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Binding-, Blocking- and Translocation-Processes Concerning Anthrax-Toxin and Related Bacterial Protein-Toxins of the AB_7-Family

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"Probleme kann man niemals mit der selben Denkweise lösen, mit der sie entstanden sind. [Deshalb ist] Phantasie [...] wichtiger als Wissen, denn Wissen ist begrenzt."

frei nach Albert Einstein

Publications included in this thesis

Christoph Beitzinger, Monica Rolando, Angelika Kronhardt, Caroline Stefani**,** Gilles Flatau, Emmanuel Lemichez, and Roland Benz, (2011) **Anthrax toxin protective antigen promotes uptake of N-terminal His_6-tag labeled polypeptides into cells in a voltage-dependent way**
(*Manuscript* submitted to *PLoS One*)

Christoph Beitzinger, Angelika Kronhardt, and Roland Benz, (2011) **Binding partners of protective antigen from *Bacillus anthracis* share certain common motives**
(*E-book chapter* published in *Toxins and Ion transfers*)

Angelika Kronhardt, Monica Rolando, Christoph Beitzinger, Caroline Stefani, Michael Leuber, Gilles Flatau, Michael R. Popoff, Roland Benz, and Emmanuel Lemichez, (2011) **Cross-reactivity of anthrax and C2 toxin: protective antigen promotes the uptake of botulinum C2I toxin into human endothelial cells**
(*Paper* published in *PLoS One*)

Christoph Beitzinger, Angelika Kronhardt, Kerstin Duscha, Holger Barth, György Hajos, and Roland Benz, (2012) **Interaction of Chloroquine-like 4-Aminoquinolines with anthrax toxin's Protective Antigen (PA) *in vitro* and *in vivo***
(*Manuscript* in preparation)

Christoph Beitzinger, Kerstin Duscha, and Roland Benz, (2012) **Anti-His antibody is able to block PA-pores in an ion-dependent manner**
(*Manuscript* in preparation)

All publications included in this thesis were used in agreement with the respecting journal and all the participating authors.

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Preface

SUMMARY

Bacterial protein toxins belong to the most potent toxins which are known. They exist in many different forms and are part of our every day live. Some of them are spread by the bacteria during infections and therefore play a crucial role in pathogenicity of these strains. Others are secreted as a defense mechanism and could be uptaken with spoiled food. Concerning toxicity, some of the binary toxins of the AB_7-type belong to the most potent and dangerous toxins in the world. Even very small amounts of these proteins are able to cause severe symptoms during an infection with pathogen species of the genus *Clostridium* or *Bacillus*. *Clostridium botulinum* for example inherits a whole arsenal of different toxins, ranging from the botulinum toxin, which was feared for causing food poisoning and is now commercially used as a neurotoxin called Botox, to binary toxins like C2-toxin.

Lately, these pathogens are not only known for nosocomial infections, which still exist in areas with low hygienic standards or bad medical care systems, but also for being used as tools for bioterrorism or biological warfare. Since the terroristic assaults of September 11th in the USA, the awareness is back to another binary toxin: The Anthrax toxin. It is produced by *Bacillus anthracis* a soil living bacteria, which is able to form lasting spores. These spores where spread by letters containing "white powder". After breathing in some of the spores the pathogen infects the lungs. Depending on the point of entrance, further organs for an infection could be the gut or the skin, where severe necrosis is one of the visible symptoms. This infection, also known as splenic fever or anthrax, is very dangerous and leads to death with a high probability if not treated properly with antibiotics on time.

Apart from the thread these toxins constitute, they exhibit a unique way of intoxication. Members of the AB_7-toxin family consist of a pore-forming subunit B, that acts as a molecular syringe to translocate the enzymatic moieties A into the cytosol of target cells. This complex mechanism does not only kill cells with high efficiency and therefore should be studied for treatment, but also displays a possibility to address certain cells with a specific protein cargo if used as a molecular delivery tool. Concerning both issues, binding and translocation of the channel are the crucial steps to either block or modify the system in the desired way.

To gain deeper insight into the transport of binary toxins the structure of the B subunit is of great importance, but being a membrane protein, no crystal could be obtained up to now for

either protective antigen (PA) of Anthrax toxin or any other AB_7-type binding domain. Therefore, the method of choice in this work is an electro-physical approach using the so-called black-lipid-bilayer system for determination of biophysical constants. Additionally, diverse cell-based assays serve as a proving method for the data gained during *in vitro* measurements. Further information was gathered with specially designed mutants of the protein channel.

The first part of this thesis focuses on the translocation process and its possible use as a molecular tool to deliver protein cargo into special cell types. The task was addressed by measuring the binding of different effector proteins related and unrelated to the AB_7-toxin family. These proteins were tested in titration experiments for the blockage of the ion current through a membrane saturated with toxin channels. Especially the influence of positively charged His-tags has been determined in detail for PA and C2II. As described in chapter 2, a **His-tag transferred the ability of being transported by PA**, but not by C2II, to different proteins like EDIN (from *S. aureus*) *in vitro* and in cell-based experiments. This process was found to change the well-known voltage-dependency of PA to a huge extend and therefore is related to membrane potentials which play a crucial role in many processes in living cells.

Chapter 3 sums up findings, which depict that **binding partners of PA share certain common motives**. These could be detected in a broad range of substrates, ranging from simple ions in an electrolyte over small molecules to complex protein effectors. The gathered information could be further used to design blocker-substrates for treatment of Anthrax infections or tags, which render PA possible as a molecular syringe for cargo proteins.

The deeper insight to homologies and differences of binary toxin components is the core of chapter 4, in which the **cross-reactivity of Anthrax and C2-toxin** was analyzed. The presented results lead to a better understanding of different motives involved in binding and translocation to and via the B components PA and C2II, as well as the enzymatically active A moieties edema factor (EF), lethal factor (LF) and C2I.

In the second part of the thesis, the blockage of intoxication is the center of interest. Therefore, chapter 5 focuses on the analysis of **specially designed blocker-substrate molecules for PA**. These molecules form a plug in the pore, abolishing translocation of the enzymatic units. Especially, if multi-resistant strains of Anthrax (said to be already produced in Russia as a biological weapon) are taken into consideration, these substrates could stop intoxication and buy time, to deal with the infection.

Chapter 6 describes the **blockage of PA-channels by anti-His antibody from the trans-side** of the porin, an effect which was not described for any other antibody before. Interestingly, even mutation of the estimated target amino acid Histidine 310 to Glycine could not interfere with this ionic strength dependent binding.

ZUSAMMENFASSUNG

Bakterielle Protein-Toxine gehören zu den wirksamsten bekannten Toxinen. In vielfältigen Variationen findet man sie in allen Bereichen des Lebens. Einige werden von den Bakterien während einer Infektion freigesetzt und übernehmen einen wichtigen Part in der Pathogenität. Andere werden zu Verteidigungszwecken sekretiert und können in verdorbenen Lebensmitteln gefunden werden. Was die Wirkung binärer Toxine der AB_7-Gruppe angeht, so gehören diese zu den potentesten und gefährlichsten Giften weltweit. Selbst kleine Mengen dieser Proteine können schwerste Symptome während einer Infektion mit Bakterien der Gattung *Clostridium* oder *Bacillus* verursachen. *Clostridium botulinum* zum Beispiel beherbergt ein ganzes Giftarsenal, welches verschiedene Toxine wie Botulinus-toxin – früher gefürchtet als Verursacher von Lebensmittelvergiftung, heute kommerziell unter dem Namen Botox als Nervengift in Verwendung – oder das binäre C2-Toxin beinhaltet.

Neuerdings registriert man diese Pathogene nicht mehr nur als nosokomiale Erreger, die immer wieder unter schlechten hygienischen Bedingungen oder bei mangelnder medizinischer Versorgung auftreten, sondern nimmt sie unter dem Gesichtspunkt des Bioterrorismus oder biologischer Kriegsführung wahr. Die Anschlägen des 11. September in den USA rückten ein weiteres binäres Toxin ins öffentliche Interesse: Das Anthrax Toxin. Der Bodenorganismus *Bacillus anthracis*, der in der Lage ist haltbare Sporen zu bilden, produziert dieses Gift. Briefe die mit „weißem Pulver" gefüllt waren dienten zur Verbreitung des Bakteriums, das nach dem Einatmen von geringen Mengen der Sporen die Lunge befällt. Weitere Organe die betroffen sein können sind – je nach dem Ort der Infektion – der Darm oder die Haut, auf der schwere Nekrosen ein sichtbares Symptom darstellen. Die Erkrankung, die unbehandelt mit hoher Wahrscheinlichkeit tödlich verläuft, ist hierzulande auch unter den Bezeichnungen Anthrax oder Milzbrand bekannt.

Abgesehen von der Bedrohung die durch diese Toxine ausgeht, zeichnen sie sich durch einen einzigartigen Intoxikationsmechanismus aus. AB_7-Toxine sind aus einer porenformenden Domäne B, die als eine Art molekulare Injektionskanüle fungiert, und enzymatisch aktiven Proteinen A zusammengesetzt. Der komplexe Wirkmechanismus ermöglicht es nicht nur Zellen in höchst effektiver Weise abzutöten und sollte deswegen zu Behandlungszwecken untersucht werden, sondern könnte auch als molekulares Werkzeug umfunktioniert werden, um spezielle Zellen mit gewünschten Proteinen zu beladen. Für beide Zwecke (Blockierung und gezielter Transport) ist die Bindung an, und der Transport durch die porenformende Domäne von größter Bedeutung.

Die Struktur der B-Domäne ist wichtig um tiefere Einsicht in den Transportprozess der binären Toxine zu ermöglichen. Leider ist es bisher nicht gelungen die Kristallstruktur des Membranproteins protective antigen (PA) von Anthrax oder irgendeiner anderen Bindedomäne eines AB_7-Toxins zu lösen. Deshalb wurde in dieser Arbeit ein elektrophysiologischer Ansatz zur Bestimmung der biophysikalischen Konstanten des Prozesses gewählt, die Black-lipid-Bilayer Methode. Zusätzliche Versuche an Zellen und mit Mutanten der Proteine dienen zur Absicherung der *in vitro* Ergebnisse.

Im ersten Teil der Arbeit wird der Translokationsmechanismus, und die mögliche Nutzung dessen als molekulares Werkzeug näher behandelt. Dies erfolgte durch Bindungsstudien an PA und C2II mit Effektoren (sowohl aus der AB_7-Familie, als auch nicht näher verwandt). In Kapitel 2 wird beschrieben, **dass ein His-Tag es EDIN (von *S. aureus*) und anderen Effektoren ermöglicht, dass ein Transport durch PA-Poren** *in vitro* und *in vivo* stattfindet (nicht aber durch C2II). Ebenfalls konnte eine starke Abweichung in der bekannten Spannungsabhängigkeit von PA festgestellt werden, die den Prozess eindeutig mit den Membranpotentialen in Verbindung bringt, die häufig eine wichtige Rolle im Metabolismus spielen.

Kapitel 3 fasst zusammen, dass **Bindungspartner von PA bestimmte Motive beinhalten**, die von Substraten wie Ionen in Elektrolyten, über kleine Moleküle, bis hin zu komplexen Proteinen reichen. Diese Erkenntnisse könnten genutzt werden um Blockersubstanzen zur Behandlung von Anthrax, oder Tags zur Aufnahme durch Anthrax zu designen.

Neueste Befunde zu Homologien und Unterschieden zwischen den Komponenten der binären Toxine sind der Kern von Kapitel 4, in dem **die Kreuzreaktivität von Anthrax und C2-Toxin** analysiert wurde. Die enthaltenen Daten ermöglichen einen tieferen Einblick in die verschiedenen Stufen der Bindung und Translokation des edema factor (EF), des lethal factor (LF) und von C2I an und durch PA und C2II.

