gels poured in petri dishes. He also stipulated the Koch's postulate where he defined a four step procedure to identify an agent causing disease, a procedure by large still followed today. Louis Pasteur made important discoveries by disproving the "theory of spontaneous generation" where he showed that microorganisms cannot start to grow in nutrition broth without inoculation of bacteria. This was an important step in the knowledge of proper hygiene, contamination and of how infectious diseases spreads. As the name implies Louis Pasteur was the inventor of Pasteurization a method in which liquids are heated in order to be cleared from microorganisms. Pasteur was also a pioneer in vaccine development, where the initial discovery was made in an infection experiment with cholera in chickens. He found that chickens that wasn't properly infected with the primary inoculum was almost impossible to re-infect despite very high doses of fresh bacteria meaning that the initial weak infection rendered the chickens immune to the disease. Pasteur continued his research and applied his discoveries to both smallpox and anthrax and he gave these

artificially weakened diseases the generic name of vaccines. Further development of the smallpox vaccine led to the official eradication of smallpox in 1979. These two initial steps was very important to increase the health standards in society and battle infectious diseases. The battle against the microbes started some 30 years earlier when Ignaz Semmelweis, a doctor at a Vienna hospital in 1847 discovered that mortality rates at birthing wards could be drastically lowered by introducing mandatory hand-washing by the doctors practicing at the ward. Using chlorinated lime solution as disinfectant he showed a drop in mortality rates from 10-35% to below 1%. Being a pioneer, his work earned widespread acceptance first after his death during the same time as the Germ Theory of Disease was acknowledged. All these discoveries in the late 19th century led to an extensive interest in microorganisms and their interactions with other forms of life; the field of cellular microbiology was born.

In the beginning of the 20th century the "Germ theory of disease" was generally accepted and research was now focused on how to battle the microbes with this newly acquired knowledge. The era of chemotherapy begun when Nobel prize winner in medicine, German chemist Paul Ehrlich 1910 discovered the first anti-bacterial agent, arsphenamine (Salvarsan®), a compound effective against *Treponema* species that cause syphilis. The drug, which was also effective against other spirochaetal infections, is no longer in use in modern medicine. The first trembling steps towards the discovery of one of the most, if not the most important drug in the history of mankind, was taken by Ernest Duchesne in 1897 when he observed some anti-bacterial properties of *Penicillin sp*, but it was not until Alexander Fleming´s accidental discovery of Penicillin in 1928, its capabilities was further investigated and found to be very effective against most Gram-positive bacteria. Ernst Chain and Howard Florey took Fleming´s research one step further and came up with the purified form of penicillin, which granted them together with Fleming the Nobel prize in Medicine 1945. The discovery of penicillin was followed by that of sulfanilamide in 1935 and streptomycin in 1943. The practical use of penicillin was spurred by the urgent need in the second world war and mass production of the drug was rushed for front line use leading to thousands and thousands of lives saved that would otherwise be lost due to simple wound infections etc. Since then penicillin's and other antibiotics have saved millions of lives to this day. During this time research has provided us with lots of knowledge within the field of microbial pathogenesis and the complexity is almost beyond the

limits of our imagination with thousands of different species of microbes known and hundreds known to cause serious human disease.

Since the discovery of penicillin, numerous other effective anti-microbial drugs and vaccines have reached the clinics and served mankind. Despite this the infectious diseases continue to cause global suffering and mortality even today. The world health organization (WHO) Global Burden of Disease (GBD) 2002 report state that a fifth of global mortality is attributed to infectious diseases meaning almost 11 million annual deaths. Today, society face acute problems of growing antibiotic resistance among bacterial pathogens due to decades of extensive overuse. The first report of antibiotic resistance was in 1947 in a strain of *Staphylococcus aureus* after only four years of commercial antibiotic use. Since then resistance has been growing and in the late 1990s strains of *Enterococcus faecium* was found to be resistant against all known antibiotics. The growing resistance and that only derivatives of already known antibiotics has been developed during the last 35 years, makes the situation critical.

The modern society also provides new niches for microbes to cause disease. This can be exemplified by Methicillin-Resistant Staphylococcus Aureus (MRSA) in hospitals and *Legionella* residing in the water pipe infrastructure and in air condition systems. The growing climate threat with global warming also poses a new threat. Geographical locations before protected by the climate environment are now potential sites for microbial epidemics. This show that the question of development of more effective therapies against infectious diseases is more important than ever.

1.2 BACTERIA – HOST INTERPLAY AND THE IMMUNE SYSTEM

In order to understand the mechanism of bacterial infections one need to understand the interplay between bacteria and host. Research in the mid 1980s started to focus on that interplay (Hagberg *et al.*, 1983). Advances within the field of microbiology, cell biology and immunology led to ability to integrate this knowledge to a new field of research termed cellular microbiology (Cossart *et al.*, 1996). Example of discoveries attributed to the field of cellular microbiology is the virulence function and mechanism of the type III secretion system of *Shigella sp, Salmonella sp* and EPEC (Kenny *et al.*, 1997; Menard *et al.*, 1994). The technical advances during over the next 10-15 years played an important part where development of novel techniques within microscopic imaging and visualization allowed the scientist to monitor mechanisms of infections in

great detail and in real-time. Bacteria and host genetics including micro-array techniques, siRNA and other knock-down techniques allowed the evaluation of involved genes in the process of infection on both the host side as well as in the virulence of bacteria. Important topics of the research in cellular microbiology includes that of molecular signalling, both in the bacteria as well as in the host cell and how these pathways cross during an infection.

During an infection the bacteria will encounter physiological barriers like skin, mucosal membranes, epithelium and pH as well as the tough defense of the immune system. Within the field of cellular microbiology it is of utmost importance to study the interaction between the bacteria and the immune system since this intrinsic system of cells, structures and molecules is the barrier that inhibits the establishment of an infection. Even though the body depends on a strictly controlled immune response as a protective measure against infection the immune system and the resulting acute inflammation can sometimes work against the host and in the favor of the invading bacteria if the control is lost. The strength of the immune system can also be its weakness when the bacteria takes advantage of a strong uncontrolled immune response often leading to tissue destruction. Example of this is endotoxic shock due to gram-negative bacterial sepsis in which strong release of acute-phase inflammatory mediators causing capillary leakage, vasodilation, organ failure and often death (Tracey *et al.*, 1986). Another example is the heat-labile toxins of the gram-positive *S. pygenes*, which works as superantigens, which induces nonspecific activation of T cells leading to a strong immune response and subsequent organ destruction and tissue necrosis(Braun *et al.*, 1993).

1.2.1 Innate Immunity

Innate immunity is characterized by a rapid action of host effector molecules and leukocytes aimed at limiting the multiplication of invading microbial organisms and destroying them. The innate immune response is the earliest response to an bacterial infection. A complex interplay between many different cell types dictates activation mechanisms and subsequent elimination of the pathogen.

A prerequisite for the swift response of the innate immunity is an efficient system to recognize a large palette of microbes with a limited number of receptors. As part of the innate immunity, the eukaryotic host have evolved a group of cellular receptors able to

recognize molecular motifs, conserved and specifically found in microbes. These are termed Pathogen Associated Molecular Patterns (PAMPs) as they are pattern molecules shared by many microorganisms. To avoid cross-reactivity with self-antigens the Pattern Recognition Receptors (PRRs) recognize PAMPs that are distinct in structure and often part of the microbial metabolism or its physical structure (e.g membrane components) (Aderem and Ulevitch, 2000). One of the most studied of this group of receptors are the Toll-like receptors (TLRs), which among many things recognize outer membrane components of bacteria (see section 1.2.1.1). Nucleotide-binding oligomerization domain proteins (NOD-like receptors, NLRs) is another group of PRRs that are able to recognize intracellular bacteria. The recognition of PAMPs leads to cellular activation and release of effector molecules, e.g of pro-inflammatory molecules, which acts as chemo-attractors for immune cells like neutrophils and Natural killer cells (NK-cells) to the local site of infection (see section 1.2.1.3). The complement system is also play a pivotal role in innate immunity (see section 1.2.1.2). Another mechanism by which the innate immune system use to eradicate bacteria is the release of antimicrobial peptides e.g defensins. LL-37 an antimicrobial peptide in the family of cathelicidins expressed in epithelial cells and in leukocytes such as monocytes, neutrophils, T cells and B cells is able to efficiently break up the cell membrane of both gram-negative and gram-positive bacteria.

1.2.1.1 Toll-like receptors (TLRs)

The Toll-receptor was discovered in the study of the fruit fly *Drosophila melanogaster* (Anderson *et al.*, 1985), which like all insects lack an adaptive immune system. Despite this the fruit fly can rally a strong immune response due to the swift recognition of microbes. Today 11 mammalian homolouges to the Toll-receptor (Toll-like receptors, TLR´s) are known, recognizing a wide flora of microbial components, by themselves or as homo or hetero dimers. The expression profile of the TLRs differ depending on the cell type and organ, and the reactivity if the innate immune system can be correlated to this expression pattern (Uhlén *et al.*, 2000). TLRs are expressed on most professional immune cells, like macrophages and dendritic cells. It is also now known that epithelial cells with their expression of TLRs is much more active in the innate immune response than previously appreciated and are for example of great importance for the hosts ability to fend of bacterial infections in the urinary tract. Activation of TLRs leads to induction of intracellular signalling cascades resulting in production of inflammatory

cytokines like tumor necrosis factor α (TNF-α), IL-6, IL-8, IL-1β, Interferons (IFN) and chemokines.

As TLR4 recognizes lipopolysaccharide (LPS) component of the outer membrane of gram-negative bacteria (Medzhitov and Janeway, 1997; Medzhitov *et al.*, 1997), it is of major importance for protection against gram-negative bacterial infections. TLR4 was established as the LPS signalling receptor based on genetic evidence from the LPS-insensitive mouse strain, C3H/HeJ, which has a single point mutation in the TIR domain of TLR4. In addition, the C57BL/10ScCr strain of mice has a null mutation in the *TLR4* gene that also confers resistance to LPS (Poltorak *et al.*, 1998). This was later elegantly confirmed with TLR4 knock-out mice that are highly susceptible to gram-negativ infections. Recognition of LPS by TLR4 signalling is a multi-step process involving initial binding of LPS by LPS-binding protein (LBP). LBP is an acute-phase protein, which role is to bring LPS to the cell surface by binding to LPS and forming a ternary complex with the LPS receptor molecule, CD14. CD14 is found in soluble form (sCD14) or as a glycosylphosphatidylinositol (GPI) anchored receptor in the membrane of the host cell. CD14 then presents LPS to the TLR4/MD-2 complex and by that induces the intracellular signalling cascade through MyD88 leading to expression of inflammatory cytokines (Kawai and Akira, 2006). Other TLRs can recognize a wide spectra of microbial components (summarized along with adaptors in Figure1).

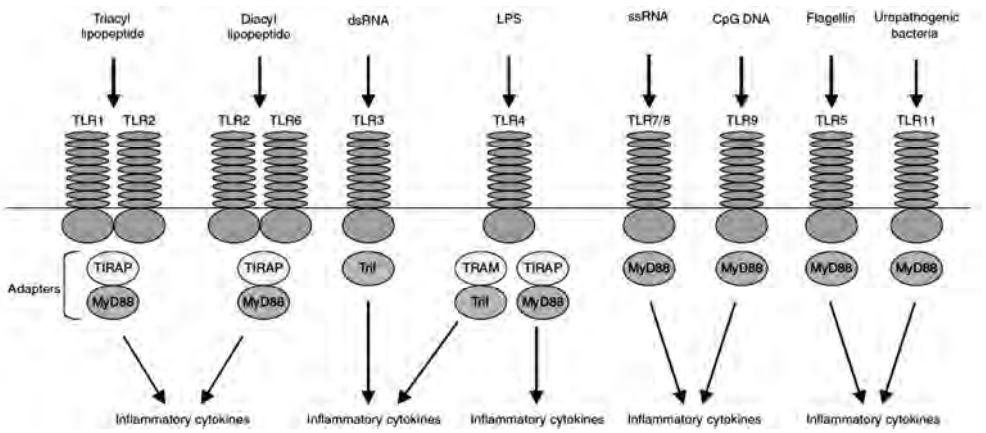


Figure1. *Summary of the 11 known human TLR´s, their adaptor proteins and ligands.*

TLR2, in concert with TLR1 or TLR6, recognizes various bacterial components, including peptidoglycan, lipopeptide and lipoprotein of Gram-positive bacteria. In particular, TLR1/2 and TLR2/6 discriminate triacyl lipopeptide and diacyl lipopeptide, respectively. TLR3 recognizes double-stranded RNA (dsRNA) that is produced from many viruses during replication. TLR5 recognizes bacterial flagellin. Mouse TLR11 recognizes yet unknown components of uropathogenic *E. coli* (UPEC) and has been shown to provide specific protection against UPEC within the kidneys (Wang *et al.*, 2004b). TLR7 and TLR8 recognizes synthetic single-stranded RNA (ssRNA) derived from human immunodeficiency virus type I (HIV-1) and some siRNAs. TLR9 recognizes bacterial and viral CpG DNA motifs.

1.2.1.2 Complement system

The complement system represents a major pathway by which antigen recognition is converted into an effective defense, especially against extracellular bacteria. The complement system consists a number of plasma proteins and can by subdivided in three different pathways of activation: The classical pathway that is triggered by antibodies, the mannan-binding lectin (MBLectin) pathway and the alternative pathway. The MBLectin and the alternative pathway is not dependant on antibody reactions and is part of innate immunity. All pathways consists of an early sequence of cleavage reactions in which the product binds to the surface of the pathogen and converge at the formation of the C3 convertase enzyme which creates the active complement component C3b. The endpoint of the complement cascade is the membrane-attack-complex (MAC) which is extremely potent and able to efficiently lyse invading bacteria. Other components in the cascade also have potent immune-stimulatory effects, e.g C5a and C3a, which mediate local inflammatory responses, recruiting fluid and activating phagcytic cells. Bacteria have evolved virulence factors to circumvent complement activation, e.g the poly-sialic acid capsule of *E. coli* K1 and *S. pneumonia* that is anti-phagocytic and protects the bacteria from recognition (Pluschke *et al.*, 1983).

1.2.1.3 Cytokines and immune cell recruitment

Cytokines plays an important role in the regulation of host defense and mediate the multi-cellular response of the immune system towards an bacterial infection. Many cytokines are endogenous pyrogens because they promote fever and enhances the inflammatory response. As with most parts in the hosts inflammatory response, a tight

regulation is crucial as overproduction of cytokines can have fatal consequences like an cytokine storm when a positive feedback loop between cytokines and activated immune cells leads to shock and tissue destruction (Suntharalingam *et al.*, 2006).

A key task of cytokines is to recruit immune cells to the site of infection. Cytokines create a chemotactic gradient leading to migration of e.g neutrophils from the blood stream towards the infection (Svanborg *et al.*, 2001b; Svanborg *et al.*, 2001a). In cystitis as well as pyelonephritis is the secretion of cytokines and the subsequent neutrophil infiltration critical for clearing an infection (Svanborg *et al.*, 2001b; Svanborg *et al.*, 2001a). Upon *E. coli* caused urinary tract infection the epithelium secretes cytokines like IL-8, IL-6, TNF-α and IL-1β inducing an early strong inflammatory response (Samuelsson *et al.*, 2004; Brauner *et al.*, 2001; Hedges *et al.*, 1995; Agace *et al.*, 1993a; Agace *et al.*, 1993b). IL-8 excert its effect through its receptors: CXCR1 and CXCR2 which both are expressed on bladder and kidney epithelium (Godaly *et al.*, 2000b; Godaly *et al.*, 2000a). CXCR1 homologue knock-out mice is defective in transepithelial neutrophil migration leading to bacteremia (Frendeus *et al.*, 2000; Godaly *et al.*, 2000b; Godaly *et al.*, 2000a). Patients with low expression of CXCR1 are more prone to pyelonephritis further emphasizing the clinical importance of intact cytokine signalling during infection (Lundstedt *et al.*, 2007a; Lundstedt *et al.*, 2007b).

Production of cytokies can also be induced by other forms of signalling. The *E. coli* exotoxin α-hemolysin (see section 2.3.1.1) induces Ca^{2+}-signalling leading to production of both IL-8 and IL-6 through activation of transcription factor NF-κB (Uhlén *et al.*, 2000).

1.2.2 Adaptive Immunity

In addition to the innate immunity the adaptive immune response has evolved, showing a more versatile means of defense that provides an increased level of protection from a subsequent re-infection with the same pathogen. The cells of the adaptive immune response consists of a clonal selection of antigen-specific lymphocytes, immunoglobulin producing B-cells and two types of T-cells. The clonal selection and expansion of antigen-specific clones gives the adaptive immunity much slower kinetics compared to the innate immunity but gains higher specificity and most important memory through the expansion of specific memory cells. Efficient signalling between the two branches of the immune system is of great importance as the cells of the innate

immune response play a crucial part in the initiation and subsequent direction of the adaptive immune response. Most important is the fact that there is a delay of 4-7 days before the adaptive immune response takes effect. Here the innate immune response has a critical role in controlling the infection during this period.

1.2.3 Urinary tract infections

The human urinary tract is normally a sterile environment, protected from pathogens by the shear flow of urine, secreted and tissue-associated antibacterial factors, and the bactericidal activities of effector immune cells. However, even in populations with these natural defenses seemingly intact, and despite the increasing use of antibiotics, bacterial infection of the urinary tract is an exceedingly common problem. Among half of the worlds women will at some point in her life suffer from an urinary tract infection (UTI). Even though women are significantly more likely to experience UTI than men, UTI is the most common bacterial infection in the world today (Foxman, 2003), this is also without consideration that many cases of UTI never get reported to the clinic making the statistics for UTI certainly on the low side. Nearly 1 in 3 women will have had at least 1 episode of UTI requiring antimicrobial therapy by the age of 24 years. Most of these episodes are cystitis-like (infection of the bladder), but some of them may become complicated by acute pyelonephritis (infection of the kidney), a serious condition that might lead to urosepsis, renal scarring and subsequent permanent damage to the kidney function. Pyelonephritis is one of the most common febril diseases in children (Wing, A. J. in *Oxford Textbook of Clinical Nephrology*). The frequency of acute cystitis among young women is 0·5–0·7 episodes per person per year in the western world (Hooton *et al.*, 1996), representing a main source of health-care costs in the population. In Sweden we have about 1 million cases per year according to the Swedish institute for infectious disease control (SMI).