Im zweiten Teil rückt die Blockierung der Intoxikation in den Fokus. Die **Analyse speziell designter Blockersubstanzen für PA** wird in Kapitel 5 behandelt. Diese formen einen Porenverschluss, der weiteren Transport von Toxinkomponenten verhindert. Eine besondere Bedeutung könnten diese Substanzen im Zusammenhang mit Multiresitenz bei Anthrax Stämmen (vermutlich in Russland als biologische Waffe hergestellt) zur Verhinderung von Symptomen und der Verlängerung der Zeit spielen, die man hat um neue Antibiotika zu erzeugen.

Kapitel 6 beschreibt zum ersten Mal **die Blockierung von PA-Poren mittels eines Anti-His Antikörpers von der trans-Seite** aus. Interessanterweise trat diese Ionenstärke abhängige Blockierung, auch bei einer Histidin zu Glycin Mutation an der Stelle 310 (vermutete Bindeposition) auf.

Chapter 1

INTRODUCTION

The routes of the word toxin are Greek, where "*toxikon*" translates as "*poison for use on arrows*". Today, a very general definition of toxins is found in many encyclopedias: "*Any substance poisonous to an organism*".

1.1 Bacterial toxins

In contrast to the general definition of toxins, bacterial toxins are defined as "*soluble substances that alter the normal metabolism of host cells with deleterious effects on the host*" (Schaechter *et al.*, 1993). The microbial toxins have been identified as the primary virulence factor of lots of different pathogens, since Roux and Yersin discovered diphtheria toxin in 1888 (Roux *et al.*, 1888). The symptoms of various severe diseases are associated with bacterial protein toxins, for example: Anthrax (*Bacillus anthracis*), Diphtheria (*Corynebacterium diphterie*), whooping cough (*Bordetella pertussis*), Cholera (*Vibrio cholera*), Tetanus (*Clostridium tetani*) and Botulism (*Clostridium botulinum*);
The toxicity of bacterial protein toxins exceeds those of chemical agents (considered toxic) by magnitudes and therefore those toxins belong to the most powerful poisons known. Botulinum toxin, for instance, possesses an LD_{50} of 0.03 ng/kg body mass, whereas a highly toxic dioxin's (TCDD) LD_{50} is 1 μg/kg and cyanide's LD_{50} is 3 mg/kg (Forth *et al.*, 1996). From an evolutionary point of view, those toxins developed due to the enhancement of survivability in gaining advantage over other microbes. Designed to damage target cells by interfering with the host's metabolism, bacterial toxins provide resources (dead cells), space for further grow and protection from immune systems as benefits.
The mode of action varies in different organisms and includes processes from simple lysis of the cells, to tissue disruption or even penetration of eukaryotic cell membranes followed by intracellular proliferation. Some bacteria produce pore-forming units to translocate specific enzymatic moieties, which than interfere with cell signaling or cytoskeleton composition, others disturb the cell cycle or protein synthesis (Schmitt *et al.*, 1999, Turk, 2007, Montecucco, 1997). The very distinct and effective intoxications compared to chemical toxic agents mentioned above originates in the enzymatic activity, which most of the bacterial toxins inherit.

Therefore, a first step to classify them on a functional level is the separation into two groups. The endotoxins act in an unspecific way, whereas exotoxins exploit enzymatically active agents to target specific structures and molecules of host cells.

Table 1.1: Differences of endo- and exotoxins.

	Endotoxin	**Exotoxin**
Chemical nature	lipopolysaccharide	protein
Molecular weight	ca. 10 kDa	> 20 kDa
Origin	part of the outer membrane of gram-negative bacteria	secreted by gram-positive and gram-negative bacteria
Heat-stable	yes	usually not
Antigenic	yes	yes
Useable as toxoids	no	yes
Toxicity	low	high
Specificity	low	high
Enzymatic activity	no	usually

Taken from (Leuber, 2007)

1.1.1 Endotoxins

Endotoxins are components of the outer membrane of Gram-negative bacteria and associated with the cell envelope of most strains irrespective if these are pathogens or not. Lipopolysaccharides (LPS) are typical endotoxins of leading pathogens like *Escherichia coli*, *Salmonella*, *Shigella*, *Pseudomonas*, *Neisseria* and many others. They consist of two components, a lipid moiety (Lipid A), which is associated with toxicity, and a polysaccharide, the immunogenic domain. The actual toxicity is caused by the host immune system's release of pro-inflammatory cytokines and nitric oxide, resulting in an endotoxic shock.

Release of these compounds happens throughout the complete lifecycle of Gram-negative cultures, but as a part of the cell envelope, huge amounts of LPS spread when the bacteria get lysed (for example by antibiotics, autolysis or in lysozyme). The general mode of action of endotoxins is less specific compared to exotoxins, as they do not feature enzymatic activity. Due to their heat stability and the lack of immunizing capacity (Trent *et al.*, 2006) the intoxication, never the less could lead to severe consequences.

Table 1.2: Examples of enzymatic properties of different bacterial protein toxins and their biological relevance.

	Enzymatic moiety	**Biological effects**
Cholera toxin	ADP-ribosylates adenylate-cyclase Gs regulatory protein	Activates adenylate-cyclase; increased cAMP promotes secretion of electrolytes and fluids in the intestine, leading to diarrhea
Diphteria toxin	ADP-ribosylates elongation factor 2	Inhibition of protein synthesis and cell death
Pertussis toxin	ADP ribosylates adenylate-cyclase Gi regulatory protein	Blocks inhibition of adenylate-cyclase; increased levels of cAMP effect hormone activity
Shiga toxin	Glycosidase cleavage of ribosomal RNA (28s rRNA)	Inactivation of ribosomal 60s subunit, stopping protein synthesis and leading to cell death
Botulinum neutrotoxin A	Zn^{2+}-dependent protease acting on synaptobrevin at motor ganglioside	Inhibition of presynaptic acetylcholine release from peripheral cholinergic neurons resulting in flaccid paralysis
Tetanus toxin	Zn^{2+}-dependent protease acting on synaptobrevin in the central nervous system	Inhibition of neurotransmitter release from inhibitory neurons in the central nervous system, resulting in spastic paralysis
Anthrax lethal toxin	Zn^{2+}-dependent protease, cleaving MAPKK	Inhibition of MAPK pathway leading to cell death
Anthrax edema toxin	Ca^{2+}- and calmodulin-dependent adenylate- cyclase	Increased intracellular cAMP interfering with cell signalling
Large clostridial toxins	Glycosylates small, monomeric G-proteins	Interference with small G-protein induced signaling pathways with effects on morphology and physiology
Clostridium C2-toxin	ADP-ribosylates G-actin at Arg177	Interference with the actin-polymerisation, disruption of the cytoskeleton
Bordetella AC toxin	Calmodulin-dependent adenylate-cyclase	Increased cAMP leading to inhibition of phagocytosis by neutrophils and macrophages; hemolysis or leukolysis

Taken from (Leuber, 2007)

1.1.2 Exotoxins

Gram-positive as well as Gram-negative bacteria produce exotoxins. Typically secreted in the surrounding media during exponential growth phase, they could be packed in spores, as well. In most cases exotoxins exhibit enzymatic activities with high specificity for certain substrates. Therefore, their efficiency is analyzed by common kinetic models. Other modes of action include specificity for special cell-types or tissues (tetanus toxin from *Clostridium tetani* only attacks neurons). Furthermore, some exotoxins are not restricted to certain cell-types or tissues, which often leads to necrosis. An attempt to fight intoxication is to use parts of these toxins as non-toxic vaccines, so called toxoids. Exotoxins and correlating bacterial strains are listed in table 1.2.

1.2 Binary toxins

The class of binary toxins is characterized by the existence of two different protein components, which are not toxic themselves. Domain A exhibits the enzymatic activity and is transferred to the cytoplasm by a binding and translocation domain B. Transport occurs via formation of a pore in the membrane or simple flip-flop in the membrane. Both domains can be located in one protein-chain or separately secreted. Diphtheria toxin or the large complexes of ToxA and ToxB are examples for the first exotoxins (Lemichez *et al.*, 2003, Egerer *et al.*, 2007), the second group comprises Anthrax and C2-toxin (Young *et al.*, 2007). Some of the B components need special receptors to bind to the cell surface of their corresponding target cells.
Nowadays, lots of crystal structures for binary toxins are solved, but exclusively in the water-soluble form (Barth *et al.*, 2004). Large hydrophobic areas in the membrane-spanning part of the B subunits prevent the formation of diffracting crystals for membrane-bound states in most of the cases. The Anthrax toxin's B component protective antigen (PA) water-soluble prepore for example is known since 1997 (Petosa *et al.*, 1997). However, only a model exists of the membrane-active β-barrel transportation unit (Nguyen, 2004). This model is derived by the only solved membrane-inserted structure of a pore-forming toxin (PFT), the α-hemolysin of *Staphylococcus aureus* (Song *et al.*, 1996). The same is true for C2-toxin's binding domain C2II, where Schleberger et al. proposed a hypothetical model (Schleberger *et al.*, 2006).

1.3 Pore-forming toxins (PFTs)

A major subgroup of bacterial exotoxins constitutes of the pore-forming toxins. As their name clearly indicates, these toxins are categorized in respect to their ability to form pores in membranes. Typically, they are secreted in the external medium in a water-soluble form and

congregate to oligomers, that assemble in α-helical channels (α-PFTs) or a membrane-spanning β-barrel build by each monomer's hydrophobic β-sheet structure (β -PFTs). Some prominent members of each PFT-type are listed in table 1.3 below.

Table 1.3: Examples of various α- and β-PFTs.

α-PFT	**Organism**	**Structural characteristics**
Colicin	*Escherichia coli*	Globular pore-forming domain, ten α-helices including two hydrophobic helices
Diphteria toxin	*Corynebacterium diphteriae*	Two disulfide-linked chains resulting from cleavage of one protein, ten α-helices
Exotoxin A	*Pseudomonas aeroginosa*	Six α-helices, not predominantly hydrophobic
Cry toxins	*Bcillus thuringensis*	Seven α-helices, five necessary for toxicity
β-PFT	**Organism**	**Structural characteristics**
α-Hemolysin	*Staphilococcus aureus*	Heptamer and prepore, 14-stranded transmembrane β-barrel, mushroom shaped
Leucocidins	*Staphilococcus aureus*	Hetero-octamer of LukF and LukS
Pneumolysin	*Streptococcus pneumoniae*	Ring of 30-50 subunits, two β-strands per unit, cholesterol-dependent
Aerolysin	*Aeromonas hydrophila*	Heptameric pores without prepore formation
Anthrax toxin	*Bacillus anthracis*	Binary toxins (see Chapter 1.4 for details)
C2-toxin	*Clostridium botulinum*	Binary toxins (see Chapter 1.4 for details)

Taken from (Leuber, 2007)

1.4 Anthrax toxin of *Bacillus anthracis*

The rod-shaped Gram-positive bacterium *Bacillus anthracis* produces Anthrax toxin as its main virulence factor. One possible Symptom of an Anthrax infection is large black necrotic patches on the skin. Therefore, the name Anthrax is derived from the Greek word for coal "*ánthrax*". An infection is caused by the uptake of *Bacillus anthracis* spores in skin bruises, the lung or gastrointestinal. Depending on the site of infection different phenotypes of the disease evolve, of which the one in the lungs is the most dangerous and leads to death with nearly 100% probability if not treated. These spores are resistant to environmental stresses and could last for more than 100 years, still able to start bacterial growth and the cycle of infection (Dixon *et al.*, 1999, Mock *et al.*, 2001).

The uptake of the spores is followed by germination and proliferation of vegetative bacteria, which invade the lymphatic system. There, they eliminate host immune cells and enter the bloodstream. Finally, death occurs due to septicemia and toxemia. With the restriction of nutrients a high amount of spores is produced afterwards. During the assaults of September 2001 in the USA, letters containing Anthrax spores have been sent to persons involved in the government and led to cases of infection and death, conjuring up Anthrax toxin in the public again (Atlas, 2002).

This toxin is classified, as a binary AB_7-type toxin comprised of three components. Protective antigen (PA) is the binding and translocation unit, which transports edema factor (EF) and lethal factor (LF) into target cell's cytosol (Collier *et al.*, 2003, Friedlander, 1986, Mock *et al.*, 2001). Thereby, in contrast to other members of the AB-family Anthrax contains two enzymatically active moieties. Additionally, another virulence factor, the poly-D-glutamyl capsule, inhibits the phagocytosis of *B. anthracis* by host immune system.

1.4.1 Protective antigen (PA) – The binding and translocation moiety

The B-component of Anthrax toxin is called protective antigen (PA) due to its use as a vaccine. It is secreted as a 83 kDa monomeric protein (PA_{83}) to the external media and consists of four domains. Correlated to their function, domain I is proteolytically cleaved by furin-like cell bound proteases during activation, domain II, a flexible loop, inserts in the membrane, oligomerization takes place in domain III and domain IV binds to the receptors (Petosa *et al.*, 1997, Mogridge *et al.*, 2002a, Mogridge *et al.*, 2002b, Lacy *et al.*, 2002). Activation of PA_{83} leads to a 20 kDa (PA_{20}) and a 63 kDa (PA_{63}) fragment, of which the larger one represents the active PA. Lately, a vital discussion in this field of work is going on about the number of monomers, which form the water-soluble so-called prepore and later on the channel. The structures of a homo-heptameric and a homo-octameric prepore were published (Petosa *et al.*, 1997, Feld *et al.*, 2010). Concerning the electrophysiological results and the possibility of crystallization artifacts, this work considers the heptameric symmetry as the prominent form. For the membrane active PA-pore only a model exists (Nguyen, 2004), based on the mushroom shaped resolved structure of α-hemolysin (Song *et al.*, 1996). Accordingly, the 14-stranded β-barrel, forming the channel is created by unfolding β-hairpins in a Greek-key motif (strands 2β1-2β4) (Benson *et al.*, 1998, Nassi *et al.*, 2002). The important process of prepore to pore transition is triggered by acid pH in the endosome (Krantz *et al.*, 2004, Krantz *et al.*, 2005). Recent progress in electron microscopy made it possible to take pictures of PA-channels in membrane disks. These show a slightly different shaped protein, especially in the head region (Fig. 1.1) (Katayama *et al.*, 2010).

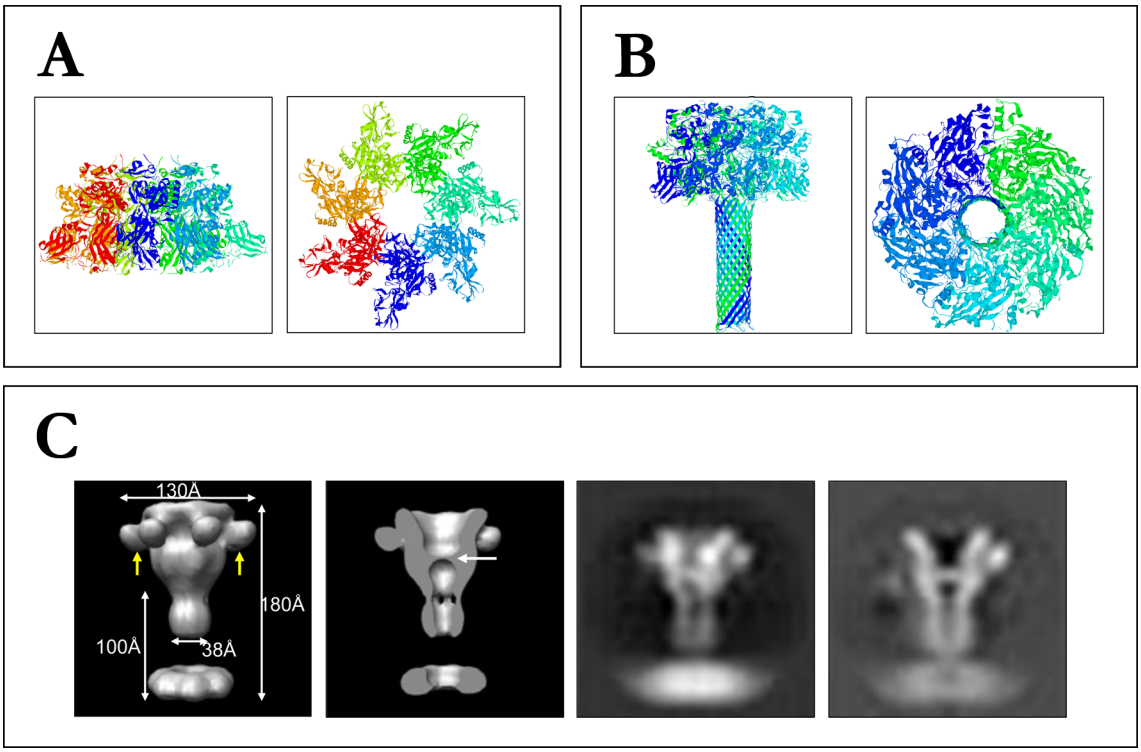

Fig. 1.1:
Structures and hypothetical models of Anthrax's protective antigen:
A: Top and side view of the heptameric PA prepore (Petosa *et al.*, 1997);
B: Hypothetical model of membrane-inserted PA-heptamer with membrane-spanning β-barrel (Nguyen, 2004);
C: Three-dimensional reconstruction of PA-pore, inserted in nano-discs (Katayama *et al.*, 2010);

1.4.2 Edema factor (EF) and Lethal factor (LF)

The 89 kDa protein edema factor (EF) is one of the enzymatically active A-moieties of Anthrax toxin and represents a calmodulin- and Ca^{2+}-dependent adenylate-cyclase. It is named edema factor because it is believed to cause edemas in cutaneous Anthrax. The toxic potential of EF reasons in the transformation of ADP in cAMP. Accumulated cAMP interferes with water homeostasis and many intracellular signaling pathways, causing the cell to die (Dixon *et al.*, 1999, Mock *et al.*, 2001, Lacy *et al.*, 2002, Turk, 2007).

Cell death is the final effect of the second A-component lethal factor (LF), as well. LF represents a 90 kDa Zn2+-dependent metalloprotease, that specifically cleaves mitogen-activated protein kinase kinases (MAPKKs), thereby not only blocking the MAPK-pathway, but also leading to a special form of apoptosis (Turk, 2007). Especially when attacking macrophages, this death does not spread chemokines and cytokines, which normally alert the host immune system (Hanna *et al.*, 1993, Pellizzari *et al.*, 1999). Even specialized killers like dendritic cells and T-cells are inhibited (Lacy *et al.*, 2002, Agrawal *et al.*, 2003, Rossi Paccani *et al.*, 2007), which explains, why antibiotics including penicillin and doxicyclin only show effect during early stages of an Anthrax infection in which no obvious symptoms could be detected.

1.4.3 Intoxication pathway of Anthrax toxin

After the release of the enzymatic components and the PA_{83}, this precursor binds to cellular expressed surface receptors. Two receptors represent prominent targets, ATR (Anthrax toxin receptor), an alternative splice product of TEM8 (tumor endothelial marker 8) and CMG2 (capillary morphogenesis gene transcript 2) (Bradley *et al.*, 2001, Scobie *et al.*, 2007), which are also called ANTXR1 and ANTXR2. Afterwards, LRP6 (low density lipoprotein receptor-related protein 6) interacts with ATR or CMG2 , on the cell surface to initiate internalization of the whole complex (Wei *et al.*, 2006). As mentioned before, PA_{83} is proteolytically activated by furin-like proteases on the cell surface, rendering it possible to form heptameric PA_{63}-prepores (Petosa *et al.*, 1997). The 20 kDa PA_{20} fragment is supposed to play a role in uptake of the complex, as well (Chvyrkova *et al.*, 2007).

Up to three EF and LF molecules attach to the prepore with their N-terminal end (Escuyer *et al.*, 1991, Elliott *et al.*, 2000, Cunningham *et al.*, 2002), which exhibits a significant homology. In other studies the steric complexity of the complex leads to the assumption that only one effector molecule is able to bind each prepore (Ren *et al.*, 2004, Chvyrkova *et al.*, 2007). It is generally proven, that ion-ion interaction between the positively charged N-terms of the A-components and the negative charges of the PA-heptamer enhance the affinity of the binding process. This is further reasoned in experiments, which use charged tags to increase this effect (Neumeyer *et al.*, 2006a, Neumeyer *et al.*, 2006b, Blanke *et al.*, 1996, Beitzinger *et al.*, 2011c).

Subsequently, the whole $(PA_{63})_7$-EF/LF-complex is endocytosed by receptor-mediated and/or clatherin-coated pits (Abrami *et al.*, 2003). During the maturation of the endosome it's acidification prompts changes in both, the prepore and the enzymatic-units. While the first undergoes a transition to a β-barrel formed pore, the later partially unfold and pass through the channel, driven by voltage and pH-gradient (Benson *et al.*, 1998, Nassi *et al.*, 2002, Young *et al.*, 2007, Beitzinger *et al.*, 2011c). The transport through the pore occurs in a molten-globular state, in which the N-terminal end of the effectors inserts in the Lumen first. Further unfolding leads - together with Brownian molecular movement and cytosolic uncharging of some amino acids – to the translocation to the cytosol, where the toxic impact unravels. The whole intoxication pathway is depicted in figure 1.2.