UTI can be caused by many bacterial species, ranging from opportunists to pathogens. The majority of cases (about 80%) (Mims, 2004; Ferry *et al.*, 1988), is caused by uropathogenic *E. coli* (UPEC). Bacteria that are responsible for the remaining cases are among others *Proteus sp*, *Klebsialla sp* and *Enterobacter sp*. There are many factors, both on the host side as well as in the pathogen that are predisposing for UTI. This can be virulence factors (see section 1.2.4.1) expressed by the pathogen. On the host side behavioral risks of the host, like voiding dysfunction, anatomic abnormalities, like urine reflux (VUR) (Finer and Landau, 2004) and genetic variations in the host, like

different expression patterns of immune receptors e.g CXCR1 influence susceptibility to infection (Lundstedt *et al.*, 2007a; Lundstedt *et al.*, 2007b).

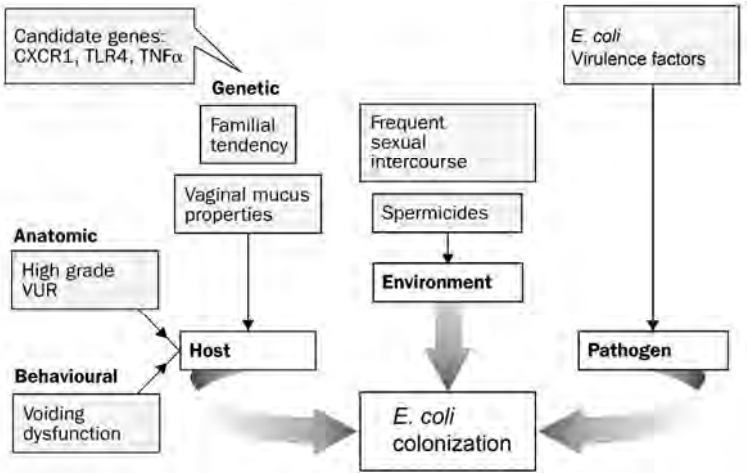


Figure 2. *Factors affecting the pathophysiology of UTI.*

1.2.4 Uropathogenic *E.coli*

Escherichia coli are a very diverse species of bacteria found naturally in the intestinal tract of all humans and many other animal species. A subset of *E. coli* are capable of causing enteric/diarrhoeal disease, and a different subset cause extra-intestinal disease (EXPEC), including urinary tract infection (UTI) and meningitis. *E. coli* that cause enteric/diarrhoeal infections are: enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC). Each pathotype of *E. coli* causes disease using different combinations of virulence factors, with different molecular pathways, and in some but not all cases resulting in disease symptoms that can be distinguished from each other

The vast majority of UTIs are caused by UPEC (within the group of EXPEC), a surprisingly heterogeneous group of pathogens. It is believed that most UPEC reside in the colon, and are later introduced through the urethra to the bladder and further to the kidneys (Jantunen *et al.*, 2001; Yamamoto *et al.*, 1997). The expression of virulence genes clustered on pathogenicity islands (PAI´s) greatly determine the region in which the *E. coli* strain cause disease. This is apparent when comparing the genome of a

pyelonephritogenic isolate (CFT073) with that of an EHEC isolate showing that they share only 39.2% of their protein coding sequences (Welch *et al.*, 2002). Many of the genomic differences among UPEC and other *E. coli* isolates likely reflect changes that have enabled UPEC isolates to better colonize and evade the immune system in the unique and varied environmental niches found within the urinary tract and by that be able to cause infection. These niches include the epithelia lining of the urethra, bladder and ureters, as well as the renal proximal and distal tubule cells and the collecting ducts of the kidneys.

1.2.4.1 Virulence factors of Uropathogenic E. coli

UPEC isolates encode a variety of virulence factors that facilitate colonization of the urinary tract. To date, however, no single virulence factor has been shown to be specifically unique to UPEC. The type III secretion systems often elaborated by enteric *E. coli* pathogens such as EHEC as a means of delivering bacterial products into target host cells are with a very few exceptions missing from UPEC isolates, as are many of the toxins encoded by enteropathogenic strains (Miyazaki *et al.*, 2002; Welch *et al.*, 2002). UPEC instead express a number of virulence factors that enteric pathogens often lack. This includes specific adhesion organelles, including P (Pap), type 1 and other fimbriae (such as F1C, S, M and Dr) that seem to aid in colonization. Type 1 fimbriae are important for cystitis as the tip, adhesin FimH bind to mannose moieties of the uroplakin receptors that coat bladder epithelial cells (Zhou *et al.*, 2001; Mulvey *et al.*, 1998). P fimbriae is the virulence factor that best correlates with pyelonephritis. The P fimbriae adhesin PapG binds to digalactoside receptors that are expressed on the kidney epithelium (Roberts *et al.*, 1994; Dowling *et al.*, 1987; Lund *et al.*, 1987).

Several toxins are produced by UPEC, including α-haemolysin (Hly), cytotoxic necrotizing factor (CNF-1) and an autotransported protease (Sat). Hly will be discussed in greater detail in section 2.3.3.1.

UPEC uses capsule-forming high-weight polysaccharides for immune evasion, The capsular antigen, which forms the basis for classification into K serotypes uses molecular mimicry to host cell components and shear bulk to shield immunogenic structures from recognition of the complement system to mention a few mechanisms. K1, K2, K, K5, K12, and K13are the most common serotypes attributed to pyelonephritogenic strains (Sandberg *et al.*, 1988).

A major virulence factor of UPEC as well as of many other gram-negativ bacteria are LPS, also referred to endotoxin in its role as an virulence factor and is highly

immunogenic. LPS is a large phosphoglycolipid, composed of the acylated, hydrophobic lipid A component, the LPS-core and the O-polysaccharide antigen (Jann and Jann, 1987). The O-polysaccharide antigen (O) together with the capsular antigen (K) and the flagellar antigen (H) determines the complete serotype of *E. coli* (O:K:H). Lipid A anchors the LPS molecule to the outer membrane via fatty acids (lauric, myristic) and is crucial for the viability of the bacteria (Wyckoff *et al.*, 1998). The Lipid A portion is the most immunogenic structure of LPS and is crucially important for the immune response in against *E. coli* infection as it is a PAMP molecule recognized by the PPR TLR4. By using mutant strains (*waaN*$^{-}$) expressing penta-acylated lipid A the host response to LPS can be experimentally abrogated (Uhlén *et al.*, 2000; Hedlund *et al.*, 1999).

Other components denoted as virulence factors are iron-acquisition systems (siderophores) and phase-switch recombinases.

All these virulence factors are found in differing percentages among various subgroups of UPEC and listed below in Table 1.

Table 1.

Virulence factor	***Function***
CNF-1	Cytotoxicity, stress fiber formation and internalization
Hly	Cytolysis and induction of cytokine production
Sat	Vacuolating cytotoxicity
Vat	Vacuolating autotransporter toxin
Curli/Cellulose	Bio-film formation, adhesion and resistance
Flagellum	Motility
P fimbriae	Adhesion to uroepithel
S fimbriae	Adhesion to epithelium and endothelium
F1C fimbriae	Adhesion to epithelium and endothelium, induction of cytokines
Type 1 fimbriae	Adhesion to epithelium and endothelium, invasion
Dr adhesion	Adhesion, ligand to decay acceleration factor
LPS	Induction of immune responses, O-antigen coupled complement resistance
Capsular antigen	Complement resistance, immune recognition and antiphagocytic
IroN	Iron siderophore receptor, iron metabolism

2 CELL SIGNALLING

Signalling within and between cells is critical for the living organism to function. The inter- and intracellular pathways have evolved to a complex system able sense and react and through positive and negative feedback-loops respond to stimuli.

The binding of most signalling molecules to their receptors initiates a series of intracellular reactions that regulates virtually all aspects of cell behavior, including metabolism, movement, proliferation and differentiation. Investigation of the molecular mechanisms of these pathways has thus become a major area of research. Within infection biology, understanding of cell signalling pathways is critical as many pathogens utilize and /or exert their effect on them. The hope is that increased knowledge of signalling occurring between bacteria and host leads to novel insights of mechanisms to target for future anti-microbial therapies.

2.1 INTRACELLULAR SIGNALLING

One of the most widespread pathways of intracellular signalling is based on second messengers. Phosphatidylinositol 4,5-bisphosphate (PIP_2) is a minor component of the plasma membrane and forms the basis for many second messengers. A number of hormones and growth factors stimulates hydrolysis of PIP_2 by phospholipas C (PLC), which produces the two second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG and IP_3 stimulate distinct downstream signalling pathways e.g activation of protein kinase C (PKC) and Ca^{2+} mobilization (see section 2.3). Thus, by alterning the kinetics of hydrolysis, PIP_2 can trigger two different intracellular signalling cascades (Oancea and Meyer, 1998; Divecha and Irvine, 1995; Maeda *et al.*, 1990). Another important target for PKC regarding the inflammatory cascade is its activation of transcripton factor NF-κB (Beg *et al.*, 1993). In its inactivation state NF-κB is retained in the cytoplasm as a complex with IκB. Activation of PKC leads indirectly to phosphorylation of IκB, which targets IκB for degradation. Destruction of IκB allows NF-κB to translocate to the nucleus where it regulates transcription of its target genes.

2.1.1 Regulation of the actin cytoskeleton

The actin cytoskeleton plays an important part in intracellular signalling by linking siganalling complexes and conveying signals within the cell. This control is achieved by the actin cytoskeleton- regulating proteins, termed small-GTPases. Three members of this family, RhoA, Rac and Cdc42 regulate different aspects of actin reorganization; filopodia (Cdc42), lamellipodia (Rac), focal adhesion and stress fibers (RhoA) (Bishop and Hall, 2000). Trancduction of signalling through RhoA is also involves PIP_2 and PLC, showing that cytoskeleton mediated signalling is coupled to Ca^{2+} mobilization (Saci and Carpenter, 2005). The ERM family of proteins (ezrin, radexin and moesin) is a group of linker proteins that mediates signals to the cytoskeleton (Bretscher *et al.*, 2002; Bobacka *et al.*, 2000; Takeuchi *et al.*, 1994b). ERM proteins have an N-terminal FERM domain and a C-terminal actin-binding domain and the intra-molecular interaction between these domains is believed to regulate ERM activity. The ERM proteins all have regulatory activity on the small-GTPases, e.g ezrin which interacts with RhoA (Speck *et al.*, 2003).

2.1.2 Lipid rafts

For signalling to occur within the cell it is critical for the signalling components to interact. Lipid rafts have been suggested to constitute a signalling platform in the plasma membrane of eukaryotic cells. High levels of sphingolipids results in a tightly packed cholesterol rich domain with high rigidity. Many groups of signalling membrane proteins have higher affinity for lipid rafts including GPI-anchored proteins, creating a high concentration of signalling molecules in so called signalosomes (Simons and Toomre, 2000). It is recently shown that CD14 directs LPS to lipid rafts for recognition by TLR4 and induction of signalling (Triantafilou *et al.*, 2002). The small-GTPase RhoA is also described to concentrate in lipid rafts (Palazzo *et al.*, 2004).

2.2 ION SIGNALLING

The smallest signalling enteties in the cell is the ion. Inorganic ions of the cell, including protons (H^+), sodium (Na^+), potassium (K^+), magnesium (Mg^{2+}), calcium (Ca^{2+}), phosphate (HPO_4^{2-}) and chloride (Cl^-) are all involved in the cell signalling and metabolism.

Despite its moderate size there are three main characteristics that give the ion its power as signallig conveyor:

1. *Speed:* Because of its small size, transport of ions is extremely rapid. More than $1x10^6$ ions can flow through an ion channel per second, a flow rate that is 1000 times faster than that of a carrier protein.

2. *Selectivity:* Signalling mediated by ion channels are highly selective as the narrow pores restrict ions of appropriate size and charge. The spatial and temporal signalling pattern further enhances the specificity of the signal.

3. *Regulation:* Ion signalling are highly regulated by ion channels and ion binding proteins. Ion channels are regulated by gates that transiently open to a specific stimuli. This can be molecular stimuli in receptor-operated channels (ROCC´s) e.g neurotransmitters, or physiological events e.g voltage-operated channels (VOCC´s) that open due to changes in the electrical potential across the cell membrane.

The action potential initiated by neurotransmitter release in the synaptic cleft of nerve cells represents one of the fastest ion signalling mechanisms. The membrane potential is altered from -60mV to +30mV in >1ms as a result from rapid sequential opening and closing of voltage-operated Na^+ and K^+ channels. The altered membrane potential opens of voltage-operated Ca^{2+} channels (VOCC´s), leading to increase of cytosolic Ca^{2+} and subsequent intracellular signalling.

2.3 CALCIUM SIGNALLING

The Ca^{2+} ion is the hallmark of an signalling ion, being an universal intracellular messenger controlling a diverse range of cellular processes (Hisatsune and Mikoshiba, 2005; West and Galloway, 1998; Bootman and Berridge, 1995). The ability of the Ca^{2+} ion to play such a pleitropic role results from the fact that cells module Ca^{2+} signals in the dimensions of space, time and amplitude using a Ca^{2+} signalling "toolkit" (Berridge *et al.*, 2003; Berridge *et al.*, 1998). The toolkit comprises of Ca^{2+} sensory mechanisms, Ca^{2+} channels and Ca^{2+} transporters that together regulate the Ca^{2+} concentration across the plasma membrane as well as between the cytosol and intracellular compartments.

The intracellular Ca^{2+} concentration [Ca^{2+}] in resting state is ~100 nM but upon activation of signalling this can increase to 1 μM or more, creating a large gradient between on and off state (~10^4 to 1). Ca^{2+} signalling manifest itself as short Ca^{2+} sparks or puffs (Tovey *et al.*, 2001), Ca^{2+} waves and Ca^{2+} oscillations to prevent the cell from sustained high [Ca^{2+}], which is toxic for the cell. Intracellular Ca^{2+} oscillations activate processes like secretion (periodicity of μs), contraction, transcription, proliferation and fertilization (periodicity of hrs). Global Ca^{2+} signals can also pass between cells via gap junctions, to coordinate the activities of whole tissues or organs. Such waves mediates e.g activation of cilia on epithelial cells along the trachea (Boitano *et al.*, 1992).

2.3.1 Regulation of Ca^{2+} signalling

Cells utilize different types of Ca^{2+} channels for its on and off mechanisms. Influx of extracellular Ca^{2+} is one source to increase cellular [Ca^{2+}] and this is mediated by channels in the plasma membrane. VOCC´s are sensitive to depolarization of the plasma membrane and are accordingly key channels in excitable cells such as neuronal or muscle cells. VOCC´s are also expressed in no-excitible cells like kidney epithelia cells where they play a pivotal role (Zhao *et al.*, 2002; Yu *et al.*, 1992). Receptor-operated Ca^{2+}-channels (ROCC´s) are activated by binding of the proper ligand to the extracellular domain of the channel. The different ROCC´s are activated by a wide variety of ligands e.g acetylcholine, glutamate and ATP. Well known ROCC´s include the nicotinic acetylcholine receptor and N-methyl-D-aspartate receptor (NMDA) (Murphy *et al.*, 1992). Store-operated Ca^{2+}-channels (SOCC´s) are activated in response to depletion of the intracellular Ca^{2+} store, either by Ca^{2+} mobilizing messengers or pharmacological agents. The mode by which SOCC´s respond to depletion is called capacitative Ca^{2+} entry (CCE) (Miyakawa *et al.*, 2001). The mechanism in which SOCC´s are able to sense the filling status of the intracellular Ca^{2+} pool is under debate and subject for intense research. One suggestion is either activation in close proximity of, or a direct coupling to the endoplasmatic reticulum (ER) (Bolotina and Csutora, 2005; Berridge *et al.*, 2000a; Berridge *et al.*, 2000b; Boulay *et al.*, 1999). Efflux of Ca^{2+} from the cytoplasm during "off" mechanisms involves ion pumps and ion exchangers e.g Ca^{2+}-ATPase and the Na^+/Ca^{2+} exchanger that transports Ca^{2+} out of cells.

Another major source of Ca^{2+} is the intracellular stores e.g ER. Release from ER is mainly mediated by the IP_3-receptor (IP_3R) or ryanodine receptors (Maeda *et al.*, 1990;

Furuichi *et al.*, 1989; Lai *et al.*, 1988). Three different subtypes of the IP_3R have been identified (IP_3R1, IP_3R2 and IP_3R3) and their expression pattern differs depending on tissue and cell type (Monkawa *et al.*, 1998; Bush *et al.*, 1994). The IP_3R subtypes have also been attributed different roles in conveying the intracellular Ca^{2+} signal (Yamada *et al.*, 1994; Yamamoto-Hino *et al.*, 1994), where the ratio aspects between the expression of the subtypes affect the amplitude and frequency of Ca^{2+} oscillations (Hattori *et al.*, 2004). IP_3 is the ligand for the IP_3R and is as previously described a product of the PIP_2 signalling pathway (see section 2.1). IP_3 induces conformational changes of the IP_3R upon activation leading to opening of the channel and outflow of Ca^{2+} into the cytosol (Sato *et al.*, 2004; Hamada *et al.*, 2003). The N-terminal domain of IP_3R contains high-affinity binding sites for Ca^{2+}. This mode of activation is called Ca^{2+} induced Ca^{2+} release (CICR) and further enhances the IP_3R´s functional diversity. To refill the intracellular stores, Ca^{2+}-ATPases located in the ER membrane pumps back Ca^{2+} from the cytosol. Mitochondria are also considered as intracellular Ca^{2+} stores. These organelles have substantial capacity for Ca^{2+} and can when needed significantly buffer cytosolic Ca^{2+} increases (Montero *et al.*, 2000).

2.3.2 Ca^{2+} signalling regulates gene transcription

One of the major functions of Ca^{2+} signalling is the activation of signalling pathways leading to gene transcription (Dolmetsch, 2003; West *et al.*, 2002). Today almost 400 genes together with over 30 transcription factors are known to be regulated by Ca^{2+} signalling (Berridge *et al.*, 2003; Dolmetsch, 2003; Feske *et al.*, 2001; Crabtree, 1999; Dolmetsch *et al.*, 1997). Ca^{2+} oscillations activate proteins that regulate transcription factors as well as modulating chromatin structure. These proteins are able to sense changes in $[Ca^{2+}]$ by various mechanisms (Berridge *et al.*, 2003; Bootman *et al.*, 2001).

Calmodulin (CaM) is a Ca^{2+} binding protein, activated by cytosolic Ca^{2+} increase of about 0.5 μM. CaM changes conformation when complexed with Ca^{2+} in its active form (See Figure 3) and binds to a variety of proteins including protein kinases, VOCC´s and the IP_3R (Zotti *et al.*, 2003; Taylor and Laude, 2002; Matters *et al.*, 1999; Zuhlke *et al.*, 1999).

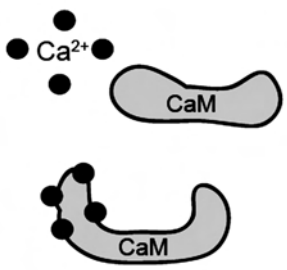


Figure 3. *CaM changes conformation when complexed with Ca^{2+} in its active form.*

CaM-dependant protein kinase II and PKC are also suggested to sense the frequency of Ca^{2+} oscillations (De Koninck and Schulman, 1998; Oancea and Meyer, 1998) and activate transcription factors CREB (Dolmetsch *et al.*, 2001; West *et al.*, 2001) and NF-κB respectively. Transcription factor NF-κB together with NF-AT and Oct/OAP was furthermore shown to be regulated differently depending on the frequency of the Ca^{2+} signal (Dolmetsch *et al.*, 1998; Dolmetsch *et al.*, 1997). The importance of Ca^{2+} signalling in inflammation was demonstrated by the finding that expression of the pro-inflammatory cytokine IL-8 depends on Ca^{2+} frequency regulated activation of NF-κB.

2.3.3 Bacterial Toxins

Bacterially expressed exotoxins are virulence factors that affect the host directly or indirectly. Although exotoxins aid in bacterial colonization and defense against neutrophils, their signalling activity also aid bacteria to reach deeper tissues as the epithelial lining loses its integrity during the inflammatory response. One group of pore-forming exotoxins have drawn much attention during recent years by being able to induce Ca^{2+} signalling in the target cell, attributing them with a more complex pathiophysiological role besides their cytolytic effect.

2.3.3.1 E. coli α–Hemolysin

The *E. coli* α-hemolysin (Hly) belongs to the family of RTX-toxins (repeats in toxin), which refers to the shared characteristics of a number of amino acid repeats, Gly-Gly-X-Gly-Asp/Asn-Asp-X (where X is any amino acid) in its Ca^{2+}-binding domain. Hly is the most studied RTX-toxin and is considered a major virulence factor of ExPEC *E. coli* (Menestrina *et al.*, 1994b; Menestrina *et al.*, 1994a). This includes UPEC, where 50% of the strains express Hly (Brauner *et al.*, 1990; Hughes *et al.*, 1983). The assumptions of its role as a virulence factor came from early reports that described Hly´s cytolytic effect on artificial lipid bilyers, erythrocytes (hence is name) and epithelial cells (Mobley *et al.*, 1990; Bhakdi and Tranum-Jensen, 1988; Hughes *et al.*, 1983). Later it was discovered that Hly have the ability to induce Ca^{2+} signalling in renal epithelial cells. Thus Hly´s mode of action is biphasic. High concentrations of Hly is cytolytic, while sublytic concentrations initiates Ca^{2+} oscillations with a periodicity of 12.0±0.7 min leading to production of IL-6 and IL-8 by frequency modulated activation of NF-κB (Uhlén *et al.*, 2000). Hly was the first pathiophisiological protein reported to induce Ca^{2+} signalling. Its role *in vivo* as

modulator of renal inflammatory responses have recently been described using multi-photon microscopy techniques (Mansson *et al.*, 2007).

Hly is genetically organized in an operon consisting of four linked genes, *hlyCABD*. The *hlyA* gene encodes the protein, while *hlyB* and *hlyD* together with the distant *tolC* encodes the type I secretion apparatus (Koronakis, 2003). Hly is synthesized as an inactive 110 kDa protein. Post-translational activation involves HlyC-mediated attachment of two fatty acyl chains (Stanley *et al.*, 1998; Stanley *et al.*, 1994; Issartel *et al.*, 1991). Upstream of *hlyCABD* is *hlyR* located, which acts as an enhancer by stabilizing the mRNA transcripts (Vogel *et al.*, 1988). The active Hly protein have three functional domains (Figure 4).

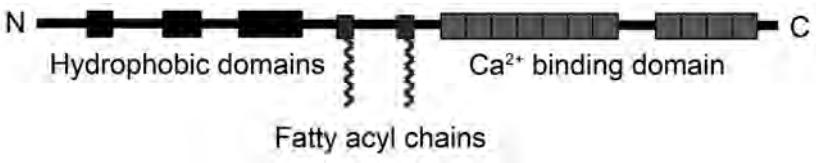


Figure 4. *The active form of E. coli Hly have three functional domains*

The hydrophobic domains (amino acids 238-410) have been suggested to be involved in pore-formation as they form transmemrane amphiphatic α-helices (Ludwig *et al.*, 1991; Boehm *et al.*, 1990a). The fatty acyl groups (myristic C_{14}) are essential for the activity but also for the stabilization of a membrane pore (Welch, 2001; Ludwig *et al.*, 1996). The Ca^{2+}-binding domain consists of 13 tandemly repeated glycin-rich units (amino acids 739-849). Binding of Ca^{2+} to this domain induces a conformational change in the toxin required for its action on the target cell membrane (Schindel *et al.*, 2001; Boehm *et al.*, 1990b; Ludwig *et al.*, 1988). The Ca^{2+}-binding domain have also been suggested to constitute a receptor-binding domain of the toxin (Ludwig *et al.*, 1988). A receptor for Hly on the cell membrane have long been debated and many suggestions are reported (Koschinski *et al.*, 2006; Cortajarena *et al.*, 2001; Lally *et al.*, 1997). It has been suggested that other bacterial components interact with Hly and take part in its action. One such component is LPS, that has been suggested to prevent aggregation and degradation of Hly, as well as to promote an active conformation of the toxin (Peyrl *et al.*, 2003; Czuprynski and Welch, 1995; Bohach and Snyder, 1985). Whether there is a molecular function of LPS in Hly induced signalling hitherto unknown.

2.3.3.2 S. pneumonia Pneumolysin

Pneumolysin (Ply) is a major virulence factor of Pneumococci. The effects of Ply are mediated by two mechanisms: binding to membranes leading to damage through pore formation, and activation of the complement system in a non-immunospecific manner (Rossjohn *et al.*, 1998; Mitchell *et al.*, 1991). Ply induces Ca^{2+} signalling in both neuronal cells and neuroblastoma, inducing apoptosis-inducing factor-dependent (AIF-dependant) apoptosis and activation of the p38 mitogen-activated protein kinase (MAPK) signalling pathway (Braun *et al.*, 2002; Stringaris *et al.*, 2002).

2.3.3.3 E. coli Cytolysin

E. coli cytolysin (ClyA) is expressed by both pathogenic and non-pathogenic *E. coli*, even though the pathogenic strains show elevated expression levels (Oscarsson *et al.*, 2002). Various serovars of *Salmonella sp* and *Shigella sp* also express the toxin (Oscarsson *et al.*, 2002; del Castillo *et al.*, 2000). ClyA is exported in outer-membrane vesicles and induces Ca^{2+} oscillations with a periodicity of 22.9±0.9 min (Wang *et al.*, 2004b; Uhlén *et al.*, 2000).

2.3.3.4 Listeria monoctytogenes Listeriolysin

Listeriolysin (LLO) is a crucial virulence factor for the pathogenicity of *Listeria monocytogenes*. It is required for invasion (Cossart *et al.*, 2003) and escape from the primary phagocytic vacuole of macrophages (Camilli *et al.*, 1993). LLO induces Ca^{2+} signalling in human embryonic kidney cells (HEK) and Hep-2 epithelial cells(Dramsi and Cossart, 2003; Repp *et al.*, 2002). Ca^{2+} signalling induced by LLO in macrophages is shown to be dependent of ROCC`s and formation of DAG (Wadsworth and Goldfine, 1999).

3 CONTEMPORARY METHODS TO REGULATE AND RECORD CELL SIGNALLING

Fluorescence-based techniques are valuable tools for studying cellular structure and function, as well as interactions of molecules in biological systems. Fluorescence forms also the foundation in the detection and quantification of nucleic acids and proteins in gel electrophoresis, microarrays, and various imaging technologies. With microscopes, it is possible to visualize and measure the amount of fluorescence in samples as small as individual cells using multiple fluorescent colors simultaneously. The combination of specialized fluorescent chemicals and instruments has given us an unprecedented detailed view of cells.

The complex interplay of ion signalling has been studied using several different technology platforms. The most common for real-time measurements is a technique based on fluorescent indicators. Cells pre-loaded with the fluorescent probe are exposed to stimuli that are manually added to the recording chamber, while the concomitant cellular response is recorded in the microscope. Another technology includes patch-clamp, as well as voltage and current clamp, where a thin electrode-pipette is used to record ion fluxes and membrane potential in single cells.

3.1 RATIOMETRIC ION IMAGING

The sensitivity of microscopy-based techniques can be enhanced by detection of fluorescence, which occur as the result of luminescense excited by radiation. Studies presented in this thesis rely on the possibility to perform real-time fluorescence imaging of cell signalling events. We have used an imaging system based on a highly sensitive, cooled CCD-camera coupled to a image-intensified microscope. A computer-controlled monochromator coupled to a high energy xenon lamp was used for probe excitation and imaging software was used for image acquisition. Fura-2 AM and PBFI are fluorescent probes detecting Ca^{2+} and K^{+} respectively. Both probes are ratiometric, meaning that the maximum excitation spectra is altered upon binding to the ion. Ratiometric measurements are advantageous as compared to the use of classic fluorescence probes, as they eliminate distortions of data caused by photobleaching and variations in probe loading/retention, as well as by instrumental factors such as

illumination stability. Fura-2 AM and PBFI have similar excitation spectra, where emission at 510 nm is maximized as a result of excitation at 340 nm (ion-bound probe) or 380 nm (only probe). The AM-form of Fura-2 has the additional advantage that it easily pass the cell membrane. Once inside, the probe is trapped because endogenous esterase's cleaves off the AM group, giving a hydrophilic nature of the probe. The temporal resolution of the real-time measurements is determined by the speed of the excitation and the sensitivity of image acquisition. Although visualization of ion fluxes is a powerful tool and as such has revolutionized the study of ion signalling, it clearly has some limitations as well. By being a passive observer, this imaging technique can only be used to study naturally occurring signalling events or those triggered by known inducers.

3.2 PATCH-CLAMP

The patch-clamp technique is the one of few contemporary methods that induce ion signalling in cells as well as record a response. A patch-clamp microelectrode is a micropipette with a relatively large tip diameter. The microelectrode is placed next to a cell, and gentle suction is applied through the microelectrode to draw a piece of the cell membrane (the "patch") into the microelectrode tip. The glass tip forms a high resistance 'seal' with the cell membrane. This configuration is the "cell-attached" mode, and it can be used for studying the activity of the ion channels that are present in the patch of membrane. If more suction is applied, the small patch of membrane in the electrode tip can be displaced, leaving the electrode sealed to the rest of the cell. This "whole-cell" mode allows very stable intracellular recording. A disadvantage is that the intracellular fluid of the cell mixes with the solution inside the recording electrode, causing dilution of the intracellular fluid. The patch-clamp technique has high sensitivity and can with great specificity induce and record events in single cell. Despite the many advantages of the "clamp" techniques they are also attributed with several disadvantages. Only highly skilled staff can use the technology, it is extremely cumbersome and expensive, and the technical devices require continuous maintenance. The "clamp" techniques can only be performed on one single cell, lacking the ability to sense the dynamics of a whole cell population. The invasiveness of the technique is also a limiting factor.

3.3 A NEED FOR NOVEL TECHNOLOGIES

Based on the above-mentioned limitations, the need for novel technologies for the studies of the complex dynamics of ion signalling is apparent. Today, stimuli are delivered to a population of cells by means of a manual pipette, which is neither exact nor especially delicate. There is a need for a simple, yet highly specific delivery system, which is tightly controlled at the temporal level and can be automated or programmed. The device should be able to achieve global signalling events in a population of cells and in addition have the sufficient resolution to address individual cells. Novel materials developed for organic electronics applications hold great promises to be integrated with biological specimens. Such organic electronic devices meet many wanted requirements for new tools and applications to study cell signalling, as they are easy to manufacture, possible to miniaturize, and they can be produced at a low cost.

4 ORGANIC ELECTRONICS – A BACKGROUND

Plastics are large molecules consisting of polymers of large carbon based monomers. Polymers are abundant in nature, where DNA is the most well known example.

Syntetic polymers revolutionized the field of materials in the beginning of the 20th century. The first commercial plastic was bakelite that started its career as a new wonder material in weapons industry, where its lightweight properties would potentially outmatch iron and steel in many applications. Bakelite also became commercially available and was first used as electrical insulators as this property proved better than anything then available at that time. Today, bakelite is still used because of its features of being electrically resistant, chemically and heat stable, and shatterproof. Today, polymers have developed into thousand of commercially available forms and is found in all aspects of our daily lives.

The second breakthrough came in 1977 when Heeger, MacDiarmid and Shirikawa achieved conductivity in polymers, for which they were awarded the Noble prize year 2000. This finding opened for the use of polymers in completely new areas such as the electronic industry. Since then the field of Organic electronics (because of its carbon based structure) have grown and today a wide array of conducting polymers are synthesized, whose different levels of conductivity varies between almost insulators to good conductors. Conductive polymers have attracted much attention because of its unique electrochemical properties leading to applications that include that of field-effect transistors (FET) (Matters *et al.*, 1999), polymer batteries (Murata *et al.*, 2000), electrochromic displays (Andersson *et al.*, 2002), sensors (Mabeck and Malliaras, 2006; Nilsson *et al.*, 2002b; Nilsson *et al.*, 2002a) and actuators (Isaksson *et al.*, 2004; Jager *et al.*, 2000). The novelty of organic electronics results in part from the fact that this technology offers new or improved electroactive and opto-electronic features compared to the inorganic counterparts as they are flexible (Gustafsson *et al.*, 1992) and can be manufactured using printing tools (Berggren *et al.*, 2007).

4.1 CONJUGATED POLYMERS

What makes a polymer conductive? In what way does it differ from the polymers used in plastic toys? The key to conductivity is alternating double bonds in the polymer. Polyethylene is a good insulator and is built of chains of monomers of two carbons

bound to each other together with four hydrogen atoms (Figure 5a). The symmetrical structure yields four equivalent hybrid orbitals resulting in that each carbon valence electron is occupied in the bonds to the surrounding atoms. In this case there is no electron left to transport current, thus rendering polyethylene non-conductive. In the case of polyacetylene, where the monomer consists of two carbons joined with a double bond and two hydrogen atoms, resulting in each carbon binds three atoms (alternating double bonds) (Figure 5b). Since carbon have four valence electrons there is one left in a non-hybridized orbital which can participate in transporting current, resulting in a conductive polymer.

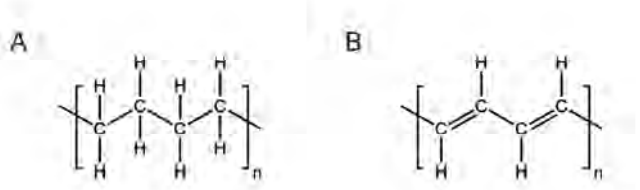


Figure 5. *A) Polyethylene **B)** Polyacetylene*

4.1.1 Doping

Most conjugated polymers are semiconductors, meaning that their conductivity is rather limited and far from that of metals. Heeger, MacDiarmid and Shirikawa discovered that by doping conjugated polymers the conductivity could be increased several orders of magnitude to almost reach the level of metals (Figure 6).

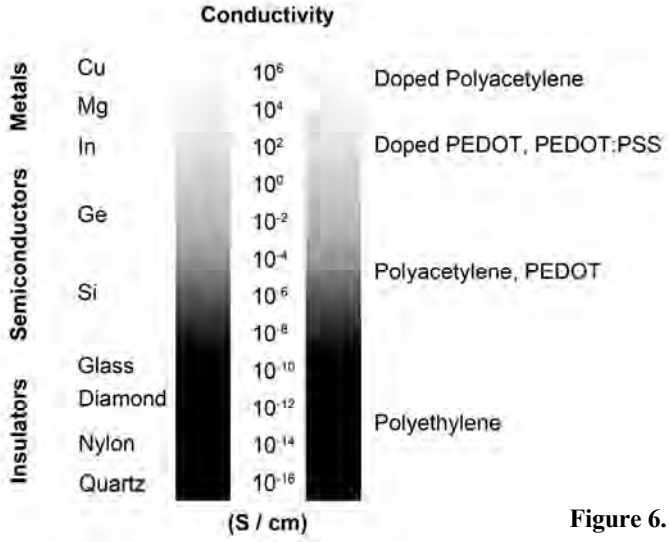


Figure 6.

Doping of a conjugated polymer means that another molecule is added that form an ionic complex with the polymer chain and either adds or frees one valence electron to the complex, to further enhancing the conductivity. Positive-doping (p-doping) of a conjugated polymer means that one electron is removed from the valence band, while addition of one electron to the valence band is termed negative-doping (n-doping).