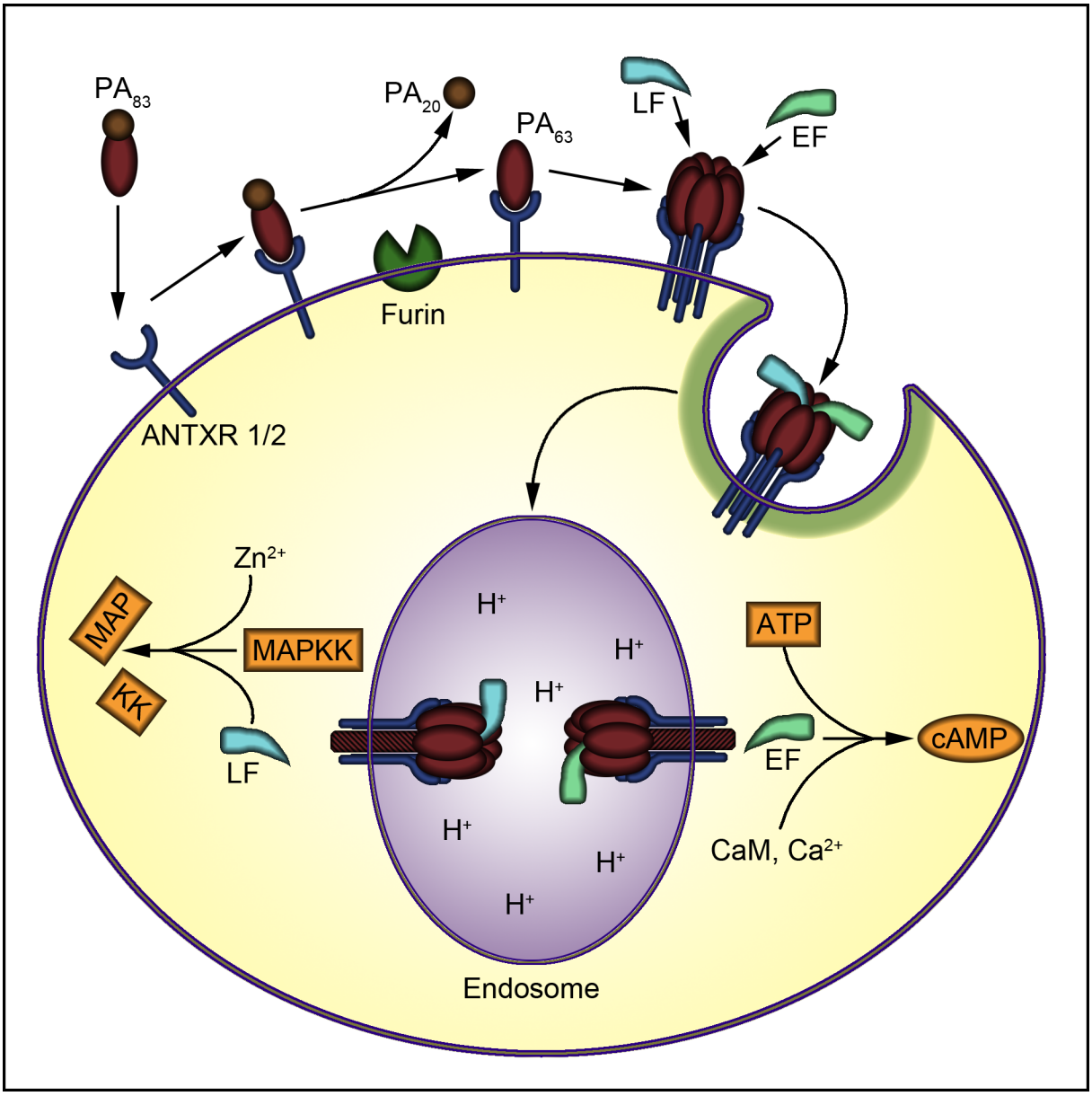

Fig. 1.2:
Intoxication pathway of Anthrax toxin. Precursor PA_{83} binds to the cellular receptor and is proteolytically activated. PA_{63} forms the heptameric prepore, which may bind 1-3 of the enzymatic components EF and/or LF. After clathrin-dependent endocytosis, acidification of the endosome leads to prepore-to-pore conversion of the PA-heptamer and subsequent translocation of the enzymatic components into the cytosol. Here, they cause either an increase of cAMP (EF) or the cleavage of MAPKKs (LF). (Adapted from Young *et al.* (2007))

The transport into CHO-K1 cells was blocked by removing the first 27 or 36 amino acids of the N-terminus (Zhang *et al.*, 2004b). In addition, the fragment LF_N (residues 1-263) was still able to block the pore and even to propel the transduction of diphtheria toxin (DTA) (Zhang *et al.*, 2004a, Halverson *et al.*, 2005, Blanke *et al.*, 1996).

Concerning the PA-channel, two important structures have to be mentioned. First on is the α-clamp, the proposed binding site for EF and LF (Feld *et al.*, 2010). The second one is the so-called ϕ-clamp, an aperture-like ring of seven phenylalanine residues (F427), which triggers

translocation and restricts ion-current (Krantz *et al.*, 2005). The ϕ-clamp is surrounded by negatively charged residues (E399 and D426), which could be the reason for His-tag-driven transport through PA (Beitzinger *et al.*, 2011c).

1.4.4 *Bacillus anthracis* - Pathogenicity

The above-mentioned difficulties in treatment of Anthrax due to the delayed appearance of symptoms imply the necessity to use a pharmacological approach of the problem. Even if antibiotics work quite well against the infection, when applied on time, and vaccination offers some protection against the intoxication, the existence of multi-resistant strains and the high probability of death reason the research for so-called blocker-substrates. These form a plug in the lumen of the pore (presumably on the ϕ-clamp), thereby hindering the effectors from being translocated. Experiments performed with anti-bodies (Little *et al.*, 1997, Kobiler *et al.*, 2002) and 4-aminoquinolines or cyclodextrins seem to be promising as a complementary medication (Karginov *et al.*, 2005, Karginov *et al.*, 2006, Orlik *et al.*, 2005, Hirsh *et al.*, 2006, Nestorovich *et al.*, 2010, Beitzinger *et al.*, 2011b).

1.5 C2-toxin of *Clostridium botulinum*

Clostridium botulinum is an anaerobic, spore-forming bacterium, producing a large variety of highly lethal exotoxins. Included in it's arsenal is botulinum toxin (BoNT) (Johnson, 1999, Bohnel *et al.*, 2005), one of the most potent and best known toxins in the world. It not only causes botulism, a disease with terrible degeneration in neural system (Cherington, 1998), nowadays it is commonly used as a cosmetic drug to remove wrinkles, the famous Botox™ (Turkoski, 2009).

Strains C and D of *C. botulinum* produce another two non-neurotoxic proteins, the binary C2- and the Rho-modifying C3-toxin (Aktories *et al.*, 1987, Bohnel *et al.*, 2005, Wilde *et al.*, 2002, Aktories *et al.*, 2004a). C2-toxin elicits an actin-ADP-ribosylation and acts lethal if applied to animals (Simpson, 1982, Ohishi *et al.*, 1984). It's toxic effects lead to necrotic and hemorrhagic lesions in the intestine due to loss of barrier function of the epithelium, followed by a decrease of blood pressure and edema (Simpson *et al.*, 1988, Considine *et al.*, 1991).

C2-toxin is a binary β-PFT of the AB_7-type and consists of the enzymatic moiety C2I and the binding domain C2II. The homology in amino acid sequence of C2II and PA from *B. anthracis* is round about 35% (Kimura *et al.*, 1998), therefore providing a possibility for comparative experiments.

1.5.1 C2II – The binding and translocation moiety

The high homology of C2- and Anthrax toxin's B-components mirrors a comparable molecular weight, which is 81 kDa for C2II. Similar problems occur for the crystallization of the membrane-bound form of C2II, for which only a model is available. The heptameric water-soluble prepore-structure has been solved (Petosa *et al.*, 1997, Schleberger *et al.*, 2006). In difference to PA, C2II binds to a complex hybrid carbohydrate receptor (Barth *et al.*, 2000, Kaiser *et al.*, 2006). C2II shares another important feature of PA, the ϕ-clamp. It is found in a phenylalanine ring in position F428 in the C2II-channel (Orlik *et al.*, 2005, Neumeyer *et al.*, 2008).

1.5.2 C2I – The enzymatic component

The A-component of C2-toxin is represented by C2I, a 49 kDa ADP-ribosyltransferase (Simpson *et al.*, 1988). Modifying actin molecules at position Arg177 in adding a ADP-ribosyl subgroup, it hinders the formation of F-actin out of G-actin (Aktories *et al.*, 1992, Aktories *et al.*, 2004a). The ADP-ribosylation also affects gelsolin-actin interaction (Wille *et al.*, 1992). Finally, the cytoskeleton is completely disturbed, which results in cell-rounding and cell-death afterwards. C2I possesses an N-terminal binding domain and a C-terminal enzymatic domain. Common structural details are a hint of a gene duplication involved in it's evolution (Schleberger *et al.*, 2006).

1.5.3 Intoxication pathway of C2-toxin

Anthrax toxin and C2-toxin belong to the same group of binary AB_7-β-PFT. Therefore, it is not surprising, that they share most of the intoxication pathway. Differences to the Anthrax intoxication described above (chapter 1.4.3) are, the formation of the prepore directly after activation by furin (Blocker *et al.*, 2000), but before the receptor-binding (Barth *et al.*, 2000), the receptor itself, and the necessity of chaperone proteins for the transport process (Haug *et al.*, 2003). As mentioned above, active C2II (also called C2IIa) binds to a complex of hybrid carbohydrates and is afterwards endocytosed into endosomes. Presumably, the acidic pH in this compartment is the trigger for pore formation, which leads to transport of C2I to the cytosolic opening of the pore. Here, heat-shock proteins assemble on the nascent chain and "pull" the effector through the channel, on the same time refolding the active enzyme (Haug *et al.*, 2003). C2I than interferes with the F-actin to G-actin homeostasis, in shifting it to the G-actin side, which leads to disruption of the actin-cytoskeleton, followed by cell-rounding and cell-death.

1.5.4 *Clostridium botulinum* - Pathogenicity

Based on the similar mode of action and potential to kill cells effectively and rapidly, comparable measures have been taken to fight the intoxication with C2-toxin. The effects of inhibitory substrates like 4-aminoquinolines and cyclodextrins have been described for C2II, as well (Bachmeyer *et al.*, 2003, Bachmeyer *et al.*, 2001, Nestorovich *et al.*, 2011). Another vital field of work is represented by experiment, which use the N-terminal, non-toxic part of C2I for directed transport of cargo proteins into certain cell-types (Barth *et al.*, 2002a). As heat-shock proteins are involved in the intoxication procedure, it could be possible to find another way of stopping the delivery of C2I to the cytosol.

1.6 General considerations and aims of this work

This thesis is composed of two major fields of studies, which correlate to one another. The first part of the work was dedicated to the topic of binding and transport, focusing on the use of the pore-forming units of binary toxins as a molecular tool. The knowledge already present for a sophisticated delivery system for cargo-proteins using PA as a molecular syringe, that attaches only to certain cell-types, could be extended, by correlating the charged tags ability of transport to a change in voltage-dependence of the whole system. The complete first part should contribute to a broadened understanding of the binding and transport processes concerning homologies and differences of PA and C2II.

Equally, the second part focuses on these two B-components. The task to block the pores with different specially designed molecules is investigated using titration and noise-analysis measurements to determine the contribution of certain chemical functional side chains. Finally, a first time proof for the blockage of PA-channels, from the cytoplasmic side, by an anti-his antibody, changes the view of the proposed model of the membrane-bound pore and opens a field of further investigations.

Chapter 2

Anthrax toxin protective antigen promotes uptake of N-terminal His_6-tag labeled polypeptides into cells in a voltage-dependent way

This work was submitted to *PlosONE* and is used here in agreement with all authors.

The use of Anthrax toxin's PA-channel as a molecular syringe for His-tagged protein-cargo, certainly represents one of the most sophisticated ways of utilizing a formerly feared biological substrate as a modern helper. Additionally, it broadens the knowledge for heptameric toxin channels in general, concerning binding and translocation processes for two homologous but different proteins.

2.1 Summary

It is of interest how to use bacterial toxins as molecular molecular-syringe devices to deliver enzymatic activities into endothelial host cells. Binary toxins of the AB_7-type are among the most potent and specialized bacterial protein toxins. The B subunits multimerize to form a pore that binds with high affinity host cell receptors and the enzymatic A subunit. This allows the endocytosis of the complex and subsequent injection of the A subunit into the cytosol of the host cells. Here we report that the addition of an N-terminal His_6-tag to various proteins increased their binding affinity to the protective antigen (PA) PA_{63}-channels, irrespective if they are related (EF, LF, C2I) or unrelated (gpJ, EDIN) to the AB_7-family of toxins. Interestingly, this affinity increase was shown to be highly voltage-dependent when the voltage at the trans-side, the side opposite to the addition of the proteins had a negative sign. His_6-EDIN exhibited voltage-dependent increase of the stability constant for binding by a factor of about 25 when the *trans*-side corresponding to the cell interior was at -70 mV. Surprisingly, the *C. botulinum* toxin C2II-channel did not share this feature of PA. Cell-based experiments demonstrated that addition of an N-terminal His_6-tag promoted also intoxication of endothelial cells by C2I or EDIN via PA but not via C2II. Our results revealed that Anthrax protective antigen, in combination with His-tagged proteins could be used to import foreign protein activities into cells.

2.2 Introduction

Gram-positive bacteria such as *Bacillus anthracis* and *Clostridium botulinum* possess special A-B toxins as one of their most potent virulence factors. These toxins are composed of two components which are nontoxic by themselves when added to the external media of target cells (Barth *et al.*, 2004). One or more A-components of the toxins feature intracellular enzymatic activity and are responsible for the toxicity. The B-component binds to cellular receptors or directly to the membrane and transports the enzymatic component into the cell. Anthrax-toxin from *B. anthracis* belongs to the AB_7-type of toxins classified by a pore forming B-component, protective antigen (PA) and two enzymes, edema factor (EF) and lethal factor (LF). PA has a 83 kDa water soluble precursor, which has to be activated by cleavage of a 20 kDa N-terminal part to form the functional PA_{63}-heptamers (Miller *et al.*, 1999, Abrami *et al.*, 2004, Abrami *et al.*, 2005, Petosa *et al.*, 1997). The proteolytic activation is performed *in vivo* by cell bound furin and renders possible pore formation and transport of the two enzymatic components EF and LF (Mock *et al.*, 2001, Ascenzi *et al.*, 2002, Young *et al.*, 2007, Turk, 2007). EF is an 89 kDa Ca^{2+}- and calmodulin-dependent adenylate cyclase which causes severe edema by uncontrolled increasing the intracellular cAMP. LF is a Zn^{2+}-binding metalloprotease that cleaves mitogen-activated protein kinase kinases (MAPK-kinases) and thereby interferes with the MAPK cascade, a major signaling pathway. It is triggered by surface receptors, controlling cell proliferation and survival and causes cell death by interfering with intracellular signaling leading to apoptosis.