4.1.2 PEDOT:PSS

One important conjugated polymer is p-doped poly(3,4-ethylenedioxythiophene) (PEDOT) (Figure 7a), which is used in electrochemical devices, such as light-emitting diodes (LED´s) (Dodabalapur, 1997), FET´s (Drury *et al.*, 1998), smart windows (Gustafsson-Carlberg et al 1995), electrochemical displays (Somani and Radhakrishnan, 2003) and transistors (Nilsson *et al.*, 2002b). PEDOT is useful because of its chemical stability and high conductivity. The conductivity can be further enhanced by addition of a counter-ion, that can be a small molecule or a polymer itself (Aleshin *et al.*, 1997a; Aleshin *et al.*, 1997b). PEDOT:PSS is a polymer-polyelectrolyte consisting of a mix of PEDOT and the charge-balanced counter-ion poly(styrene sulphonate) (PSS^-) (Figure 7b), which offers high stability, high conductivity as well as water solubility (Groenendaal *et al.*, 2000). PEDOT:PSS is commercially available from different companies e. g Bayer (Baytron P™, PEDOT:PSS solution) and AGFA (Orgacon™, PEDOT:PSS thin film on polyester carrier).

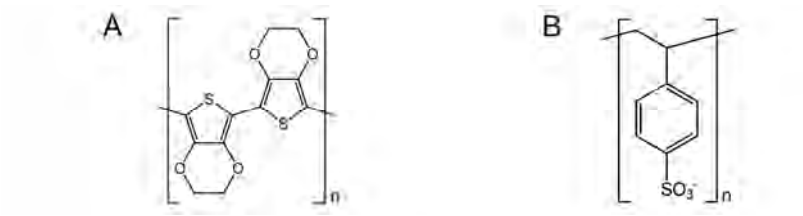


Figure 7. ***A)*** *poly(3,4-ethylenedioxythiophene) (PEDOT)*
B) *poly(styrene sulphonate) (PSS^-)*

4.1.3 Ionic conductivity

PEDOT:PSS and other conjugated polymers can in addition to electrons also conduct ions when doped. This is in sharp contrast to metal and inorganic electronics that purely rely on electrons as charge carriers.

The ability of PEDOT:PSS to conduct ions comes from the fact that when biased in a electrochemical cell, PEDOT:PSS undergo the following half-reaction:

$$PEDOT^{+}\ PSS^{-} + M^{+} + e^{-} \leftrightarrow PEDOT^{0} + M^{+}{:}PSS^{-}$$

where M^{+} represents a monovalent cation and e^{-} an electron. The arrow to the right indicates reduction of PEDOT, while the arrow to the left indicates oxidation of PEDOT. Thus, switching PEDOT between reduced and oxidized state requires transport of both cations and electrons to the reaction site, whereas the polymer chains themselves are effectively immobile. The red-ox switch can therefore be used to initially charge the polymer with ions and then subsequently switch them out e.g in a aqueous solution. The ion conductivity of PEDOT:PSS have to a lesser extent been studied in different experimental setups (Wang *et al.*, 2004a; Lisowska-Oleksiak and Kupniewska, 2003).

4.2 ORGANIC BIOELECTRONICS; PRESENT APPLICATIONS

Ever since Luigi Galvani performed his famous experiments in the 17th century where he successfully used charges of lightning to induce muscle tension in frog legs, scientist have continued to develop electronic devices to integrate with biological systems. The properties of organic electronic devices have gained much attention as possible candidates for such bio-electronic devices. Conjugated polymers have the advantage of being carbon based (organic) which suggest a possibility to achieve high level of biocompatibility. Other special characteristics that make organic electronic materials truly unique and promising as active material in bioelectronics include (Adopted from Berggren and Richter-Dahlfors, 2007):

1. Functionality can easily be defined at the materials level, giving that chemical biosignals can be translated into electronics signatures or signals within the material itself.

2. In the thin-film state organic electronic materials are often transparent, which allows optical transmission imaging and use of various microscopy-based techniques when analyzing biological specimens interacting with the device.

3. Organic electronic materials are soft and can be (self-)assembled and (self-)organized to mimic biological structures.

4. Organic electronic materials conduct electrons as well as ions.

5. Organic electronic materials can be decorated with (bio-)molecular side-groups to promote cell viability.

The field of organic bioelectronics is still in its cradle but collectively hold great promises for the discovery of novel tools to study biology and its mechanisms.

5 AIM

The aim of this thesis is to study various aspects of Ca^{2+} signalling induced by bacterial toxin or organic bioelectronic devices. More specifically my work includes:

I. Investigate the molecular mechanisms behind *E. coli* Hly induced Ca^{2+} signalling.

II. Develop an organic electronic bio-interface that can work as an ion pump device.

III. Use this ion pump device to integrate electronic control to biological cell signalling events.

6 RESULTS AND DISCUSSION

6.1 CA^{2+} SIGNALLING INDUCED BY *E.COLI* HLY (PAPER I AND II)

UPEC Hly was among the earliest exotoxins that was characterized as a virulence factor (Bhakdi *et al.*, 1986; Felmlee *et al.*, 1985; Hacker *et al.*, 1983; Welch *et al.*, 1981). Since then new findings have shed light on the effect of Hly, attributing it novel signalling roles besides that of cytolysis. Hly was the first pathophysiologically relevant protein demonstrated to induce intracellular Ca^{2+} oscillations (Uhlén *et al.*, 2000). Althoughit was shown that Hly induced Ca^{2+} oscillations induce production of the pro-inflammatory cytokines IL-6 and IL-8 (Uhlén *et al.*, 2000), few details are known regarding the signalling mechanism. In paper I and II we investigate this signalling mechanism, using fura-2 AM based Ca^{2+} imaging (Figure 8) of a physiological relevant model system based on primary preparations of rat renal proximal tubule cells (PTC).

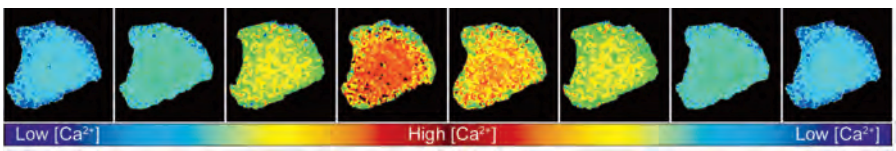


Figure 8. *Intracellular [Ca^{2+}] fluctuation visualized by ratiometric imaging in a single PTC upon Hly stimulation. The pseudo-colored cell shows the basal level of intracellular [Ca^{2+}] as blue, while increases in intracellular Ca^{2+} are depicted as yellow, red, and black.*

Our studies focus on bacteria, recruitment of Hly to the cell membrane, signalling at the cell membrane to the identification of components involved in the intracellular signalling cascade. As it previously has been shown that mutations within the three functional domains of Hly affects cytolysis of erythrocytes exposed to high concentrations of the toxin (Ludwig *et al.*, 1988; Vogel *et al.*, 1988; Ludwig *et al.*, 1987). We analyzed whether these domains also were involved in the induction of Ca^{2+} signallig. In paper II we used three set of mutants each harboring defined deletions within the hydrophobic domain, fatty-acyl groups or the Ca^{2+} binding domain (Paper II Table 1). When analyzing their capacity to promote Ca^{2+} oscillations in PTC, we found that neither one were able to induce Ca^{2+} oscillations. Next, competition experiments

where performed cells Ca^{2+} was recorded in cells exposed to mutant Hly followed by the addition of wild-type (wt) Hly. Hly with mutations within the hydrophobic domain, or lacking the fattyl acyl groups, both prevented subsequent induction of Ca^{2+} oscillation by wt Hly. As these mutants harbours an intact Ca^{2+} binding domain these results indicated that these mutants are able to interact with the target cell membrane, thus preventing wt Hly to exert its effect (Ludwig *et al.*, 1991). The opposite was found for Hly containing a deletion within the Ca^{2+} binding domain which could not block subsequent induction of Ca^{2+} oscillations by wt Hly. This suggests that this mutant Hly fails to bind to the cell. This is in accordance with previous discussions that Ca^{2+} binding domain is important for binding to a putative receptor on the cell membrane (Cortajarena *et al.*, 2003; Ludwig *et al.*, 1988) (Figure 10).

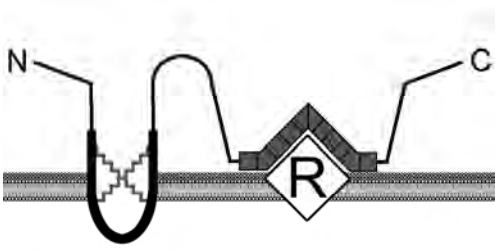


Figure 10. *Schematic model of Hly's interaction with the cell membrane, where the Ca^{2+}-binding domain interacts with a membrane receptor (R).*

Hly is found associated with the outer membrane of bacteria, in the supernatant of bacterial cultures, and in outer membrane vesicles. In all these situations Hly is tightly associated with LPS (Balsalobre *et al.*, 2006; Oropeza-Wekerle *et al.*, 1989). To further study the mechanisms of Hly interaction with the target cell we investigated the role of LPS for Hly induced Ca^{2+} signalling (paper I). We show that ultra-pure preparations of Hly, with extremely low amounts of contaminating LPS failed to induce Ca^{2+} oscillations. In addition, induction of Ca^{2+} oscillations by the "normal" LPS-containing Hly preparation was abrogated if the toxin was treated with excess amounts of LPS or polymyxinB. This shows that the tight interaction between Hly and LPS must be maintained for Ca^{2+} signalling to occur. Since LPS is essential for induction of Ca^{2+} oscillations it is tempting to analyze the role of the LPS recognition system in this context. Using increasing amounts of recombinant CD14 or blocking antibodies directed towards the binding sites for CD14 and LBP on LPS we show that these

components are essential for recruiting the Hly-LPS complex to the cell membrane where Ca^{2+} signalling is initiated. Interestingly, we found that Hly-LPS mediated Ca^{2+} signalling occur independently of TLR4. We conclude that CD14/LBP facilitates Hly´s recruitment to the cell membrane where interaction with a hitherto unknown receptor induces Ca^{2+} signalling. It is, however, unlikely that CD14 functions as a signalling receptor since this GPI-anchored protein lacks an intracellular signalling domain (Triantafilou and Triantafilou, 2002).

It has previously been reported that a receptor is needed for Hly to initiate Ca^{2+} signalling in PTC (Laestadius *et al.*, 2002). The integral membrane protein glycophorin has been suggested as a receptor for Hly in erythrocytes (Cortajarena *et al.*, 2003; Cortajarena *et al.*, 2001). We show that glycophorin indeed is expressed on PTC as well. Blocking with glycophorin-specific antibodies intervene with Hly´s action, thereby blocking induction of Ca^{2+} oscillations. Also, sequestration of Hly with soluble glycophorin had the same effect. As glycophorin is a highly glycosylated membrane protein, we chemically depleted glycophorin of its carbohydrate structure and found that this treatment prohibited Hly from induction of Ca^{2+} oscillations, while the cytolytic effect of Hly was unaltered. Taken together, these findings support the model proposed by Moayeri and Welch, depicting that two distinct conformations of cell-associated Hly exists when the toxin-binding results in a non-lytic versus lytic event on the target cells (Moayeri and Welch, 1997). Collectively we suggest that glycophorin is the receptor for Hly on PTC leading to induction of Ca^{2+} oscillations (Paper II). Recent data explain that the promiscuity of Hly depends on 9 amino-acid shift domains in the protein sequence that gives the toxin specificity for different cell types (Herlax and Bakas, 2007). This may explain the diverse suggestions previously reported for a membrane receptor for Hly (Koschinski *et al.*, 2006; Valeva *et al.*, 2005; Cortajarena *et al.*, 2001; Lally *et al.*, 1997).

Hly induced Ca^{2+} oscillations have been shown to affect gene expression (Uhlén *et al.*, 2000). Consequently we analysed the effect of Hly-induced Ca^{2+} oscillations on the global gene expression pattern in PTC. We found in three separate microarray-based experiments that the mRNA encoding ezrin was consistently increased three-fold, as compared to control cells exposed to heat-inactivated toxin (no Ca^{2+} oscillations). This observation was confirmed and quantified in ribonuclease protection assays, showing that Hly-induced Ca^{2+} oscillations caused a 2.9-fold increase of ezrin-specific mRNA,

as compared to cells exposed to heat-inactivated toxin. Using anti-ezrin antibodies in a western blot experiment revealed that the total ezrin concentration was increased 1.4 and 1.8 times 1h and 3h after onset of the Hly-induced Ca^{2+} oscillations. Interestingly studies in erythrocytes reveal that proteins belonging to the ERM family links the glycophorin to the cytoskeleton (Bobacka *et al.*, 2000; Takeuchi *et al.*, 1994a). Ezrin anchors trans-membrane proteins to the underlying filamentous actin in the cortex (Bretscher *et al.*, 2002).To investigate whether the actin cytoskeleton was important for transduction of the Hly-induced signal, the dynamics of the cytoskeleton was destroyed with jasplakinolide (Paper I). Performing Ca^{2+} imaging on jasplakinolide-treated cells revealed that this abrogates Hly-induced Ca^{2+} oscillations.

Modulation of the actin cytoskeleton in response to extracellular signals involves the regulatory action of the small GTPases RhoA, Rac and Cdc42. This can be exemplified by RhoA, which is an important activator of ezrin (Speck *et al.*, 2003; Matsui *et al.*, 1998). RhoA is also the target of the *E. coli* toxin CNF-1.To investigate whether any of the small GTPases are involved as mediators in Hly-induced Ca^{2+} signalling, affinity-precipitation was performed on cells exposed to sub-lytic concentrations of Hly. We found that RhoA was up-regulated 2-fold, while no effect was observed on Rac or Cdc42. To confirm the molecular involvement of RhoA aa well as ezrin in Hly induced signalling, functional assays were performed on cells transfected with EGFP-tagged constructs expressing either the dominant negative form of RhoA or of ezrin (Paper I and II). In both situations cells transfected with the dominant negative constructs became non-responsive to Hly.
RhoA has been shown to concentrate in lipid rafts (Speck *et al.*, 2003; Matsui *et al.*, 1998). This membrane domain is a hot-spot for signal-transduction events to occur. CD14 is known to direct LPS to lipid rafts for induction of cell signalling, and the same is true for several bacterial toxins, e.g cholera toxin and LLO (Gekara *et al.*, 2005; Abrami *et al.*, 2003; Triantafilou *et al.*, 2002). We therefore investigated whether Hly-induced Ca^{2+} signalling requires intact lipid rafts using cholesterol-depleting agent methyl-β-cyclodextrin. As this treatment abrogated the response we conclude that the signal transduction initiated by Hly occurs in lipid rafts.

One key effector in transducing extracellular signals into Ca^{2+} oscillations is IP_3. Previously, we have shown that interaction of this PLC-derived second messenger with IP_3R in ER is required to obtain Ca^{2+} oscillations (Uhlén *et al.*, 2000). However, the

IP_3R inhibitor we used in that study, 2-aminoethyl diphenylborate (2-APB), was later shown as somewhat unspecific, as it also inhibits store operated Ca^{2+} channels (SOCCs) and capacitative Ca^{2+} entry (Iwasaki *et al.*, 2001). Instead, in paper II, we used an approach based on transfection of PTC with a EGFP-tagged plasmid expressing the IP_3 high-affinity binding site from IP_3R1. Ratiometric Ca^{2+} measurements of Hly-treated cells revealed that sequestration of IP_3 inhibited Ca^{2+} oscillations, while an oscillatory response was readily detected in non-transfected cells present in the same cell cluster (Figure 11). Thus, two different methods, both points to the importance of IP_3 for Hly induced Ca^{2+} oscillations.

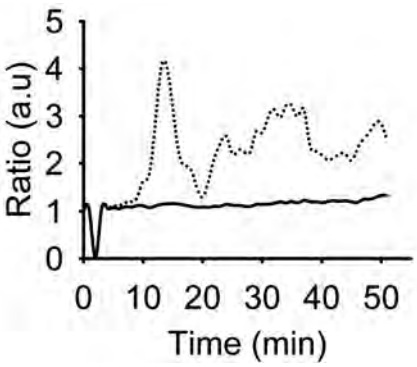


Figure 11. *Sequestration of IP_3 prohibits Hly-induced Ca^{2+} oscillations (solid line) while non-transfected cells (dotted line) recorded in the same experiment respond with Ca^{2+} oscillations.*

6.1.1 An integrated picture of signalling pathways activated by Hly

Although the picture is still incomplete, the data reported in paper I and II show that we have identified several key effector molecules involved in Hly-induced Ca^{2+} signalling. We show that all functional domains of the Hly protein are essential for initiation of signalling and that the Ca^{2+} binding domain of the toxin is important for the interaction with the receptor glycophorin. To approximate Hly to the receptor, we demonstrate a novel TLR4-independent function of the LPS-recognition system as it recruits Hly to the cell membrane (Figure 12). This may represent a general mechanism for activation of signal-transduction events by other LPS-associated microbial molecules as well.