C. botulinum, well known for the production of potent neurotoxins, also produces other protein toxins such as the binary C2-toxin and the single-component C3 exoenzyme (Aktories *et al.*, 2004a, Aktories *et al.*, 2004b, Boquet *et al.*, 2003). The homologue pore forming B-component to PA is C2II. After proteolytic cleavage with trypsin (60 kDa) it forms heptamers that insert into biological and artificial membranes at an acidic pH and promotes the translocation of the 45 kDa enzymatic component C2I. Similar to Anthrax-toxin the receptor-mediated endocytotic pathway of the cell is used (Barth *et al.*, 2000, Blocker *et al.*, 2000). C2I acts as an NAD-dependent ADP-ribosyltransferase on arginine177 of monomeric G-actin, causing disruption of the actin cytoskeleton of eukaryotic cells (Blocker *et al.*, 2003b, Considine *et al.*, 1991).

The toxins of the AB-type represent simple but sophisticated molecular syringes for protein delivery into target cells. This means that they could be important systems for development of new strategies for efficient injection of polypeptides into target cells. Possible Trojan Horses could be binary toxins of the AB_7 type such as Anthrax- and C2-toxin because they represent highly potent bacterial toxins composed of two polypeptide chains that are secreted in the external media of Gram-positive bacteria (Barth *et al.*, 2004). The binding of the N-terminal ends

of the enzymatic components to the heptameric channel formed by the binding components is followed by receptor-mediated endocytosis, acidification of the endosomes and final release of the enzymatic components into the cytosol of target cells, where they exert their enzymatic activities (Abrami *et al.*, 2004, Abrami *et al.*, 2005, Wei *et al.*, 2006). Interestingly, the amino-terminal part of LF is sufficient to confer the ability to associate with PA_{63}-heptamers on LF. It can be used to drive the translocation of unrelated polypeptides fused to LF_{1-254} into target cells in a PA_{63}-dependent manner (Leppla *et al.*, 1999). Although the enzymatic components of Anthrax- and C2-toxin differ considerably in their enzymatic activity and in their primary structures as well, the binding components PA and C2II share a significant overall sequence homology of about 35%, which means that they are closely related in structure and probably also in function (Young *et al.*, 2007, Petosa *et al.*, 1997, Schleberger *et al.*, 2006, Neumeyer *et al.*, 2006a). Important for the binding of channel blockers and enzymes to be delivered into the target cells are besides the so-called phi-clamp - F427 in PA and F428 in C2II - two rings of seven negatively charged amino acids - E399 and D428 in PA and E398 and D427 in C2II (Krantz *et al.*, 2005, Melnyk *et al.*, 2006). These negatively charged amino acids seem to interact with the positively charged N-terminal ends of the enzymatic components (Neumeyer *et al.*, 2008, Krantz *et al.*, 2004).

In this study we have investigated the influence of additional charges on the N-terminal end on binding of the enzymatic factors to the channels formed by PA_{63} and C2II. First results in the field were found with polycationic peptides fused to LF_N and EF_N (Blanke *et al.*, 1996, Neumeyer *et al.*, 2006a). The results suggested that the binding of LF and EF to C2II is possible and that C2I binds to PA_{63} in the black lipid bilayer assay as well. The most significant result that was observed was a preferential binding of His_6-C2I to PA_{63}. Interestingly, PA_{63} is able to transport His_6-C2I into target cells with high efficiency, exhibiting host cell toxicity, whereas C2II does not transport His_6-EF or His_6-LF. This prompted us to investigate whether a His_6-tag might also lead to an increase in binding affinity for heterologous polypeptides to PA_{63}. In fact, we could demonstrate that the epidermal cell differentiation inhibitor EDIN of *Staphylococcus aureus* fused to a His_6-tag enters cells via PA. Both EDIN and His_6-EDIN bind *in vitro* to PA_{63}. In addition, the binding constant of His_6-EDIN and not that of EDIN to PA_{63}-channels was found to be highly voltage-dependent.

2.3 Material and Methods

2.3.1 Materials

Protective antigen encoding gene was cloned with *Bam*HI-*Sac*I restriction sites into pET22 (Novagen) as previously described (Rolando *et al.*, 2009). The translocation-defective PA mutant F427A (Sellman *et al.*, 2001, Krantz *et al.*, 2005) was constructed by site-directed mutagenesis using the QuickChange™ kit (Stratagene) according to the manufacturer´s instructions. The PA-gene cloned in the plasmid pET19 (Novagen) (Cataldi *et al.*, 1990, Tonello *et al.*, 2004), was used as a template. The construct was confirmed by DNA sequencing. The protein was expressed with an N-terminal His_6-tag in BL21 (DE3) (Novagen) and purified by HiTrap chelating (Pharmacia) charged with Ni^{2+} ions.

C2I and C2II genes were PCR-amplified from genomic DNA of *Clostridium botulinum* D strain 1873 and cloned into pET22 (Novagen) and pQE30 (Qiagen) expression plasmids with *Bam*HI-*Sac*I restriction sites.

The plasmid coding for the chimera protein MBP-gpJ (maltose-binding-protein attached to amino acids 684-1132 of Lambda phage tail protein J) was a kind gift of Alain Charbit, Paris, France. Expression and purification of MBP-gpJ was performed as described previously (Wang *et al.*, 2000). gpJ was obtained by treatment of MBP-gpJ bound to starch column beads (amylose-Sepharose, New England Biolabs) with factor X_a (Invitrogen). His_6-gpJ (684-1132) was obtained as described previously (Berkane *et al.*, 2006).

The DNA encoding EDIN (NCBI M63917) was cloned into pET28a vector using *Bam*HI-*Eco*RI restriction site as described previously (Boyer *et al.*, 2006). Recombinant toxins containing His_6-tags were expressed in *E. coli* BL21 (DE3) and purified on a Chelating Sepharose Fast Flow column previously chelated with nickel (Amersham Biosciences) as recommended by the manufacturer. Fractions containing toxin were pooled and dialyzed over night against 250 mM NaCl and 25 mM Tris-HCl, pH 8. The N-terminal His_6-tag was removed by incubation with thrombin. Nicked Anthrax PA_{63} from *B. anthracis* was obtained from List Biological Laboratories Inc., Campbell, CA. One mg of lyophilized protein was dissolved in 1 ml 5 mM HEPES, 50 mM NaCl, pH 7.5 complemented with 1.25% trehalose. Aliquots were stored at -20°C. Channel formation by PA_{63} was stable for months under these conditions.

2.3.2 Cell culture and biochemical products

HUVECs (human umbilical vein endothelial cells, a human primary cell line obtained from PromoCell) were grown in serum-free medium (SFM) supplemented with 20% FBS (Invitrogen),

20 ng/ml basic ßFGF (Invitrogen), 10 ng/ml EGF (Invitrogen) and 1 µg/ml heparin (Sigma-Aldrich) as described previously (Doye *et al.*, 2006). Monoclonal antibodies used were: anti-RhoA (BD Biosciences, [clone 26C4]); anti- ß-actin (SIGMA, [clone AC9-74]); anti-His-tag (Qiagen, [Penta-His]). Primary antibodies were visualized using goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (DakoCytomation), followed by chemiluminescence detection ECL (GE Healthcare). Levels of active Rho were determined by GST-rhotekin RBD pull-down that was modified as described previously (Doye *et al.*, 2006).

2.3.3 Lipid bilayer experiments

Black lipid bilayer measurements were performed as described previously (Benz *et al.*, 1978). The instrumentation consisted of a Teflon chamber with two aqueous compartments connected by a small circular hole. The hole had a surface area of about 0.4 mm^2. Membranes were formed by painting a 1% solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) in n-decane onto the hole. The aqueous salt solutions (Merck, Darmstadt, Germany) were buffered with 10 mM MES to pH 5.5 to pH 6. Control experiments revealed that the pH was stable during the time course of the experiments. The binding components of the binary toxins were reconstituted into the lipid bilayer membranes by adding concentrated solutions to the aqueous phase on one side (the *cis*-side) of a black membrane. The temperature was kept at 20°C throughout. Membrane conductance was measured after application of a fixed membrane potential with a pair of silver/silver chloride electrodes inserted into the aqueous solutions on both sides of the membrane. Membrane current was measured using a homemade current-to-voltage converter combined with a Burr Brown operational amplifier. The amplified signal was monitored on a storage oscilloscope and recorded on a strip chart recorder.

2.3.4 Binding experiments

The binding of the His-tagged proteins to the C2II-channel and the binding component PA_{63} was investigated with titration experiments similar to those performed previously to study the binding of 4-aminoquinolones to the C2II- and PA_{63}-channels and EF and LF to the PA_{63}-channel in single- or multi-channel experiments (Bachmeyer *et al.*, 2003, Neumeyer *et al.*, 2006b, Orlik *et al.*, 2005). The C2II- and PA_{63}-channels were reconstituted into lipid bilayers. About 60 minutes after the addition of either activated C2II or PA_{63} to the *cis*-side of the membrane, the rate of channel insertion in the membranes was very small. Then concentrated solutions of His-tagged proteins were added to the *cis*-side of the membranes while stirring to allow equilibration. The results of the titration experiments, i.e. the blockage of the channels, were analyzed using Langmuir

adsorption isotherms (Benz *et al.*, 1987, Neumeyer *et al.*, 2006a). The conductance as a function of the concentration of the enzymatic components was analyzed using Lineweaver-Burke plots. *K* is the stability constant for binding of the enzymatic components of the binary toxins to the PA_{63}- or C2II-channels. The half saturation constant K_s is given by the inverse stability constant $1/K$.

$$\frac{(G_{max} - G(c))}{G_{max}} = \frac{K \cdot c}{(K \cdot c + 1)} \qquad [2.1]$$

2.4 Results

2.4.1 Interaction of PA_{63}-pores with His_6-C2I in artificial black lipid bilayer membranes

We compared the binding affinity of different proteins with and without a His_6-tag to the PA_{63}- and C2II-channels. Taking into account that positive charges seem to have a huge influence in binding to the PA_{63}-pore but only less to the C2II-pore (Orlik *et al.*, 2005, Leuber *et al.*, 2008), we chose the enzymatic component C2I as the first substrate. In a previous study we could show that it binds to PA_{63}-pores and could even be translocated into cells albeit with very low efficiency (Rolando *et al.*, 2010). We now addressed the question, if binding and translocation are enhanced by addition of a His_6-tag to C2I.

The stability constants K and K_s for the binding of His_6-C2I to the PA_{63}-channel were measured in multichannel experiments, performed as described previously (Neumeyer *et al.*, 2006b). A receptor is required for the binding and oligomerization of PA_{63} on the surface of mammalian cells (Young *et al.*, 2007). However, this is not necessary for reconstitution of PA_{63}-channels in artificial lipid bilayers, where channel formation is obtained under mildly acidic conditions (Finkelstein, 1994). 60 minutes after the addition of the protein to the *cis*-side of the lipid bilayer, the rate of conductance increase had slowed down considerably at an applied membrane potential of 20 mV. At that time, small amounts of a concentrated protein solution were added to the *cis*-side of the membrane and the PA_{63}-induced membrane conductance decreased in a stepwise manner.