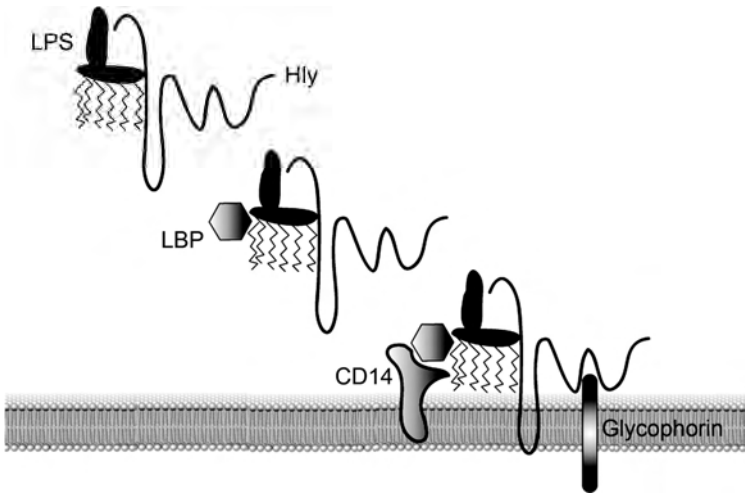


Figure 12. *We propose a model in which the Hly-LPS complex is recruited by LBP and CD14 to the cell membrane where interaction with the receptor glycophorin occur.*

The specialized cell membrane microdomains termed lipid rafts are shown to be essential for the initiation of Hly-induced signalling, suggesting that recruitment of Hly by CD14, and binding of Hly to the receptor glycophorin is initiated within this signalosome. We find that the small GTPase RhoA is concentrated in lipid rafts, where it is likely to activate the protein-cytoskeleton linker protein ezrin. Indeed, we find that ezrin and RhoA are up-regulated in cells exposed to Hly and both are required to convey Hly-induced Ca^{2+} signalling. The ERM family of proteins have been shown to link glycophorin to the cytoskeleton, suggesting a similar function for ezrin in Hly-glycophorin mediated signalling. This picture is further enhanced by the fact that hyper-polymerization of the actin filaments blocks the Hly-induced signalling cascade. Activation of ERM proteins requires local elevations of PIP_2, a second-messenger that is generated by PLC. Previously reported data from our group shows that PLC is involved in Hly-induced Ca^{2+} signalling (Fievet *et al.*, 2004; Uhlén *et al.*, 2000). Collectively, these findings efficiently connects these components to those involved in intracellular Ca^{2+} signalling. The cytoskeleton is also a key player in Ca^{2+} signalling, as it has been reported to be involved in CCE and IP_3R –induced Ca^{2+} release from the ER, as well as in store operated Ca^{2+} release (Wang *et al.*, 2002). We further demonstrate a central role of the Ca^{2+} regulating second-messenger IP_3 in Hly induced Ca^{2+} oscillations. This suggests that release of Ca^{2+} from intracellular stores is critical to maintain Ca^{2+} oscillations, in addition to the initial influx of Ca^{2+} that occur via

VOCC´s presumably activated by transient pore-formation generated by the pore-forming domain of Hly (Uhlén *et al.*, 2000). These new details of the signalling mechanism of Hly is especially interesting in the context of the *in vivo* situation, where a recent publication from our group demonstrates *i)* that Hly is expressed *in vivo* when bacteria reside inside the renal tissue, and that *ii)* a striking immune-modulatory effect of the toxin is demonstrated (Mansson *et al.*, 2007).

6.2 CA^{2+} SIGNALLING INDUCED BY AN ORGANIC ELECTRONIC ION PUMP (PAPER III AND IV)

Ca^{2+} is a key second-messenger in cell signalling. This signal acquires its specificity by varying its spatial and temporal properties, locally and globally. Although many stimuli are known to induce Ca^{2+} signals, the spatial and temporal features of induced signals have only been assigned to a fraction of them. Furthermore, knowledge of the down-stream effects the signals have, e.g. on transcription and fertilization, is limited. Researchers are currently investing a lot of time and efforts to map these unknown areas to further their understanding of the dynamics of Ca^{2+} signalling. To aid in this process, we decided to make an attempt to develop a novel technology, where specific Ca^{2+} signalling patterns could be induced electronically. This would generate a system where intracellular Ca^{2+} oscillations could be induced with defined spatio-temporal properties. The ensuing cellular out-put could then be recorded by molecular biology tools.

Paper III and IV describe our development of an electrophoretic ion pump that functions as an actuator to pump H^+, K^+ and Ca^{2+}. This prototypic device is designed as a bio-interface to convert an electronic signal to a biological output. The conjugated polymer PEDOT:PSS is used as the active material in the device because it has good electronic and ionic conductivity, it is a stable, yet flexible material which is easy to manufacture. A critical concern when selecting a material is the biocompatible features. Many conjugated polymers have been shown to be biocompatible; e.g poly-pyrrole (PPy) (George *et al.*, 2005; Wang *et al.*, 2004b) and PEDOT:PSS (Cui and Martin, 2003; Nyberg *et al.*, 2002). To analyze the biocompatible potential of PEDOT:PSS in this application, the adhesion, morphology and viability of cells were studied. The neuronal cell line HCN-2 were chosen as these cells are known to be easily excitable, and they have retained many properties of primary neurons, such as cell morphology

and expression of neuronal markers (Peyrl *et al.*, 2003; Ronnett *et al.*, 1994). In biocompatibility experiments, cells cultivated on PEDOT:PSS for 24 and 48 h were analyzed. We found that cell adhesion and cell viability on PEDOT:PSS were identical to those on silica and polystyrene plastic. These biocompatible features of the organic electronic material were found to be generally applicable for a variety of different cell types, as epithelial cells (HeLa), endothelial cells (BCE-hTERT+), fibroblasts (TGR1), macrophage-like cells (THP-1) and T cells (Jurkat) all showed similar results. Morphological analysis of neuronal cells cultured on PEDOT:PSS indicates that neurites are developed without the formation of stress fibers, suggesting that this polymer exhibits proper biocompatibility for the current application. Another conjugated polymer; poly-aniline (PANI) was also tested as a candidate material for the ion pump, however, poor cell viability disqualified this material (Peter Kjäll, *unpublished observation*).

The goal for the ion pump is to utilize electronic addressing signals to control and drive an ionic current. Such translation of an electronic signal into ion fluxes allows the delivery of ions to cells growing on the polymer without any convection effects (Paper III). A schematic picture of the ion pump developed in paper III is shown in Figure 13a and 13b. The four 200 nm thin polymer electrodes denoted A-D are biased with three voltages (V_{AB}, V_{BC} and V_{CD}). The source electrolyte, AB, is the reservoir containing high concentration of the ion to be transported into the receiving electrolyte, CD. The CD electrolyte contains either other cation(s) or it can be replaced by a electrolyte of choice for the specific application e.g cell culturing media such as RPMI-1640. To restrict the conductivity between the AB and CD electrode to ions, the electrodes are separated by an over-oxidized PEDOT:PSS barrier unable to conduct electrons (Figure 13). The remaining parts of the ion pump are coated with the hydrophobic photoresist SU-8 to keep the AB and CD electrolytes separated. When applying the V_{BC} potential, the B electrode is oxidized while the C electrode is reduced. Consequently, cations in the AB reservoir are transported in the electric field through the solid state polymer electrolyte into the target electrolyte CD. When the ions reach the edge of the polymer electrolyte, they are released from the solid state polymer and spread by diffusion into the CD electrolyte. V_{AB} and V_{CD} are used to continuously regenerate electrode B and prevent electrode C from becoming completely reduced.

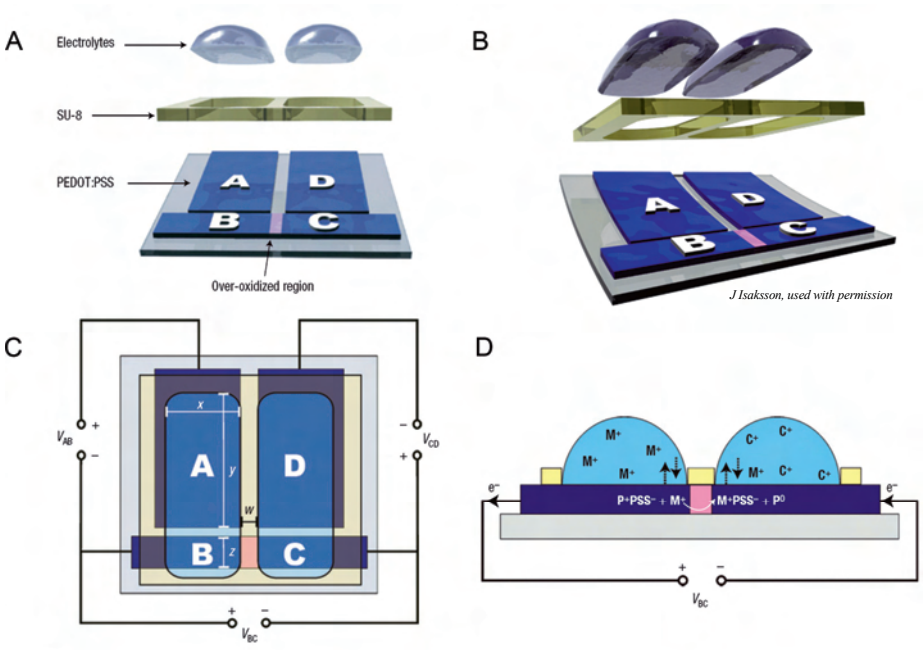


Figure 13. ***A)****and* ***B)****Schematic view of the ion pump shown as separated layers. The four PEDOT:PSS electrodes are labeled A to D. PEDOT:PSS in the over-oxidized region (pink) between B and C conducts ions but not electrons.* ***C)****Top view with voltages applied, dimensions: x =7 mm, y =12 mm, z =4mm or 50 µm and w =2 mm.* ***D)*** *Schematic cross-section of electrodes B and C. M^+ and C^+ are cations, P=PEDOT.*

The capabilities / characteristics of the ion pump were assessed by *i)* measuring transported charges in relation to applied voltages using atomic absorption spectroscopy of the CD electrolyte and *ii)* by real-time fluorescence microscopy using ion-specific fluorescent probes in the CD electrolyte. Both techniques allowed us to quantify the increased concentration of transported ions in the CD electrolyte (Paper III). A high starting concentration of the cation in AB increases the transport rate of that particular ion. The initial cation concentration in CD on the other hand, does not influence the transport at all, as the diffusion rate through the polymer is almost negligible as compared to electro-migration. This is particularly interesting when the initial ion concentrations in AB and CD are equal. Figure 14a shows that it is possible to pump ions against a concentration gradient, denoting the device's true pumping properties. The correlation between total charge and the amount of transported ions after 10 min of operation is shown in Figure 14b. If no voltages are applied, the diffusion/leakage of K^+ from the AB electrolyte to the target electrolyte is very small. Thus, the on/off ratio of the ion transport exceeds 300.

The relatively low optical absorbance of PEDOT:PSS thin films makes this material suitable for microscopy studies. To measure the increase of $[K^+]$ in real-time in the target electrolyte, the K^+ sensitive probe PBFI was added to the CD electrolyte. As the ion pump was activated, the altered fluorescence of PBFI was measured at the polymer/electrolyte release barrier. Figure 14c shows that the $[K^+]$ starts to increase after only a few minutes (solid line). This increase is stopped when the voltages are turned off, while it subsequently starts to increase 10 min later when voltages again are applied (dashed line). This corroborates the previous results, showing that leakage of ions in the polymer electrolyte bridge between B and C is close to zero. By integrating the BC current, it is possible to count the number of transported ions. Interestingly, the tight correlation of charge and transported ions show a transporting efficiency close to 100%, a unique number compared to other molecular deliver systems. Characterization of Ca^{2+} transport through the pump was shown using a similar approach.

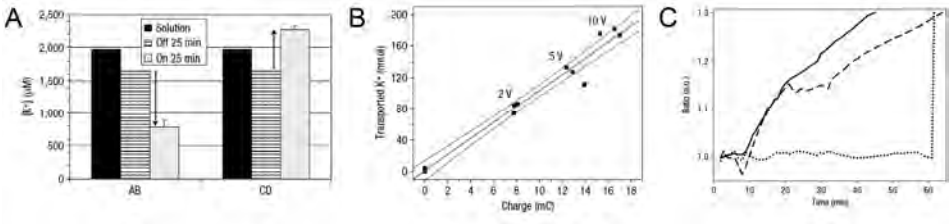


Figure 14. ***A)*** *Comparison of $[K^+]$ in AB and CD when starting with identical electrolyte solutions in both. Off indicates that no voltages were applied and on means V_{AB} =1 V, V_{BC} =10 V, V_{CD} =1 V.**B)** Corresponding relationship between transported K^+ and total B–C charge after 10min. The middle line shows the linear fit and the outer lines represent the 95% confidence interval.* ***C)*** *Time-lapse microscopy of ion-pump-mediated K^+ transport from AB (0.1M KCl) to CD electrolyte (0.1M Ca^{2+} acetate containing the K^+-sensitive probe PBFI) at V_{BC} =10 V (solid line). Dotted line: dH_2O as AB electrolyte, where increased fluorescence at 60 min results from manual addition of 50mM KCl directly to the CD electrolyte. Dashed line: Activation of the ion pump for 15 min, followed by a switch to the off state causes the fluorescence increase to stop. Reactivation of the pump 10 min later causes the $[K^+]$ increase to continue.*

K^+ is an activator of excitable cells such as HCN-2 neuronal cells. High extracellular $[K^+]$ depolarizes the plasma membrane, and subsequently activates VOOC´s in the cell membrane. This allows for a rapid influx of Ca^{2+} from the extracellular environment into the cytoplasm (Bootman *et al.*, 2001; Eckert and Ewald, 1982). Neurons cultivated on the C electrode in cell-culturing medium were prepared for

single-cell recordings of intracellular [Ca^{2+}] by loading the cells with the Ca^{2+}-sensitive probe FURA-2 AM. Using fluorescence imaging, the basal intracellular level of Ca^{2+} was initially recorded. Thereafter the ion pump was activated by applying constant V_{BC} =10 V (V_{AB} and V_{CD} =off). Approximately 15 min later, an increase of the intracellular [Ca^{2+}] was observed indicating that increased [K^+] caused depolarization of the membrane and subsequent influx of Ca^{2+} via VOCCs occurred (Figure 15a solid line). The 15 min lag time was reduced to 2–4 min when increasing the K^+ transport rate by application of all three potentials (V_{BC} =10 V, V_{AB} and V_{CD} = 1 V) (Figure 15a dotted line). The amplitude of the Ca^{2+} increase resembled the response obtained by manually increasing the extracellular [K^+] to the physiological concentration of 50mM in the CD electrolyte (Figure 15a dashed line).

To exclude the possibility that the Ca^{2+} response was induced by the applied potentials rather than ion transport, the same experiment was repeated using dH_2O as AB electrolyte; however, no Ca^{2+} response was observed (Figure 15a dashed-dotted line). To analyze the physiological relevance of Ca^{2+} influx in response to ion-pump-transported K^+, we used pharmacological substances known to inhibit ion channels in the cell membrane. Gd^{3+}, commonly used to block VOCC´s and SOCC´s (Flemming *et al.*, 2003), abrogated extracellular Ca^{2+} influx when cells were exposed to increased [K^+] (Figure 15b solid line) A similar effect was observed using nifedipine, an inhibitor of L-type VOCC´s (Miller, 1987) (Figure 15b dotted line).

Besides temporal control, spatial control of ion fluxes is of utmost importance for conveying the signal to cells. The ion pump delivers ions to the target electrolyte at the boundary between the polymer /liquid electrolyte, resulting in a high local concentration at this interface. This effect is qualitatively visualized using H^+ transport from HCl in the AB electrolyte (pH 1) to the CD electrolyte (KCl, pH 5). The altered color of a pH-sensitive indicator paper placed on top of electrode C in the CD electrolyte indicates delivery of protons (Figure 15c). A continuous increase of H^+ close to the delivery line occurs, whereas diffusion slowly creates a pH gradient along the C electrode. To investigate whether spatial control also can be achieved when K^+ is used to induce Ca^{2+} fluxes in cells, Ca^{2+} imaging of FURA-2 AM-loaded cells located at different distances from the delivery line was performed. Cells located within a few μm of the barrier responded as expected with increased intracellular [Ca^{2+}] (Figure 15d solid line), whereas cells located 1 mm and 2 mm away from the barrier were unresponsive (Figure 15d dotted and dashed line).

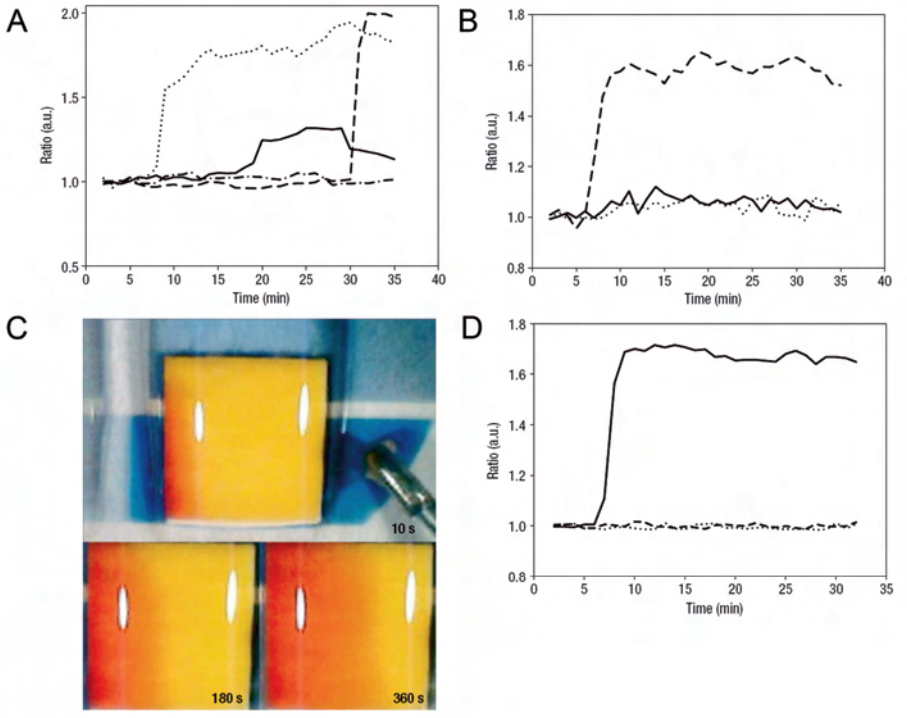


Figure 15. ***A)*** *Intracellular Ca^{2+} fluxes in FURA-2-AM-loaded HCN-2 cells in cell-culturing medium on the CD electrode resulting from ion pump delivery of K^+ from the AB electrolyte. Solid line: Intracellular Ca^{2+} response when only V_{BC} =10 V is applied. Dotted line: Increased K^+ transport rate (V_{BC} =10 V, V_{AB} and V_{CD} =1 V) causes a pronounced Ca^{2+} response in cells. Dashed line: Inactive ion pump causes no cellular response (off state), whereas increased fluorescence at 30 min results from manual addition of 50mM KCl to CD electrolyte. Dashed–dotted line: dH_2O as AB electrolyte causes no Ca^{2+} response in HCN-2 cells.* ***B)*** *Intracellular Ca^{2+} fluxes result from K^+-mediated depolarization of the cell membrane. Dashed line: Ca^{2+} response in FURA-2 AM-loaded cells on the CD electrode results from K^+ transported from AB to CD electrolyte. Solid line: Experiment in the presence of $GdCl_3$ in the CD electrolyte. Dotted line: Experiment in the presence of nifedipine in the CD electrolyte.* ***C)*** *Visualization of the pH gradient formed in the CD electrolyte during pumping of protons. Deep red color indicates pH~2 and clear yellow pH~5.* ***D)*** *The ion pump provides spatial control of cellular responses. Solid line: Time-lapse microscopy of intracellular [Ca^{2+}] in FURA-2-AM-loaded HCN-2 cells located adjacent to the 4-mm-wide barrier. Dotted and dashed lines: Cells located 1mm (dotted line) and 2mm (dashed line) from the barrier.*

As ion transport is currently confined to the width of the channel between electrodes B and C (4 mm in the present device), the ion pump device must be further developed to obtain spatial control at the cellular level. By fabricating a 50-µm-wide microchannel, we showed that stimulation at the single cell level could be achieved. This represents a first step towards miniaturization of the ion pump. Depending on the processing conditions of the conducting polymer, PEDOT and PSS form segregated phases ranging from approximately three to tenths of nanometers

(Greczynski *et al.*, 2001), a length scale that will define the lower limit for miniaturization, as ion migration is expected to occur primarily in the PSS phase.