Figure 1A shows an experiment of this type in which increasing concentrations of His_6-C2I (arrows) were added to the *cis*-side of a membrane containing about 300 PA_{63}-channels. The membrane conductance decreased as a function of the His_6-C2I concentration. The data of Figure 1A and of similar experiments were analyzed using equation 2.1 assuming Langmuir isotherms for binding (Benz *et al.*, 1987, Neumeyer *et al.*, 2006b, Benz *et al.*, 1986). Lineweaver-Burke plots were used to calculate the stability constant K for binding as shown in Figure 2.1B for the data of Figure 2.1A. The resulting curve corresponds to a stability constant K of $(3.85 \pm 0.52) \times 10^7\ M^{-1}$ for His_6-C2I binding to PA_{63}-pores.

At least three individual experiments were used to calculate the stability constant K of His_6-C2I binding to the PA_{63}-channel. The average of the stability constant K of C2I-binding was $(6.8 \pm 4.2) \times 10^6\ M^{-1}$ [half-saturation constant K_s = 150 nM] whereas the stability constant K for His_6-C2I to PA_{63}-channels averaged to $(6.2 \pm 4.2) \times 10^7\ M^{-1}$ [K_S = 16 nM] in 150 mM KCl. This means that the stability constant K for binding of His_6-C2I was roughly ten times higher than for C2I without His_6-tag (Table 2.1). Titration experiments with artificial bilayer membranes of the

wildtype A-B components C2II and C2I of C2-toxin revealed a binding constant K of (3.7 ± 0.4) × 10^7 M^{-1}, with a half saturation constant K_S of 27 nM. Interestingly, a His_6-tag attached to the N-terminal end had no obvious effect on binding of C2I to C2II-pores (Table 2.1).

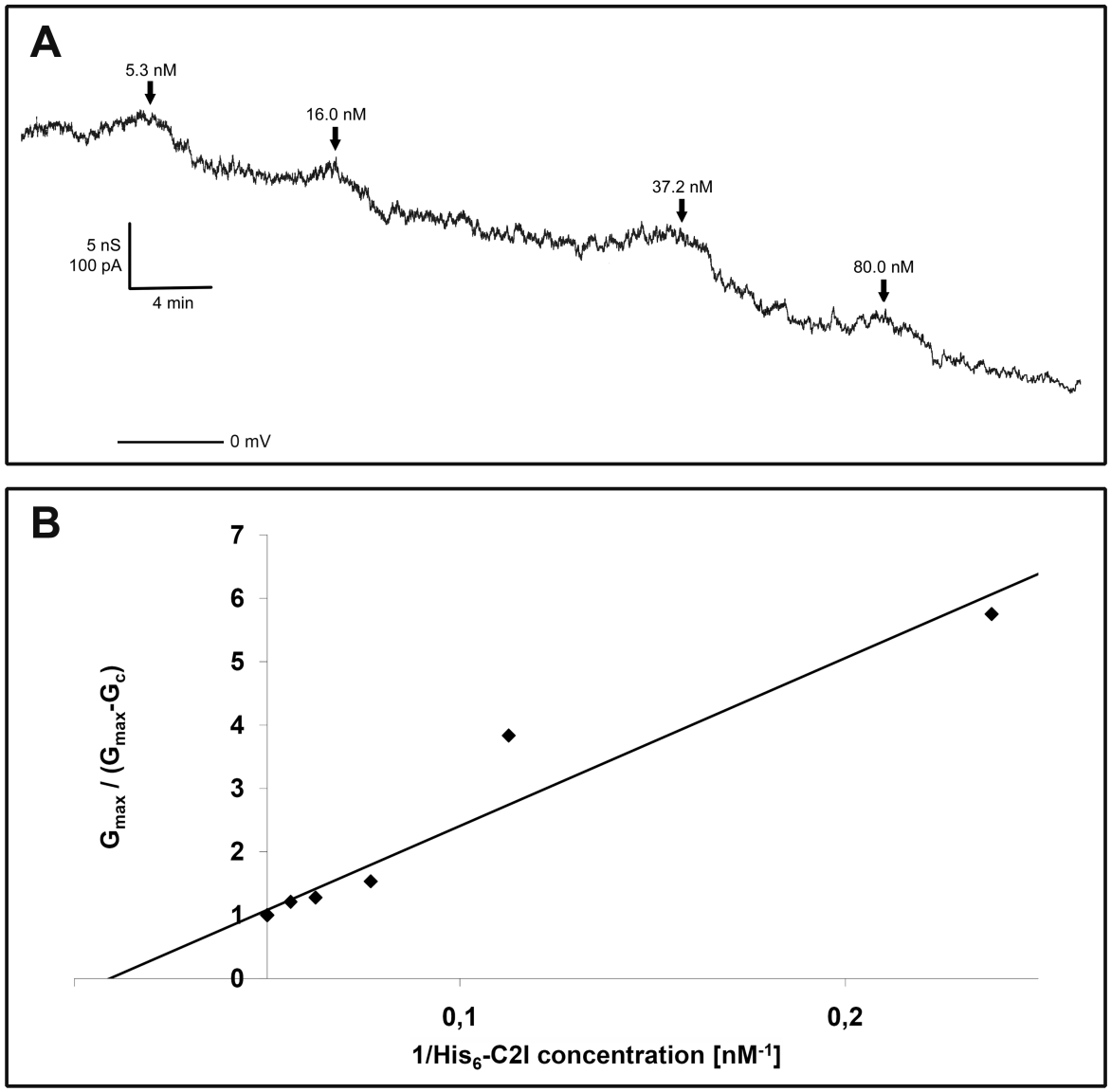

Fig. 2.1:
A: Titration of PA_{63} induced membrane conductance with His_6-C2I. The membrane was painted from diphytanoyl phosphatidylcholine/n-decane. It contained about 300 PA_{63}-channels. His_6-C2I was added at the concentrations shown at the top of the panel. Finally, about 83% of the PA_{63}-channels were blocked. The aqueous phase contained 1 ng/ml activated PA_{63} (added only to the *cis*-side of the membrane), 150 mM KCl, 10 mM MES pH 6. The temperature was 20°C and the applied voltage was 20 mV.
B: Lineweaver-Burke plot of the inhibition of the PA_{63}-induced membrane conductance by His_6-C2I. The fit was obtained by linear regression of the data points taken from Figure 1A and corresponds to a stability constant K for His_6-C2I binding to PA_{63} of (3.85 ± 0.52) × 10^7 M^{-1} (half saturation constant K_s = 180 nM).

Table 2.1: Stability constants K_s for the binding of either C2I, gpJ or EDIN to PA_{63}- or C2II-channels in lipid bilayer membranes.

		K_s [nM]			K_s [nM]
PA_{63} with	EF*	6.9		His_6-EF*	0.2
	LF*	2.8		His_6-LF*	0.2
	C2I	147.7		His_6-C2I	16
	gpJ	> 10 μM		His_6-gpJ	5
	EDIN	**2,700**		**His_6-EDIN**	**700**
		K_s [nM]			K_s [nM]
C2II with	EF**	13		His_6-EF	19.2
	LF**	49.9		His_6-LF	29
	C2I	27.2		His_6-C2I	29.1
	gpJ	no binding		His_6-gpJ	no binding
	EDIN	23,000		His_6-EDIN	900

The membranes were painted from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained 150 mM KCl, buffered to pH 5.5 to 6 using 10 mM MES-KOH; T = 20°C. Measurements were performed at a membrane potential of 20 mV. The data represent the means of at least three individual titration experiments. K_s is the half saturation constant, i.e. $1/K$. Stability constants given in bold were adjusted to the voltage dependent behavior of binding. (* taken from (Neumeyer *et al.*, 2006a) ** taken from (Rolando *et al.*, 2010))

2.4.2 Addition of His_6-tag to C2I potentiates its transfer via PA_{63}

In further experiments we tested if addition of His_6-tag to C2I triggers its entry into cells via PA_{63}-channels *in vivo*. C2I acts as an ADP-ribosyltransferase, targeting cellular G-actin (Aktories *et al.*, 1986). Therefore, successful delivery of this enzymatic component into target cells can be detected by disruption of the cytoskeleton followed by rounding up and detachment of target cells from the extracellular matrix, defined as intoxicated cells (Blocker *et al.*, 2003b). HUVECs were intoxicated with C2I and His_6-C2I driven by PA, as indicated, and the number of intoxicated cells was directly assessed by counting (Fig. 2.2A). These results were compared to that of native toxin combination C2I and C2II. We observed a cytotoxic effect with the combination of His_6-C2I and PA. No effect could be detected for C2I and PA under the same conditions. The specificity of this internalization was verified by using a mutant of PA_{63}: PA F427A. This mutant is competent for receptor binding and internalization, but defective in the pH-dependent functions: pore formation and ability to translocate bound ligand (Sun *et al.*, 2008). Intoxication of cells with His_6-C2I and PA F427A did not induce any cellular effect (Fig. 2.2B). Thus, the increase of affinity between PA and C2I, upon addition of His_6-tag to C2I allows His_6-C2I to efficiently intoxicate cells via PA_{63}-channels.

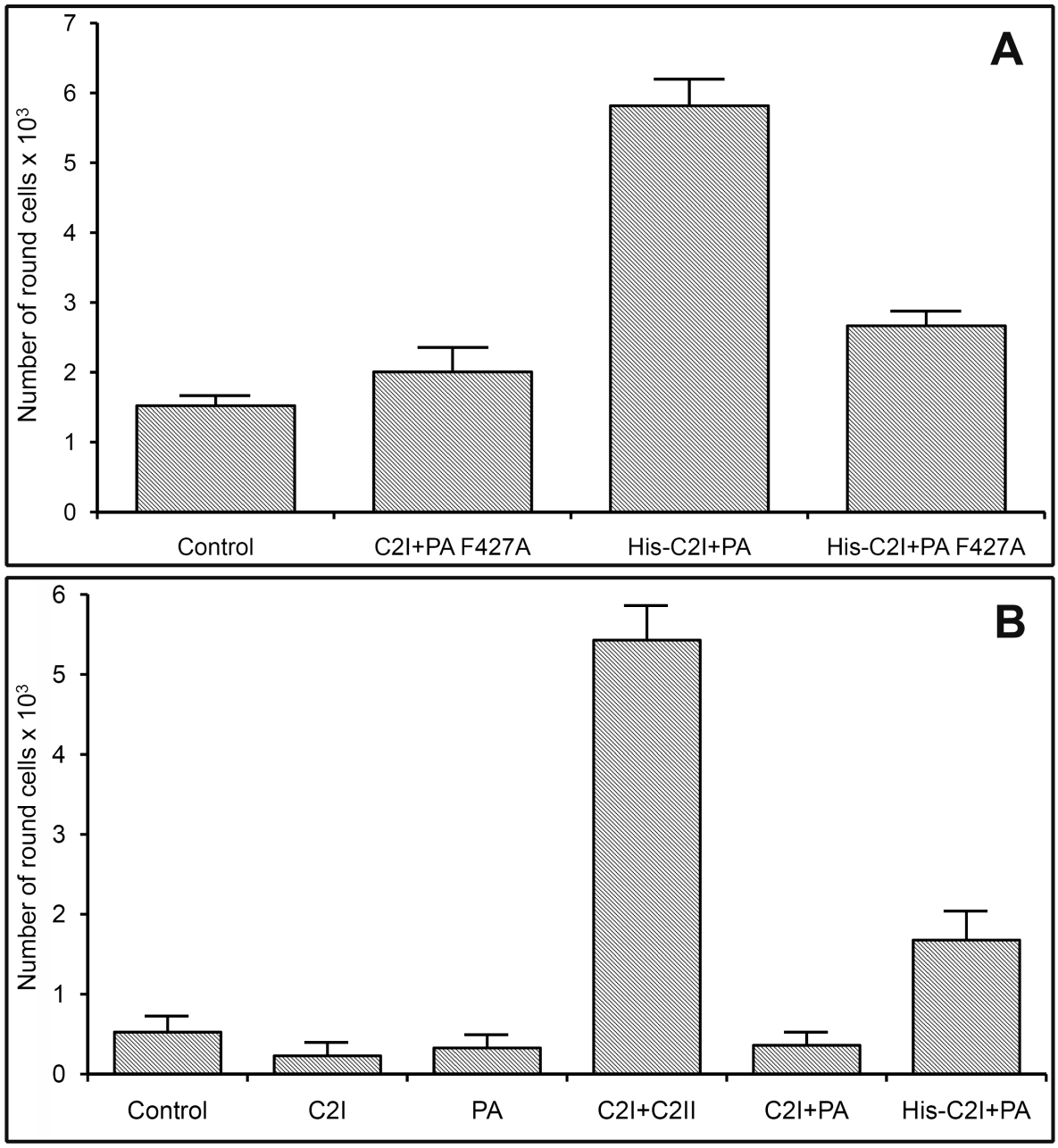

Fig. 2.2:
HUVECs (5×10^5 cells/100 mm well) were intoxicated during 24 hours and the number of intoxicated cells (round cells) was assessed by counting floating cells.
A: PA and C2II at 5 μg/ml and C2I and His$_6$-C2I at 2 μg/ml. One representative experiment showing mean values of 5 independent counting for each condition. ± SD *p < 0.05 versus control condition.
B: PA and PA F427A at 50 μg/ml and C2I and His$_6$-C2I at 2 μg/ml. mean values are of n = 3 experiments ± SD, *p < 0.05 versus control condition.