In a majority of cellular Ca^{2+} signalling events, the ion flux shows an oscillating character with repetitive increase and decrease of intracellular [Ca^{2+}] occurring at a specific frequency. In paper IV we investigate the ion pumps ability to transport and deliver ionic species in such an oscillatory pattern using H^+ transport and detection. By placing a pH-sensitive paper on top of the C electrode in the CD electrolyte and by biasing the electrodes in square-wave pulses, we detected oscillation of the H^+ concentration and subsequently the pH in the target electrolyte dropped. The oscillations showed a periodicity of 5 min and an approximate amplitude of pH 0.5 (Figure 16). This proof-of-principle demonstrates that the ion pump can be used to create artificial ion oscillations, which can be applied to cell or tissue systems.

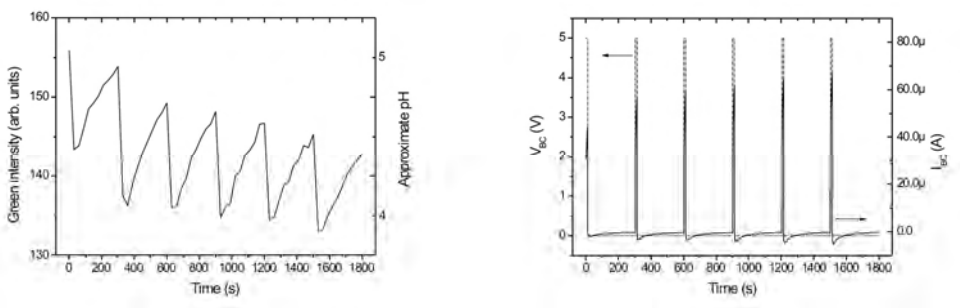


Figure 16. *Electronically controlled oscillating ion signal. Short pulses creates proton oscillations in the CD electrolyte close to the release line with a periodicity of ~300 seconds.*

Collectively, data presented in paper III and IV demonstrates the development of a novel technology platform to be used in cell signalling research. The ion pump is manufactured by standard printing and photolithography patterning techniques of the conjugated polymer PEDOT:PSS. The material is cheap, flexible and bio-compatible, giving this organic bio-electronic device many advantages compared to those composed of inorganic material. The intrinsic properties of PEDOT:PSS also gives it ionic conductive powers, thus makes the device capable of direct electronic delivery of charged species to a population of cells or down to single cell level. The feature of

non-convection delivery, almost 100% efficiency, high transport rates, unmatched ON/OFF ratio, temporal as well as spatial resolution down to 0.1μm is unique by today's standard. Further development and refinement of the technique hold great promises for the ion pump device as a potent cell signalling research tool, and many new areas of use in biology and medicine can be envisaged.

7 CONCLUDING REMARKS AND FUTURE DIRECTIONS

In this thesis we have studied various aspects of Ca^{2+} signalling. We have shown that the UPEC exotoxin Hly is a potent inducer of Ca^{2+} signalling and that this toxin induces novel signalling pathways leading to pro-inflammatory responses. The work presented here advances our understanding of the molecular details related to toxin-induced signalling *in vitro*, however, it would be interesting to study the relevance this have *in vivo*, as Hly is one of few pathophysiologically relevant proteins known to exert this kind of cellular responses. We envisage that Hly has a more complex signalling role during the infection process than earlier appreciated. Recent data shows that Hly have an immune-modulatory effect during the first hours of renal cortical infections (Mansson *et al.*, 2007). It would be highly interesting to use the 2-photon microscopy technique to monitor Hly's effect on Ca^{2+} signalling in the proximal tubule cells in real-time *in vivo*, and to simultaneously observe the effects on the tissue homeostasis. Dissection of the site of infection could be used as a source of total RNA for further microarray analysis to dissect out important signalling components at the various stages of the progression of infection.

The Ca^{2+} ion uses frequency modulation to achieve specificity of the signal, but today few known inducers of intracellular Ca^{2+} signalling are spatio-temporally characterized. To aid in this research, we developed an organic electronic ion pump as a bio-interface to electronically induce Ca^{2+} signalling in neuronal cells. Our data suggest that there is a huge potential for this group of devices as a tool for inducing artificial Ca^{2+} signalling patterns with specified frequencies in biological specimens.

The ion pump device interacts with cells as a planar bio-interface. However, *in vivo* tissues have a 3-dimensional architecture that are neither planar nor static. We aim to develop the ion pump structure to a tissue integrated device. To achieve this, one has to improve the softness and flexibility as well as the bio-compatibility and bio-stability of the organic electronic devices. We foresee that many future applications in life science and medicine will be based on organic bioelectronic devices.

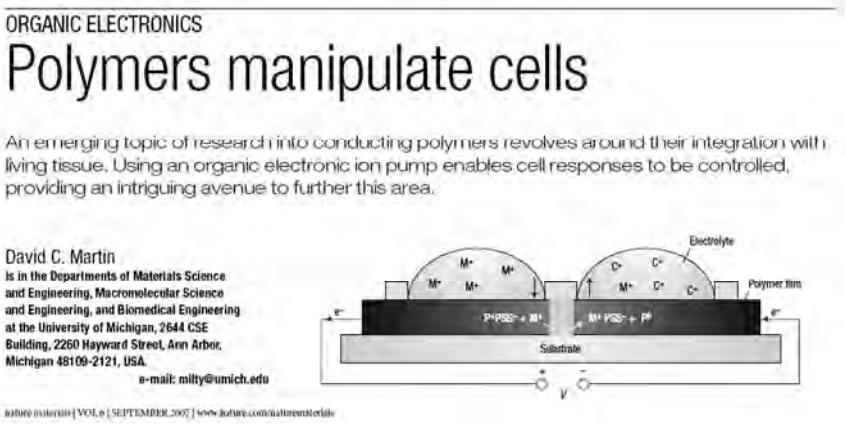
8 MY SCIENTIFIC CONTRIBUTION

During my thesis work we have studied different aspects of Ca^{2+} signalling. The bacterial toxin Hly, a major virulence factor of UPEC is shown to induce a complex signalling cascade in host cells leading to intracellular Ca^{2+} oscillations. We show a novel molecular mechanism for toxin delivery where Hly´s is recruited to the cell membrane by TLR4-independent use of the LPS-recognition system to and subsequent binding to its receptor glycophorin. This enhances our knowledge of the dynamics of infection and the bacteria host-interplay in UTI.

Our initiation of inter-disciplinary research led to the development of an ion pump device by which we have shown a novel principle, where an electronic signal is though ionic conductivity converted to a biological output in cells. This device by its intrinsic ion transporting properties as well as great potential of being miniaturized show huge potential as a tool in cell signalling research as well as in other future delivery applications where strict control of non-convection transport is needed. I feel privileged to be part of the initiation of the novel field of organic bioelectronics.

Paper III

Electronic control of Ca^{2+} signalling in neuronal cells using an organic electronic ion pump.
Isaksson J*, **Kjäll P***, Nilsson D, Robinson N. D, Berggren M and Richter-Dahlfors A. Nature Materials 2007, 673 - 679.
*contributed equally.
Was highlighted in the "news and views" in the same issue of *Nature Materials*



ORGANIC ELECTRONICS

Polymers manipulate cells

An emerging topic of research into conducting polymers revolves around their integration with living tissue. Using an organic electronic ion pump enables cell responses to be controlled, providing an intriguing avenue to further this area.

David C. Martin
is in the Departments of Materials Science and Engineering, Macromolecular Science and Engineering, and Biomedical Engineering at the University of Michigan, 2644 CSE Building, 2260 Hayward Street, Ann Arbor, Michigan 48109-2121, USA.
e-mail: milty@umich.edu

nature materials | VOL 6 | SEPTEMBER 2007 | www.nature.com/naturematerials

8.1 RELATED WORK NOT INCLUDED IN THE THESIS

Torstensson E, **Kjäll P** and Richter-Dahlfors A. (2005). *Chapter 2.2.6*, Imaging Techniques for the study of Escherichia coli and Salmonella infections. In R Curtiss III (Editor in chief), EcoSal-*Escherichia* and *Salmonella*. Cellular and Molecular Biology. [Online] http://www.ecosal.org. ASM Press.

Kjäll P and Richter-Dahlfors A. (2004) Toxin-Induced Activation of the Innate Immune Response Via Intracellular Ca^{2+} Signaling. Immunology 2004, International Proceedings 493-496.

Ries J, **Kjäll P**, Beiter K, Wartha F, Syk A, Richter-Dahlfors A, Morfeldt E, Normark S, B Henriques-Normark. (2007) Lytic antiobiotics affect cell wall pH of Streptococcus pneumoniae dependent upon endogenous H_2O_2 production.
Submitted manuscript

8.2 PATENTS

Electrically Controlled Ion Transport Device (Patent pending)

9 ACKNOWLEDGEMENTS

Sitting here thinking back on the years as a PhD student gets you so full of memories. My time as a PhD student have been full of challenges, most of them inspiring and some of them, well, were just challenges that you had to go through. Collectively I have learned so much more during these years than I could ever imagine. This is thanks to a lot of people both within the scientific world and outside. I would not have managed without your support!

The first person I want to thank is my supervisor Professor **Agneta Richter-Dahlfors**. Agneta have by her straight thru scientific enthusiasm inspired me tremendously. Thanks you for all the brainstorming scientific discussions, your support and guidance when I have needed it the most and for letting me try my wings within the field of organic bioelectronics.

Thanks to **Marget Wahlström** for being the best support, whatever the subject might have been.

Professor Magnus Berggren my mentor in organic electronics. Thanks for all the giving discussions, your never ending enthusiasm and "positivism" for our projects together.

Past and present members of Magnus group in Norrköping
Joakim Isaksson for being a nice friend, for all the discussions inside or outside the calcium room and of course for fruitful collaboration and a steady supply of ion pumps from the clean-room. **David Nilsson**, **Klas Tybrandt**, **Nathaniel Robinson** and **Daniel Simon** for teaching me everything about conductive polymers and organic electronics.

As a PhD student you tend to spend a most of the days 24hrs in the lab. I have been very lucky to be surrounded by so nice people that can wash away all problems with a laugh. Thank you!: **Lisa** for being such a good friend, being supportive when I needed it. **Camilla** for all the fun and discussions about everything. **Tomas** for the best of times, always. **Fredrik** that was my definition of how to become a successful scientist. **Anders**/Tomas/Fredrik for all the fun and drunken evenings (not what it sounds like!) I hope that I have carried the α-male legacy with distinction! Thank you **Anna-Karin** for learning me to streak bacteria in 2001. **Juan-Carlos** you was not only a very good friend, but also a crappy badminton player (Ha Ha!)
,**Kalle** for being so nice and helpful and being the new OBOE recruit. **Jorrit** for the team spirit and the foul cursing in the lab. I also wish to thank all the past and present members of G5 by letting it stay the best corridor at MTC! **Sindhu**, for all the new input to the project.

Micke and **Henrik** for being the best of friends, without you I wouldn't have manage all these years. That you also ended up at MTC was the best strike of faith ever. Not only at work but at all times have you been there, well you know.. cannot mention it all.

Thanks **Zambie**, **Bruno** and **Alex** of the SP crew for all the fun!

Thanks all the rest of my friends at MTC/SMI for the Pubs and all the fun at the network activities, there are too many to mention!

Thanks **It-department**, **Birgitta**, **Greger** and **Torbjörn** för ni alltid varit så hjälpsamma!

I have been especially lucky to be surrounded by scientist that have inspired me a lot: **Teresa Frisan** (Thanks for all the scientific discussions and help), **Stafan Arvidsson**, **Mikael Rhen**, **Staffan Normark, Katrin Pütsep, Mattias Hornef**, **Monica Thelestam**, **K Mikoshiba** and **Ola Hermansson** (and the rest of OBOE of course).

For collaboration: **Teresa Frisan**, **Johannes Ries**, **S** and **B Normark**, **K Mikoshiba** and **D Dalay** (well we tried the BN).

On the outside I wish to thank all my friends, no one mentioned no one forgotten for the fun times and with the hopes that we will see each other much more often now when this piece of work is done.

Tack till min bonusfamilj i Arvika för allt, till **Micke** och **Monica** och the **Dahlgren sisters**

Tack **Mamma** och **Pappa** för ert stöd i allt, kan inte beskriva det bättre än så, ni vet hur mycket ni betyder!!

Slutligen och viktigast av allt vill jag tacka **Anna**, för ditt stöd, för din kärlek och din tro på mig när ja haft min dagar. I mars får vi bebis och allt annat bleknar i styrkan för vad jag känner för det! Älskar dig!!

Thank you all!!

REFERENCES

Abrami, L., Liu, S., Cosson, P., Leppla, S.H. and van der Goot, F.G. (2003) Anthrax toxin triggers endocytosis of its receptor via a lipid raft-mediated clathrin-dependent process. *J Cell Biol*. **160**: 321-328.

Aderem, A. and Ulevitch, R.J. (2000) Toll-like receptors in the induction of the innate immune response. *Nature*. **406**: 782-787.

Agace, W., Hedges, S., Andersson, U., Andersson, J., Ceska, M. and Svanborg, C. (1993a) Selective cytokine production by epithelial cells following exposure to Escherichia coli. *Infect Immun*. **61**: 602-609.

Agace, W.W., Hedges, S.R., Ceska, M. and Svanborg, C. (1993b) Interleukin-8 and the neutrophil response to mucosal gram-negative infection. *J Clin Invest*. **92**: 780-785.

Aleshin, A., Kiebooms, R., Menon, R. and Heeger, A.J. (1997a) Electronic transport in doped poly(3,4-ethylenedioxythiophene) near the metal-insulator transition. *Synth Met*. **90**: 61-68.

Aleshin, A., Kiebooms, R., Menon, R., Wudl, F. and Heeger, A.J. (1997b) Metallic conductivity at low temperatures in poly(3,4-ethylenedioxythiophene) doped with PF6. *Physical Review B*. **56**: 3659-3663.

Anderson, K.V., Bokla, L. and Nusslein-Volhard, C. (1985) Establishment of dorsal-ventral polarity in the Drosophila embryo: the induction of polarity by the Toll gene product. *Cell*. **42**: 791-798.

Andersson, P., Nilsson, D., Svensson, P.-O., Chen, M., Malmstrom, A., Remonen, T*., et al* (2002) Active matrix displays based on all-organic electrochemical smart pixels printed on paper. *Advanced Materials*. **14**: 1460-1464.

Balsalobre, C., Silvan, J.M., Berglund, S., Mizunoe, Y., Uhlin, B.E. and Wai, S.N. (2006) Release of the type I secreted alpha-haemolysin via outer membrane vesicles from Escherichia coli. *Mol Microbiol*. **59**: 99-112.

Beg, A.A., Finco, T.S., Nantermet, P.V. and Baldwin, A.S., Jr. (1993) Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I kappa B alpha: a mechanism for NF-kappa B activation. *Mol Cell Biol*. **13**: 3301-3310.

Berggren, M., Nilsson, D. and Robinson, N.D. (2007) Organic materials for printed electronics. *Nature Materials*. **6**: 3-5.

Berridge, M.J., Bootman, M.D. and Lipp, P. (1998) Molecular biology: Calcium - a life and death signal. *Nature*. **395**: 645.

Berridge, M.J., Lipp, P. and Bootman, M.D. (2000a) The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol*. **1**: 11-21.

Berridge, M.J., Lipp, P. and Bootman, M.D. (2000b) Signal transduction. The calcium entry pas de deux. *Science*. **287**: 1604-1605.

Berridge, M.J., Bootman, M.D. and Roderick, H.L. (2003) Calcium signalling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol*. **4**: 517-529.

Bhakdi, S. and Tranum-Jensen, J. (1988) Damage to cell membranes by pore-forming bacterial cytolysins. *Prog Allergy*. **40**: 1-43.

Bhakdi, S., Mackman, N., Nicaud, J.M. and Holland, I.B. (1986) Escherichia coli hemolysin may damage target cell membranes by generating transmembrane pores. *Infect Immun*. **52**: 63-69.

Bishop, A.L. and Hall, A. (2000) Rho GTPases and their effector proteins. *Biochem J*. **348 Pt 2**: 241-255.

Bobacka, J., Lewenstam, A. and Ivaska, A. (2000) Electrochemical impedance spectroscopy of oxidized poly(3,4-ethylenedioxythiophene) film electrodes in aqueous solutions. *Journal of Electroanalytical Chemistry*. **489**: 17-27.

Boehm, D.F., Welch, R.A. and Snyder, I.S. (1990a) Domains of Escherichia coli hemolysin (HlyA) involved in binding of calcium and erythrocyte membranes. *Infect Immun*. **58**: 1959-1964.

Boehm, D.F., Welch, R.A. and Snyder, I.S. (1990b) Calcium is required for binding of Escherichia coli hemolysin (HlyA) to erythrocyte membranes. *Infect Immun*. **58**: 1951-1958.