2.4.3 His$_6$-tag does not facilitate binding of EF and LF to C2II-channels

To examine whether the N-terminal His$_6$-tag of EF and LF have a similar effect on binding kinetics to the C2II-channel, as previously shown for His$_6$-EF and His$_6$-LF and PA$_{63}$ (Neumeyer *et al.*, 2006a), we omitted the cleavage of the His$_6$-tag after the affinity purification and studied binding to C2II-channels. Interestingly, His$_6$-EF and His$_6$-LF did not exhibit any significant

changes of their affinity to C2II-channels as compared to EF and LF. The binding constants *K* of the interactions between His_6-EF and His_6-LF and the C2II-channels were $(5.2 \pm 1.6) \times 10^7\ M^{-1}$ and $(3.4 \pm 1.9) \times 10^7\ M^{-1}$, respectively. The half saturation constants K_S were calculated to be 19 nM for His_6-EF and about 29 nM for His_6-LF (Table 2.1).

2.4.4 Binding of His_6-gpJ and gpJ proteins to PA_{63}- and C2II-channels

The His_6-tag had a remarkable influence on binding of enzymatic components to the PA_{63}-channel but not to the C2II-channel. To check if this interaction was specific for the presence of the His_6-tag we performed titration experiment with a His-tagged protein that is not related to the effectors EF, LF or C2I. gpJ is a 447 amino acids C-terminal fragment of protein J (amino acids 684-1131), which is responsible for binding of bacteriophage Lambda to LamB on the surface of *E. coli* K-12 (Berkane *et al.*, 2006). His_6-gpJ exhibited high affinity binding (block) to the PA_{63}-channel. The half saturation constant K_S for binding of His_6-gpJ to PA_{63} was calculated to be (5.0 ± 1.5) nM in 150 mM KCl, 10 mM MES, pH 6.0 (mean of three measurements) (Table 2.1). Similar experiments with gpJ obtained by cleavage of MBP-gpJ with factor X_a (i.e. without His_6-tag) did not exploit any binding of gpJ to the PA_{63}-channel. This implies half saturation constants K_s of gpJ-binding to PA_{63} were much higher than 10 μM. We could not detect any binding of His_6-gpJ nor of gpJ to the C2II-channel (Table 2.1). These results indicate the substantial role of the His_6-tag at the N-terminal end of polypeptides for their binding to the PA_{63}- but not to the C2II-channel.

2.4.5 Binding of EDIN and His_6-EDIN to PA_{63}- and C2II-channels

To further test if transport through PA_{63}-pores is enabled for unrelated proteins we investigated binding of the epidermal cell differentiation inhibitor EDIN of *Staphylococcus aureus* as well as His_6-EDIN. EDIN is a *Staphylococcus aureus* exoenzyme with ADP-ribosylating activity on RhoA. EDIN targets RhoA in cells for inactivation producing actin cable disruption in target cells (Boyer *et al.*, 2006). Interestingly, PA_{63}-pores bound both EDIN and His_6-EDIN with stability constants that were considerably lower than those reported before for the crossing over of the AB_7 types of toxin (Rolando *et al.*, 2010). The stability constant *K* for EDIN binding to PA_{63}-channels was on average $(4.0 \pm 1.1) \times 10^5\ M^{-1}$ ($K_S = 2.7$ μM) in 150 mM KCl, whereas this constant increased to $(1.4 \pm 0.15) \times 10^6\ M^{-1}$ ($K_S = 0.7$ μM) for His_6-EDIN. The results of these experiments are summarized in Table 1 and demonstrate that EDIN without His_6-tag bound at low trans-membrane voltage (5 mV) with a roughly three-fold lower affinity to the PA_{63}-channels than His_6-EDIN. When higher voltages were applied we noticed a remarkable effect of voltage

on His_6-EDIN binding (see below). The affinity of EDIN to the C2II-channels (K_s = 23 μM) was by a factor of about eight lower as compared to binding to the PA_{63}-channels. Surprisingly, we observed a considerable effect when the His_6-tag was attached to the N-terminal end of EDIN. The half saturation constant dropped in this case to 0.9 μM for its binding to C2II-pores (Table 2.1).

2.4.6 His_6-tag promotes EDIN internalization via PA_{63}-pores

We tested the affinity of the exoenzyme EDIN, a C3-like protein, which is unrelated to the AB_7-toxin family, to PA_{63}-channels. We next verified the role of His_6-tag in the uptake of EDIN into cells. After purification the His_6-tag was cleaved as described in the material and methods section. We verified the cleavage by immuno-bloting the purified proteins using an antibody against the His_6-tag (Fig. 2.3A). The efficiency of RhoA targeting by EDIN was assessed by GST-Rhotekin pull down of active RhoA (GTP-bound RhoA). No effect on cells was measured with His_6-EDIN (1 and 10 μg/ml) alone. A decrease of RhoA activity of 36% could be achieved at a higher dose of 100 μg/ml His_6-EDIN (Fig. 2.3B).

We then intoxicated cells with His_6-EDIN in the presence of PA. Strikingly, this revealed that the addition of PA with EDIN (10 μg/ml) increased the capacity of EDIN to intoxicate cells. This led us to test the role of His_6-tag on this effect. Cells were intoxicated with PA together with EDIN or His_6-EDIN. This clearly established that addition of His_6-tag to EDIN in presence of PA produced a 78% decrease of RhoA activation specifically (Fig. 2.3C). In conclusion, addition of His_6-tag to EDIN triggers its internalization via PA.

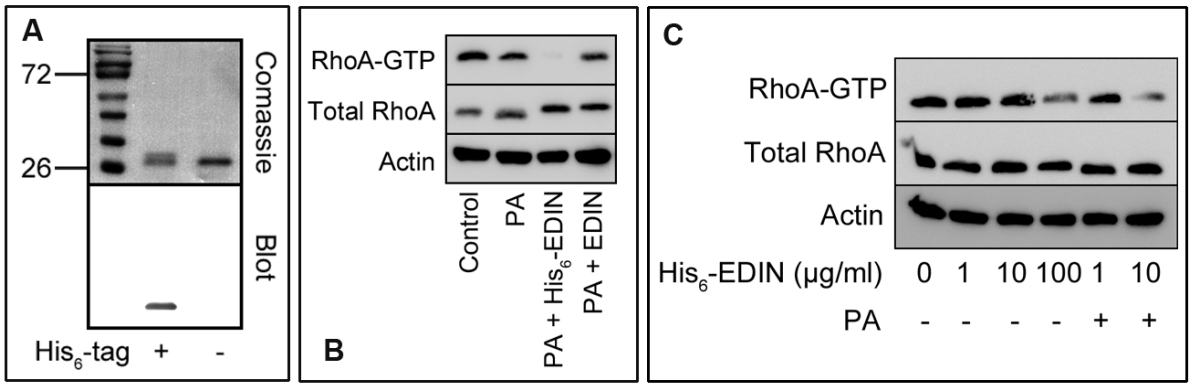

Fig. 2.3:
A: Upper panel: SDS-PAGE of recombinant His-tagged EDIN before and after thrombin treatment. (Lane Mw) 10-120 kDa pre-stained protein marker (Fermentas).
Lower panel: immuno-blot anti-His-tag on His-tagged EDIN before and after cleavage by thrombin.
B, C: Immuno-blots showing cellular levels of active RhoA (RhoA-GTP) in HUVECs determined by GST-Rhotekin RBD pull-down (labeled RhoA-GTP). Cellular content of RhoA (Total RhoA) was assessed by anti-RhoA on 2% of total protein extracts. Immuno-blot anti-actin antibody exhibits equal protein loading. **(B)** cells were intoxicated with different quantities of EDIN, His_6-EDIN, as indicated, and 3μg/ml of PA. **(C)** cell intoxicated with 10μg/ml of EDIN, His_6-EDIN and 3μg/ml of PA, as indicated.

2.4.7 The voltage dependency of PA_{63}-channels is changed when His_6-EDIN is bound to the pore

PA_{63}-channels exhibit a well described voltage dependency (Blaustein *et al.*, 1989). If only added to the *cis*-side, PA_{63}-induced conductivity decreases when applied voltage is higher than +50 mV or lower than -20 mV at the *cis*-side. It is also known that His_6-EF bound to the channel changes the voltage dependency (Neumeyer *et al.*, 2006a). When different potentials were applied to membranes after the titration of PA_{63}-pores with EDIN, there was only little change in voltage dependency of the channel (Fig. 2.4A). On the other hand, His_6-EDIN bound to PA_{63}-channels induced dramatic responses even at low positive voltages (Fig. 2.4B).

Starting at +10 mV, the conductivity decreased exponentially immediately after the onset of the voltage with a voltage-dependent exponential relaxation time. Its time constant decreased with higher positive potentials at the cis-side (negative at the trans-side). This result indicated that channels, which were not blocked before by His_6-EDIN at low voltage bound this compound and closed as a result of the higher voltage. This result suggested an increase of the stability constant of binding up to very high voltages an effect that has already been observed with full length EF (Neumeyer *et al.*, 2006a).

The increase of the stability constant for binding could be calculated from the data of Figures 2.4A and 2.4B and similar experiments by dividing the initial current (which was a linear function of voltage) by the stationary current after the exponential relaxation and multiplying the ratio with the stability constant derived at 5 mV. Figure 2.5 summarizes the effect of the positive membrane potential on the stability constant K for EDIN and His_6-EDIN binding as a function of the voltage. Starting already with -10 mV at the trans-side the stability constant K for His_6-EDIN binding started to increase and reached with about 60 to 70 mV a maximum. At that voltage K was roughly 25 times greater than at 5 mV. For higher voltages the stability constant saturated probably because of secondary effects of the high voltage on the PA_{63}-channel or on His_6-EDIN binding. Figure 2.5 shows also the effect of the positive membrane potential at the *cis*-side on the stability constant K for EDIN binding to PA_{63}-pores as a function of the voltage. Interestingly, EDIN binding was only little affected by voltage as Figure 2.5 clearly indicated.