Bohach, G.A. and Snyder, I.S. (1985) Chemical and immunological analysis of the complex structure of Escherichia coli alpha-hemolysin. *J Bacteriol*. **164**: 1071-1080.

Boitano, S., Dirksen, E.R. and Sanderson, M.J. (1992) Intercellular propagation of calcium waves mediated by inositol trisphosphate. *Science*. **258**: 292-295.

Bolotina, V.M. and Csutora, P. (2005) CIF and other mysteries of the store-operated Ca2+-entry pathway. *Trends Biochem Sci*. **30**: 378-387.

Bootman, M.D. and Berridge, M.J. (1995) The elemental principles of calcium signaling. *Cell*. **83**: 675-678.

Bootman, M.D., Lipp, P. and Berridge, M.J. (2001) The organisation and functions of local Ca(2+) signals. *J Cell Sci*. **114**: 2213-2222.

Boulay, G., Brown, D.M., Qin, N., Jiang, M., Dietrich, A., Zhu, M.X*., et al* (1999) Modulation of Ca(2+) entry by polypeptides of the inositol 1,4, 5-trisphosphate receptor (IP3R) that bind transient receptor potential (TRP): evidence for roles of TRP and IP3R in store depletion-activated Ca(2+) entry. *Proc Natl Acad Sci U S A*. **96**: 14955-14960.

Braun, J.S., Sublett, J.E., Freyer, D., Mitchell, T.J., Cleveland, J.L., Tuomanen, E.I. and Weber, J.R. (2002) Pneumococcal pneumolysin and H(2)O(2) mediate brain cell apoptosis during meningitis. *J Clin Invest*. **109**: 19-27.

Braun, M.A., Gerlach, D., Hartwig, U.F., Ozegowski, J.H., Romagne, F., Carrel, S*., et al* (1993) Stimulation of human T cells by streptococcal "superantigen" erythrogenic toxins (scarlet fever toxins). *J Immunol*. **150**: 2457-2466.

Brauner, A., Katouli, M., Tullus, K. and Jacobson, S.H. (1990) Production of cytotoxic necrotizing factor, verocytotoxin and haemolysin by pyelonephritogenic Escherichia coli. *Eur J Clin Microbiol Infect Dis*. **9**: 762-767.

Brauner, A., Soderhall, M., Jacobson, S.H., Lundahl, J., Andersson, U. and Andersson, J. (2001) Escherichia coli-induced expression of IL-1 alpha, IL-1 beta, IL-6 and IL-8 in normal human renal tubular epithelial cells. *Clin Exp Immunol*. **124**: 423-428.

Bretscher, A., Edwards, K. and Fehon, R.G. (2002) ERM proteins and merlin: integrators at the cell cortex. *Nat Rev Mol Cell Biol*. **3**: 586-599.

Bush, K.T., Stuart, R.O., Li, S.H., Moura, L.A., Sharp, A.H., Ross, C.A. and Nigam, S.K. (1994) Epithelial inositol 1,4,5-trisphosphate receptors. Multiplicity of localization, solubility, and isoforms. *J Biol Chem*. **269**: 23694-23699.

Camilli, A., Tilney, L.G. and Portnoy, D.A. (1993) Dual roles of plcA in Listeria monocytogenes pathogenesis. *Mol Microbiol*. **8**: 143-157.

Cortajarena, A.L., Goni, F.M. and Ostolaza, H. (2001) Glycophorin as a receptor for Escherichia coli alpha-hemolysin in erythrocytes. *J Biol Chem*. **276**: 12513-12519.

Cortajarena, A.L., Goni, F.M. and Ostolaza, H. (2003) A receptor-binding region in Escherichia coli alpha-haemolysin. *J Biol Chem*. **278**: 19159-19163.

Cossart, P., Pizarro-Cerda, J. and Lecuit, M. (2003) Invasion of mammalian cells by Listeria monocytogenes: functional mimicry to subvert cellular functions. *Trends Cell Biol*. **13**: 23-31.

Cossart, P., Boquet, P., Normark, S. and Rappuoli, R. (1996) Cellular microbiology emerging. *Science*. **271**: 315-316.

Crabtree, G.R. (1999) Generic signals and specific outcomes: signaling through Ca2+, calcineurin, and NF-AT. *Cell*. **96**: 611-614.

Cui, X. and Martin, D.C. (2003) Electrochemical deposition and characterization of poly(3,4-ethylenedioxythiophene) on neural microelectrode arrays. *Sens Actuators, B*. **89**: 92-102.

Czuprynski, C.J. and Welch, R.A. (1995) Biological effects of RTX toxins: the possible role of lipopolysaccharide. *Trends Microbiol*. **3**: 480-483.

De Koninck, P. and Schulman, H. (1998) Sensitivity of CaM kinase II to the frequency of Ca2+ oscillations. *Science*. **279**: 227-230.

del Castillo, F.J., Moreno, F. and del Castillo, I. (2000) Characterization of the genes encoding the SheA haemolysin in Escherichia coli O157:H7 and Shigella flexneri 2a. *Res Microbiol*. **151**: 229-230.

Divecha, N. and Irvine, R.F. (1995) Phospholipid signaling. *Cell*. **80**: 269-278.

Dodabalapur, A. (1997) Organic light emitting diodes. *Solid State Commun*. **102**: 259-267.

Dolmetsch, R. (2003) Excitation-transcription coupling: signaling by ion channels to the nucleus. *Sci STKE*. **2003**: PE4.

Dolmetsch, R.E., Xu, K. and Lewis, R.S. (1998) Calcium oscillations increase the efficiency and specificity of gene expression. *Nature*. **392**: 933-936.

Dolmetsch, R.E., Lewis, R.S., Goodnow, C.C. and Healy, J.I. (1997) Differential activation of transcription factors induced by Ca2+ response amplitude and duration. *Nature*. **386**: 855-858.

Dolmetsch, R.E., Pajvani, U., Fife, K., Spotts, J.M. and Greenberg, M.E. (2001) Signaling to the nucleus by an L-type calcium channel-calmodulin complex through the MAP kinase pathway. *Science*. **294**: 333-339.

Dowling, K.J., Roberts, J.A. and Kaack, M.B. (1987) P-fimbriated Escherichia coli urinary tract infection: a clinical correlation. *South Med J*. **80**: 1533-1536.

Dramsi, S. and Cossart, P. (2003) Listeriolysin O-mediated calcium influx potentiates entry of Listeria monocytogenes into the human Hep-2 epithelial cell line. *Infect Immun*. **71**: 3614-3618.

Drury, C.J., Mutsaers, C.M.J., Hart, C.M., Matters, M. and de Leeuw, D.M. (1998) Low-cost all-polymer integrated circuits. *Appl Phys Lett*. **73**: 108-110.

Eckert, R. and Ewald, D. (1982) Residual calcium ions depress activation of calcium-dependent current. *Science*. **216**: 730-733.

Felmlee, T., Pellett, S. and Welch, R.A. (1985) Nucleotide sequence of an Escherichia coli chromosomal hemolysin. *J Bacteriol*. **163**: 94-105.

Ferry, S., Burman, L.G. and Holm, S.E. (1988) Clinical and bacteriological effects of therapy of urinary tract infection in primary health care: relation to in vitro sensitivity testing. *Scand J Infect Dis*. **20**: 535-544.

Feske, S., Giltnane, J., Dolmetsch, R., Staudt, L.M. and Rao, A. (2001) Gene regulation mediated by calcium signals in T lymphocytes. *Nat Immunol*. **2**: 316-324.

Fievet, B.T., Gautreau, A., Roy, C., Del Maestro, L., Mangeat, P., Louvard, D. and Arpin, M. (2004) Phosphoinositide binding and phosphorylation act sequentially in the activation mechanism of ezrin. *J Cell Biol*. **164**: 653-659.

Finer, G. and Landau, D. (2004) Pathogenesis of urinary tract infections with normal female anatomy. *Lancet Infect Dis*. **4**: 631-635.

Flemming, R., Xu, S.Z. and Beech, D.J. (2003) Pharmacological profile of store-operated channels in cerebral arteriolar smooth muscle cells. *Br J Pharmacol*. **139**: 955-965.

Foxman, B. (2003) Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. *Dis Mon*. **49**: 53-70.

Frendeus, B., Godaly, G., Hang, L., Karpman, D., Lundstedt, A.C. and Svanborg, C. (2000) Interleukin 8 receptor deficiency confers susceptibility to acute experimental pyelonephritis and may have a human counterpart. *J Exp Med*. **192**: 881-890.

Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N. and Mikoshiba, K. (1989) Primary structure and functional expression of the inositol 1,4,5-trisphosphate-binding protein P400. *Nature*. **342**: 32-38.

Gekara, N.O., Jacobs, T., Chakraborty, T. and Weiss, S. (2005) The cholesterol-dependent cytolysin listeriolysin O aggregates rafts via oligomerization. *Cell Microbiol*. **7**: 1345-1356.

George, P.M., Lyckman, A.W., LaVan, D.A., Hegde, A., Leung, Y., Avasare, R*., et al* (2005) Fabrication and biocompatibility of polypyrrole implants suitable for neural prosthetics. *Biomaterials*. **26**: 3511-3519.

Godaly, G., Hang, L., Frendeus, B. and Svanborg, C. (2000a) Transepithelial neutrophil migration is CXCR1 dependent in vitro and is defective in IL-8 receptor knockout mice. *J Immunol*. **165**: 5287-5294.

Godaly, G., Bergsten, G., Frendeus, B., Hang, L., Hedlund, M., Karpman, D., *et al* (2000b) Innate defences and resistance to gram negative mucosal infection. *Adv Exp Med Biol*. **485**: 9-24.

Greczynski, G., Kugler, T., Keil, M., Osikowicz, W., Fahlman, M. and Salaneck, W.R. (2001) Photoelectron spectroscopy of thin films of PEDOT-PSS conjugated polymer blend: a mini-review and some new results. *J Electron Spectrosc Relat Phenom*. **121**: 1-17.

Groenendaal, B.L., Jonas, F., Freitag, D., Pielartzik, H. and Reynolds, J.R. (2000) Poly(3,4-ethylenedioxythiophene) and its derivatives: Past, present, and future. *Advanced Materials*. **12**: 481-494.

Gustafsson, T., Thomas, J.O., Koksbang, R. and Farrington, G.C. (1992) The Polymer Battery as an Environment for Insitu X-Ray-Diffraction Studies of Solid-State Electrochemical Processes. *Electrochimica Acta*. **37**: 1639-1643.

Hacker, J., Hughes, C., Hof, H. and Goebel, W. (1983) Cloned hemolysin genes from Escherichia coli that cause urinary tract infection determine different levels of toxicity in mice. *Infect Immun*. **42**: 57-63.

Hagberg, L., Hull, R., Hull, S., Falkow, S., Freter, R. and Svanborg Eden, C. (1983) Contribution of adhesion to bacterial persistence in the mouse urinary tract. *Infect Immun*. **40**: 265-272.

Hamada, K., Terauchi, A. and Mikoshiba, K. (2003) Three-dimensional rearrangements within inositol 1,4,5-trisphosphate receptor by calcium. *J Biol Chem*. **278**: 52881-52889.

Hattori, M., Suzuki, A.Z., Higo, T., Miyauchi, H., Michikawa, T., Nakamura, T., *et al* (2004) Distinct roles of inositol 1,4,5-trisphosphate receptor types 1 and 3 in Ca2+ signaling. *J Biol Chem*. **279**: 11967-11975.

Hedges, S., Svensson, M., Agace, W. and Svanborg, C. (1995) Cytokines induce an epithelial cell cytokine response. *Adv Exp Med Biol*. **371A**: 189-193.

Hedlund, M., Wachtler, C., Johansson, E., Hang, L., Somerville, J.E., Darveau, R.P. and Svanborg, C. (1999) P fimbriae-dependent, lipopolysaccharide-independent activation of epithelial cytokine responses. *Mol Microbiol*. **33**: 693-703.

Herlax, V. and Bakas, L. (2007) Fatty acids covalently bound to alpha-hemolysin of Escherichia coli are involved in the molten globule conformation: implication of disordered regions in binding promiscuity. *Biochemistry*. **46**: 5177-5184.

Hisatsune, C. and Mikoshiba, K. (2005) Novel compartment implicated in calcium signaling--is it an "induced coupling domain"? *Sci STKE*. **2005**: pe53.

Hooton, T.M., Scholes, D., Hughes, J.P., Winter, C., Roberts, P.L., Stapleton, A.E., *et al* (1996) A prospective study of risk factors for symptomatic urinary tract infection in young women. *N Engl J Med*. **335**: 468-474.

Hughes, C., Hacker, J., Roberts, A. and Goebel, W. (1983) Hemolysin production as a virulence marker in symptomatic and asymptomatic urinary tract infections caused by Escherichia coli. *Infect Immun*. **39**: 546-551.

Isaksson, J., Tengstedt, C., Fahlman, M., Robinson, N. and Berggren, M. (2004) A Solid-state Organic Electronic Wettability Switch. *Advanced Materials*. **16**: 316-320.

Issartel, J.P., Koronakis, V. and Hughes, C. (1991) Activation of Escherichia coli prohaemolysin to the mature toxin by acyl carrier protein-dependent fatty acylation. *Nature*. **351**: 759-761.

Iwasaki, H., Mori, Y., Hara, Y., Uchida, K., Zhou, H. and Mikoshiba, K. (2001) 2-Aminoethoxydiphenyl borate (2-APB) inhibits capacitative calcium entry independently of the function of inositol 1,4,5-trisphosphate receptors. *Receptors Channels*. **7**: 429-439.

Jager, E.W.H., Smela, E. and Inganas, O. (2000) Microfabricating conjugated polymer actuators. *Science*. **290**: 1540-1545.

Jann, K. and Jann, B. (1987) Polysaccharide antigens of Escherichia coli. *Rev Infect Dis*. **9 Suppl 5**: S517-526.

Jantunen, M.E., Saxen, H., Lukinmaa, S., Ala-Houhala, M. and Siitonen, A. (2001) Genomic identity of pyelonephritogenic Escherichia coli isolated from blood, urine and faeces of children with urosepsis. *J Med Microbiol*. **50**: 650-652.

Kawai, T. and Akira, S. (2006) TLR signaling. *Cell Death Differ*. **13**: 816-825.

Kenny, B., DeVinney, R., Stein, M., Reinscheid, D.J., Frey, E.A. and Finlay, B.B. (1997) Enteropathogenic E. coli (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell*. **91**: 511-520.

Koronakis, V. (2003) TolC--the bacterial exit duct for proteins and drugs. *FEBS Lett*. **555**: 66-71.

Koschinski, A., Repp, H., Unver, B., Dreyer, F., Brockmeier, D., Valeva, A.*, et al* (2006) Why Escherichia coli alpha-hemolysin induces calcium oscillations in mammalian cells--the pore is on its own. *Faseb J*. **20**: 973-975.

Laestadius, A., Richter-Dahlfors, A. and Aperia, A. (2002) Dual effects of Escherichia coli alpha-hemolysin on rat renal proximal tubule cells. *Kidney Int*. **62**: 2035-2042.

Lai, F.A., Erickson, H.P., Rousseau, E., Liu, Q.Y. and Meissner, G. (1988) Purification and reconstitution of the calcium release channel from skeletal muscle. *Nature*. **331**: 315-319.

Lally, E.T., Kieba, I.R., Sato, A., Green, C.L., Rosenbloom, J., Korostoff, J.*, et al* (1997) RTX toxins recognize a beta2 integrin on the surface of human target cells. *J Biol Chem*. **272**: 30463-30469.

Lisowska-Oleksiak, A. and Kupniewska, A. (2003) Transport of alkali metal cations in poly(3,4-ethylenethiophene) films. *Solid State Ionics*. **157**: 241-248.

Ludwig, A., Vogel, M. and Goebel, W. (1987) Mutations affecting activity and transport of haemolysin in Escherichia coli. *Mol Gen Genet*. **206**: 238-245.

Ludwig, A., Jarchau, T., Benz, R. and Goebel, W. (1988) The repeat domain of Escherichia coli haemolysin (HlyA) is responsible for its Ca2+-dependent binding to erythrocytes. *Mol Gen Genet*. **214**: 553-561.

Ludwig, A., Schmid, A., Benz, R. and Goebel, W. (1991) Mutations affecting pore formation by haemolysin from Escherichia coli. *Mol Gen Genet*. **226**: 198-208.

Ludwig, A., Garcia, F., Bauer, S., Jarchau, T., Benz, R., Hoppe, J. and Goebel, W. (1996) Analysis of the in vivo activation of hemolysin (HlyA) from Escherichia coli. *J Bacteriol*. **178**: 5422-5430.

Lund, B., Lindberg, F., Marklund, B.I. and Normark, S. (1987) The PapG protein is the alpha-D-galactopyranosyl-(1----4)-beta-D-galactopyranose-binding adhesin of uropathogenic Escherichia coli. *Proc Natl Acad Sci U S A*. **84**: 5898-5902.

Lundstedt, A.C., Leijonhufvud, I., Ragnarsdottir, B., Karpman, D., Andersson, B. and Svanborg, C. (2007a) Inherited susceptibility to acute pyelonephritis: a family study of urinary tract infection. *J Infect Dis*. **195**: 1227-1234.

Lundstedt, A.C., McCarthy, S., Gustafsson, M.C., Godaly, G., Jodal, U., Karpman, D.*, et al* (2007b) A genetic basis of susceptibility to acute pyelonephritis. *PLoS ONE*. **2**: e825.

Mabeck, J. and Malliaras, G. (2006) Chemical and biological sensors based on organic thin-film transistors. *Analytical and Bioanalytical Chemistry*. **384**: 343-353.

Maeda, N., Niinobe, M. and Mikoshiba, K. (1990) A cerebellar Purkinje cell marker P400 protein is an inositol 1,4,5-trisphosphate (InsP3) receptor protein. Purification and characterization of InsP3 receptor complex. *Embo J*. **9**: 61-67.

Mansson, L.E., Melican, K., Boekel, J., Sandoval, R.M., Hautefort, I., Tanner, G.A.*, et al* (2007) Real-time studies of the progression of bacterial infections and immediate tissue responses in live animals. *Cell Microbiol*. **9**: 413-424.

Matsui, T., Maeda, M., Doi, Y., Yonemura, S., Amano, M., Kaibuchi, K.*, et al* (1998) Rho-kinase phosphorylates COOH-terminal threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to-tail association. *J Cell Biol*. **140**: 647-657.

Matters, M., de Leeuw, D.M., Vissenberg, M.J.C.M., Hart, C.M., Herwig, P.T., Geuns, T.*, et al* (1999) Organic field-effect transistors and all-polymer integrated circuits. *Optical Materials*. **12**: 189-197.

Medzhitov, R. and Janeway, C.A., Jr. (1997) Innate immunity: the virtues of a nonclonal system of recognition. *Cell*. **91**: 295-298.

Medzhitov, R., Preston-Hurlburt, P. and Janeway, C.A., Jr. (1997) A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature*. **388**: 394-397.

Menard, R., Sansonetti, P. and Parsot, C. (1994) The secretion of the Shigella flexneri Ipa invasins is activated by epithelial cells and controlled by IpaB and IpaD. *Embo J*. **13**: 5293-5302.

Menestrina, G., Schiavo, G. and Montecucco, C. (1994a) Molecular mechanisms of action of bacterial protein toxins. *Mol Aspects Med*. **15**: 79-193.

Menestrina, G., Moser, C., Pellet, S. and Welch, R. (1994b) Pore-formation by Escherichia coli hemolysin (HlyA) and other members of the RTX toxins family. *Toxicology*. **87**: 249-267.

Miller, R.J. (1987) Multiple calcium channels and neuronal function. *Science*. **235**: 46-52.

Mims, C.A. (2004) *Medical microbiology*. Edinburgh ; New York: Mosby.

Mitchell, T.J., Andrew, P.W., Saunders, F.K., Smith, A.N. and Boulnois, G.J. (1991) Complement activation and antibody binding by pneumolysin via a region of the toxin homologous to a human acute-phase protein. *Mol Microbiol*. **5**: 1883-1888.

Miyakawa, T., Mizushima, A., Hirose, K., Yamazawa, T., Bezprozvanny, I., Kurosaki, T. and Iino, M. (2001) Ca(2+)-sensor region of IP(3) receptor controls intracellular Ca(2+) signaling. *Embo J*. **20**: 1674-1680.

Miyazaki, J., Ba-Thein, W., Kumao, T., Akaza, H. and Hayashi, H. (2002) Identification of a type III secretion system in uropathogenic Escherichia coli. *FEMS Microbiol Lett*. **212**: 221-228.

Moayeri, M. and Welch, R.A. (1997) Prelytic and lytic conformations of erythrocyte-associated Escherichia coli hemolysin. *Infect Immun*. **65**: 2233-2239.

Mobley, H.L., Green, D.M., Trifillis, A.L., Johnson, D.E., Chippendale, G.R., Lockatell, C.V., *et al* (1990) Pyelonephritogenic Escherichia coli and killing of cultured human renal proximal tubular epithelial cells: role of hemolysin in some strains. *Infect Immun*. **58**: 1281-1289.

Monkawa, T., Hayashi, M., Miyawaki, A., Sugiyama, T., Yamamoto-Hino, M., Hasegawa, M., *et al* (1998) Localization of inositol 1,4,5-trisphosphate receptors in the rat kidney. *Kidney Int*. **53**: 296-301.

Montero, M., Alonso, M.T., Carnicero, E., Cuchillo-Ibanez, I., Albillos, A., Garcia, A.G., *et al* (2000) Chromaffin-cell stimulation triggers fast millimolar mitochondrial Ca2+ transients that modulate secretion. *Nat Cell Biol*. **2**: 57-61.

Mulvey, M.A., Lopez-Boado, Y.S., Wilson, C.L., Roth, R., Parks, W.C., Heuser, J. and Hultgren, S.J. (1998) Induction and evasion of host defenses by type 1-piliated uropathogenic Escherichia coli. *Science*. **282**: 1494-1497.

Murata, K., Izuchi, S. and Yoshihisa, Y. (2000) An overview of the research and development of solid polymer electrolyte batteries. *Electrochim Acta*. **45**: 1501-1508.

Murphy, T.H., Blatter, L.A., Wier, W.G. and Baraban, J.M. (1992) Spontaneous synchronous synaptic calcium transients in cultured cortical neurons. *J Neurosci*. **12**: 4834-4845.

Nilsson, D., Kugler, T., Svensson, P.-O. and Berggren, M. (2002a) An all-organic sensor-transistor based on a novel electrochemical transducer concept printed electrochemical sensors on paper. *Sens Actuators, B*. **86**: 193-197.

Nilsson, D., Chen, M., Kugler, T., Remonen, T., Armgarth, M. and Berggren, M. (2002b) Bi-stable and dynamic current modulation in electrochemical organic transistors. *Advanced Materials*. **14**: 51-54.

Nyberg, T., Inganäs, O. and Jerregård, H. (2002) Polymer hydrogel microelectrodes for neural communication. *Biomedical Microdevices*. **4**: 43-52.

Oancea, E. and Meyer, T. (1998) Protein kinase C as a molecular machine for decoding calcium and diacylglycerol signals. *Cell*. **95**: 307-318.

Oropeza-Wekerle, R.L., Muller, E., Kern, P., Meyermann, R. and Goebel, W. (1989) Synthesis, inactivation, and localization of extracellular and intracellular Escherichia coli hemolysins. *J Bacteriol*. **171**: 2783-2788.

Oscarsson, J., Westermark, M., Beutin, L. and Uhlin, B.E. (2002) The bacteriophage-associated ehly1 and ehly2 determinants from Escherichia coli O26:H- strains do not encode enterohemolysins per se but cause release of the ClyA cytolysin. *Int J Med Microbiol*. **291**: 625-631.

Palazzo, A.F., Eng, C.H., Schlaepfer, D.D., Marcantonio, E.E. and Gundersen, G.G. (2004) Localized stabilization of microtubules by integrin- and FAK-facilitated Rho signaling. *Science*. **303**: 836-839.

Peyrl, A., Krapfenbauer, K., Slavc, I., Strobel, T. and Lubec, G. (2003) Proteomic characterization of the human cortical neuronal cell line HCN-2. *J Chem Neuroanat*. **26**: 171-178.

Pluschke, G., Mayden, J., Achtman, M. and Levine, R.P. (1983) Role of the capsule and the O antigen in resistance of O18:K1 Escherichia coli to complement-mediated killing. *Infect Immun*. **42**: 907-913.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., *et al* (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science*. **282**: 2085-2088.

Repp, H., Pamukci, Z., Koschinski, A., Domann, E., Darji, A., Birringer, J., *et al* (2002) Listeriolysin of Listeria monocytogenes forms Ca2+-permeable pores leading to intracellular Ca2+ oscillations. *Cell Microbiol*. **4**: 483-491.

Roberts, J.A., Marklund, B.I., Ilver, D., Haslam, D., Kaack, M.B., Baskin, G., *et al* (1994) The Gal(alpha 1-4)Gal-specific tip adhesin of Escherichia coli P-fimbriae is needed for pyelonephritis to occur in the normal urinary tract. *Proc Natl Acad Sci U S A*. **91**: 11889-11893.

Ronnett, G.V., Hester, L.D., Nye, J.S. and Snyder, S.H. (1994) Human cerebral cortical cell lines from patients with unilateral megalencephaly and Rasmussen's encephalitis. *Neuroscience*. **63**: 1081-1099.

Rossjohn, J., Gilbert, R.J., Crane, D., Morgan, P.J., Mitchell, T.J., Rowe, A.J., *et al* (1998) The molecular mechanism of pneumolysin, a virulence factor from Streptococcus pneumoniae. *J Mol Biol*. **284**: 449-461.

Saci, A. and Carpenter, C.L. (2005) RhoA GTPase regulates B cell receptor signaling. *Mol Cell*. **17**: 205-214.

Samuelsson, P., Hang, L., Wullt, B., Irjala, H. and Svanborg, C. (2004) Toll-like receptor 4 expression and cytokine responses in the human urinary tract mucosa. *Infect Immun*. **72**: 3179-3186.

Sandberg, T., Kaijser, B., Lidin-Janson, G., Lincoln, K., Orskov, F., Orskov, I., *et al* (1988) Virulence of Escherichia coli in relation to host factors in women with symptomatic urinary tract infection. *J Clin Microbiol*. **26**: 1471-1476.

Sato, C., Hamada, K., Ogura, T., Miyazawa, A., Iwasaki, K., Hiroaki, Y., *et al* (2004) Inositol 1,4,5-trisphosphate receptor contains multiple cavities and L-shaped ligand-binding domains. *J Mol Biol*. **336**: 155-164.

Schindel, C., Zitzer, A., Schulte, B., Gerhards, A., Stanley, P., Hughes, C., *et al* (2001) Interaction of Escherichia coli hemolysin with biological membranes. A study using cysteine scanning mutagenesis. *Eur J Biochem*. **268**: 800-808.

Simons, K. and Toomre, D. (2000) Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol*. **1**: 31-39.

Somani, P.R. and Radhakrishnan, S. (2003) Electrochromic materials and devices: present and future. *Mater Chem Phys*. **77**: 117-133.

Speck, O., Hughes, S.C., Noren, N.K., Kulikauskas, R.M. and Fehon, R.G. (2003) Moesin functions antagonistically to the Rho pathway to maintain epithelial integrity. *Nature*. **421**: 83-87.

Stanley, P., Koronakis, V. and Hughes, C. (1998) Acylation of Escherichia coli hemolysin: a unique protein lipidation mechanism underlying toxin function. *Microbiol Mol Biol Rev*. **62**: 309-333.

Stanley, P., Packman, L.C., Koronakis, V. and Hughes, C. (1994) Fatty acylation of two internal lysine residues required for the toxic activity of Escherichia coli hemolysin. *Science*. **266**: 1992-1996.

Stringaris, A.K., Geisenhainer, J., Bergmann, F., Balshusemann, C., Lee, U., Zysk, G., *et al* (2002) Neurotoxicity of pneumolysin, a major pneumococcal virulence factor, involves calcium influx and depends on activation of p38 mitogen-activated protein kinase. *Neurobiol Dis*. **11**: 355-368.

Suntharalingam, G., Perry, M.R., Ward, S., Brett, S.J., Castello-Cortes, A., Brunner, M.D. and Panoskaltsis, N. (2006) Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. *N Engl J Med*. **355**: 1018-1028.

Svanborg, C., Frendeus, B., Godaly, G., Hang, L., Hedlund, M. and Wachtler, C. (2001a) Toll-like receptor signaling and chemokine receptor expression influence the severity of urinary tract infection. *J Infect Dis*. **183 Suppl 1**: S61-65.

Svanborg, C., Bergsten, G., Fischer, H., Frendeus, B., Godaly, G., Gustafsson, E*., et al* (2001b) The 'innate' host response protects and damages the infected urinary tract. *Ann Med*. **33**: 563-570.

Takeuchi, K., Kawashima, A., Nagafuchi, A. and Tsukita, S. (1994a) Structural diversity of band 4.1 superfamily members. *J Cell Sci*. **107 (Pt 7)**: 1921-1928.

Takeuchi, K., Sato, N., Kasahara, H., Funayama, N., Nagafuchi, A., Yonemura, S*., et al* (1994b) Perturbation of cell adhesion and microvilli formation by antisense oligonucleotides to ERM family members. *J Cell Biol*. **125**: 1371-1384.

Taylor, C.W. and Laude, A.J. (2002) IP3 receptors and their regulation by calmodulin and cytosolic Ca2+. *Cell Calcium*. **32**: 321-334.

Tovey, S.C., de Smet, P., Lipp, P., Thomas, D., Young, K.W., Missiaen, L*., et al* (2001) Calcium puffs are generic InsP(3)-activated elementary calcium signals and are downregulated by prolonged hormonal stimulation to inhibit cellular calcium responses. *J Cell Sci*. **114**: 3979-3989.

Tracey, K.J., Beutler, B., Lowry, S.F., Merryweather, J., Wolpe, S., Milsark, I.W*., et al* (1986) Shock and tissue injury induced by recombinant human cachectin. *Science*. **234**: 470-474.

Triantafilou, M. and Triantafilou, K. (2002) Lipopolysaccharide recognition: CD14, TLRs and the LPS-activation cluster. *Trends Immunol*. **23**: 301-304.

Triantafilou, M., Miyake, K., Golenbock, D.T. and Triantafilou, K. (2002) Mediators of innate immune recognition of bacteria concentrate in lipid rafts and facilitate lipopolysaccharide-induced cell activation. *J Cell Sci*. **115**: 2603-2611.

Uhlén, P., Laestadlus, Å., Jahnukalnen, T., Söderblom, T., Bäckhed, F., Celsi, G*., et al* (2000) a-Haemolysin of uropathogenic E. coli induces Ca^{2+} oscillations in renal epithelial cells. *Nature*. **405**: 694-697.

Wadsworth, S.J. and Goldfine, H. (1999) Listeria monocytogenes phospholipase C-dependent calcium signaling modulates bacterial entry into J774 macrophage-like cells. *Infect Immun*. **67**: 1770-1778.

Valeva, A., Walev, I., Kemmer, H., Weis, S., Siegel, I., Boukhallouk, F*., et al* (2005) Binding of Escherichia coli hemolysin and activation of the target cells is not receptor-dependent. *J Biol Chem*. **280**: 36657-36663.

Wang, X., Shapiro, B. and Smela, E. (2004a) Visualizing ion currents in conjugated polymers. *Advanced Materials*. **16**: 1605-1609.

Wang, X., Gu, X., Yuan, C., Chen, S., Zhang, P., Zhang, T*., et al* (2004b) Evaluation of biocompatibility of polypyrrole in vitro and in vivo. *Journal of Biomedical Materials Research - Part A*. **68**: 411-422.

Wang, Y., Mattson, M.P. and Furukawa, K. (2002) Endoplasmic reticulum calcium release is modulated by actin polymerization. *J Neurochem*. **82**: 945-952.

Welch, R.A. (2001) RTX toxin structure and function: a story of numerous anomalies and few analogies in toxin biology. *Curr Top Microbiol Immunol*. **257**: 85-111.

Welch, R.A., Dellinger, E.P., Minshew, B. and Falkow, S. (1981) Haemolysin contributes to virulence of extra-intestinal E. coli infections. *Nature*. **294**: 665-667.

Welch, R.A., Burland, V., Plunkett, G., 3rd, Redford, P., Roesch, P., Rasko, D*., et al* (2002) Extensive mosaic structure revealed by the complete genome sequence of uropathogenic Escherichia coli. *Proc Natl Acad Sci U S A*. **99**: 17020-17024.

West, A.E., Chen, W.G., Dalva, M.B., Dolmetsch, R.E., Kornhauser, J.M., Shaywitz, A.J*., et al* (2001) Calcium regulation of neuronal gene expression. *Proc Natl Acad Sci U S A*. **98**: 11024-11031.

West, A.R. and Galloway, M.P. (1998) Nitric oxide and potassium chloride-facilitated striatal dopamine efflux in vivo: role of calcium-dependent release mechanisms. *Neurochem Int*. **33**: 493-501.

West, A.R., Galloway, M.P. and Grace, A.A. (2002) Regulation of striatal dopamine neurotransmission by nitric oxide: effector pathways and signaling mechanisms. *Synapse*. **44**: 227-245.

Vogel, M., Hess, J., Then, I., Juarez, A. and Goebel, W. (1988) Characterization of a sequence (hlyR) which enhances synthesis and secretion of hemolysin in Escherichia coli. *Mol Gen Genet*. **212**: 76-84.

Wyckoff, T.J., Raetz, C.R. and Jackman, J.E. (1998) Antibacterial and anti-inflammatory agents that target endotoxin. *Trends Microbiol*. **6**: 154-159.

Yamada, N., Makino, Y., Clark, R.A., Pearson, D.W., Mattei, M.G., Guenet, J.L*., et al* (1994) Human inositol 1,4,5-trisphosphate type-1 receptor, InsP3R1: structure, function, regulation of expression and chromosomal localization. *Biochem J*. **302 (Pt 3)**: 781-790.

Yamamoto-Hino, M., Sugiyama, T., Hikichi, K., Mattei, M.G., Hasegawa, K., Sekine, S*., et al* (1994) Cloning and characterization of human type 2 and type 3 inositol 1,4,5-trisphosphate receptors. *Receptors Channels*. **2**: 9-22.

Yamamoto, S., Tsukamoto, T., Terai, A., Kurazono, H., Takeda, Y. and Yoshida, O. (1997) Genetic evidence supporting the fecal-perineal-urethral hypothesis in cystitis caused by Escherichia coli. *J Urol*. **157**: 1127-1129.

Yu, A.S., Hebert, S.C., Brenner, B.M. and Lytton, J. (1992) Molecular characterization and nephron distribution of a family of transcripts encoding the pore-forming subunit of Ca2+ channels in the kidney. *Proc Natl Acad Sci U S A*. **89**: 10494-10498.

Zhao, P.L., Wang, X.T., Zhang, X.M., Cebotaru, V., Cebotaru, L., Guo, G*., et al* (2002) Tubular and cellular localization of the cardiac L-type calcium channel in rat kidney. *Kidney Int*. **61**: 1393-1406.

Zhou, G., Mo, W.J., Sebbel, P., Min, G., Neubert, T.A., Glockshuber, R*., et al* (2001) Uroplakin Ia is the urothelial receptor for uropathogenic Escherichia coli: evidence from in vitro FimH binding. *J Cell Sci*. **114**: 4095-4103.

Zotti, G., Zecchin, S., Schiavon, G., Louwet, F., Groenendaal, L., Crispin, X*., et al* (2003) Electrochemical and XPS studies toward the role of monomeric and polymeric sulfonate counterions in the synthesis, composition, and properties of poly(3,4-ethylenedioxythiophene). *Macromolecules*. **36**: 3337-3344.

Zuhlke, R.D., Pitt, G.S., Deisseroth, K., Tsien, R.W. and Reuter, H. (1999) Calmodulin supports both inactivation and facilitation of L-type calcium channels. *Nature*. **399**: 159-162.