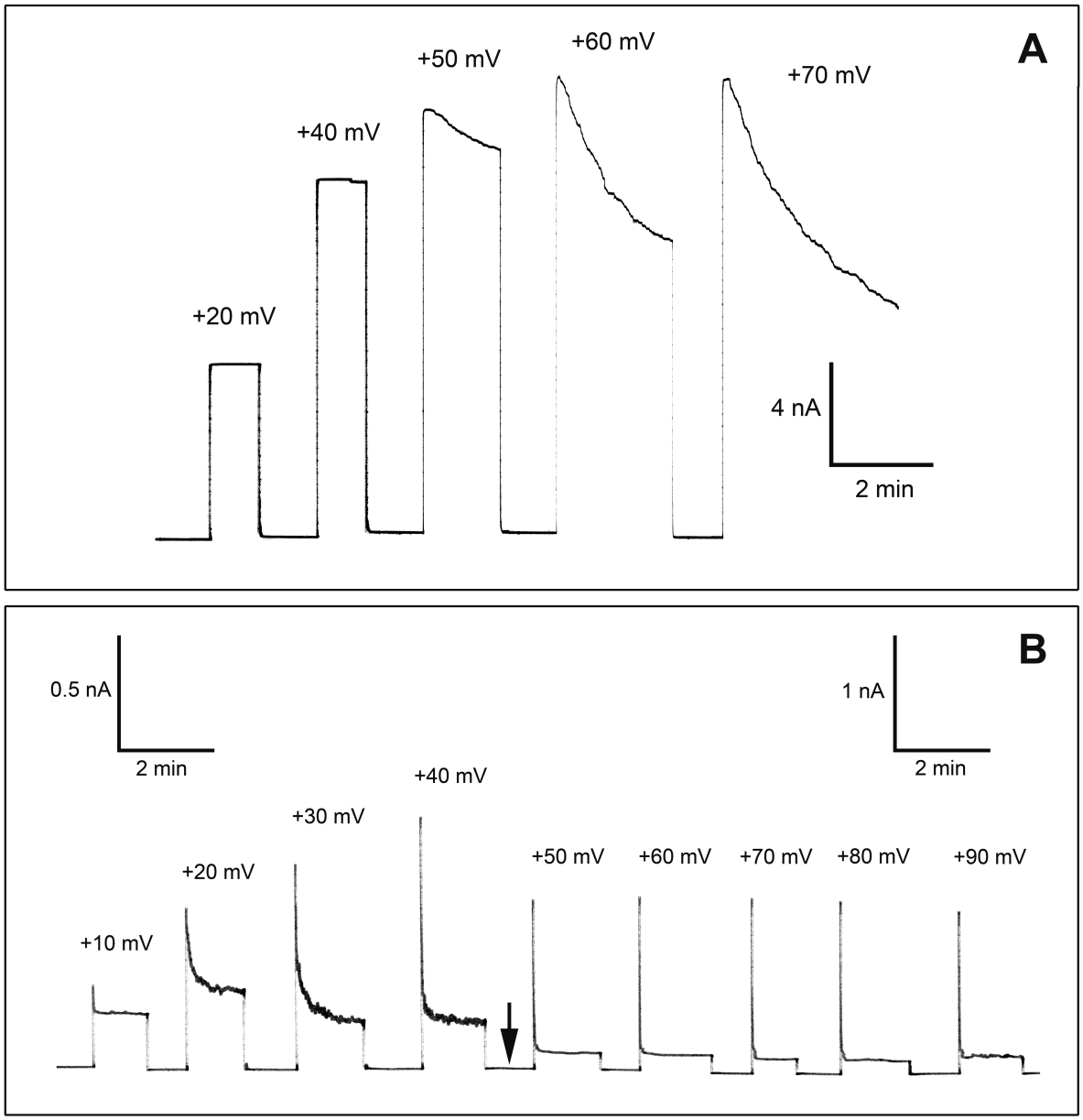

Fig. 2.4:

A: Current response of PA_{63}-channels in presence of EDIN. Voltage pulses between +20 and +70 mV were applied to a diphytanoyl phosphatidylcholine/n-decane membrane in the presence of PA_{63}-pores and EDIN (both added only to the cis side of the membrane). The aqueous phase contained 150 mM KCl, 10 mM MES, pH 6. The temperature was 20 °C.

B: Current response of PA channels in the presence of His_6-EDIN. Voltage pulses between +10 and +90 mV were applied to a diphytanoyl phosphatidylcholine/n-decane membrane in the presence of PA_{63}-pores and His_6-EDIN (both added only to the cis side of the membrane). The aqueous phase contained 150mM KCl, 10 mM MES, pH 6. The temperature was 20 °C. Note the change of the scale (Arrow).

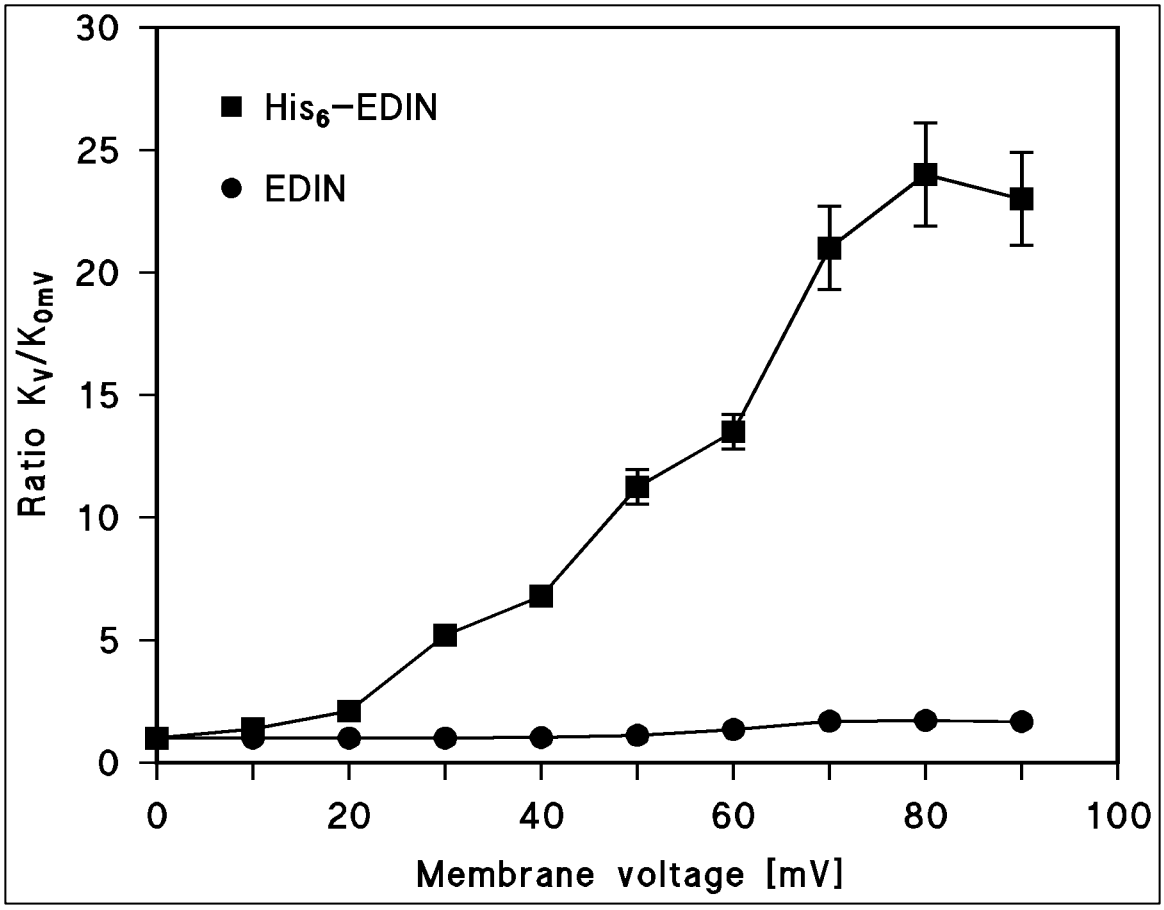

Fig. 2.5:
The stability constants of EDIN and His_6-EDIN binding to the PA_{63}-channel are given as a function of the applied membrane potential taken from experiments similar to that shown in Fig. 4A/B. Means of three experiments are shown.

2.5 Discussion

2.5.1 His_6-tag addition to several bacterial factors increased the protein binding affinity to PA_{63}- but not to C2II channels

Recent studies demonstrated that negatively charged amino acids in the vestibule of the PA_{63}-channel play a crucial role in binding of effector molecules (Orlik *et al.*, 2005, Leuber *et al.*, 2008). Thus, it is possible that a His_6-tag, which adds positive charges under mildly acidic conditions to the N-terminal end of His_6-EF and His_6-LF affects binding and transport. This has indeed been shown for the native combinations of EF+PA_{63} or LF+PA_{63} and the potential ion-ion interaction discussed with EF_N (Neumeyer *et al.*, 2006a, Neumeyer *et al.*, 2006b, Zhang *et al.*, 2004b, Zhang *et al.*, 2004a). Recently, we could show that C2I binds to PA_{63} and may even be transported into target cells albeit at high PA_{63} concentration and with very low efficiency compared with the native combination of C2I with C2II (Kronhardt *et al.*, 2011). Here we studied the cross reactivity of Anthrax- and C2-toxin in more detail and found a strong relation between binding affinity and the presence of a His_6-tag at the N-terminal end of the enzymatic components. The addition of positive charges at the N-terminal end of C2I (due to the partially charged histidines) enhanced binding to and translocation into target cells via PA_{63}-pores and agreed very well with the findings previously reported for His_6-tags attached to EF and to LF (Neumeyer *et al.*, 2006a, Neumeyer *et al.*, 2006b). Binding to PA_{63}-channels was found to be strongly enhanced for the three enzymatic components EF, LF and C2I when they contained a His_6-tag at the N-terminal end.

Interestingly, we did not observe major effects if these His-tagged proteins were combined with C2II-channels. The results of binding experiments with His_6-EF and His_6-LF to C2II-channels suggested that the increased positive charge at the N-terminal end, due to the partially charged histidines, did not increase the binding of these enzymatic components to the C2II-channels. In agreement with these observations the C2II-channel was not able to transport His_6-EF and His_6-LF into cells. These results definitely imply that binding of the enzymatic components to PA_{63}-channels occurs in a different way than binding to C2II-channels.

To gain deeper insight in the influence of N-terminal His_6-tags on binding of proteins to PA_{63}-channels we choose a protein that was not related to any of the enzymatic components used in this study. gpJ-protein is a truncated form (amino acids 684-1132) of the tail protein J of the Lambda phage, which is responsible for the binding of the phage tail to LamB-porin on the cell surface of enteric gram-negative bacteria (Berkane *et al.*, 2006). As expected, we did not observe any binding of gpJ to PA_{63}- or C2II-channels (K_S >>10 μM). However, the whole story changed completely when a His_6-tag was attached to the N-terminal end of gpJ. This protein had a half-

saturation constant for binding to the PA_{63}-channels of 5 nM, which suggested that the affinity of His_6-gpJ to PA_{63}-channels was almost the same as that of LF and EF (Neumeyer *et al.*, 2006a, Neumeyer *et al.*, 2006b). This means that the affinity increase is mainly determined by the positive charges of the His_6-tag. It is interesting to note that His_6-gpJ did accordingly not interact with C2II-channels; revealing again for a somewhat different process for binding of His_6-tagged proteins to PA_{63}-channels than to C2II-channels.

2.5.2 Influence of the His_6 tag on uptake of EF, LF and C2I into cells

The binding step is necessary, but not sufficient for the delivery of enzymatic subunits into target cells. Thus, in order to complement the results of binding studies we went on to investigate the translocation by analyzing the enzymatic activity in a cellular system. We verified that a His_6-tag attached to the N-terminal end of C2I increased its transport by PA_{63}-channels, which correlates with the difference of 10-fold measured between the binding constants of C2I and His_6-C2I. No difference in transport was observed by using EF or LF with or without a His_6-tag in combination with PA_{63}-channels. This discrepancy may be due to the already existing high affinity of LF/EF N-terminal domains to PA_{63}-heptamers (Neumeyer *et al.*, 2008, Neumeyer *et al.*, 2006a). Although the binding of EF_N and LF_N (truncated forms of EF and LF) to the PA_{63}-channel is substantially weaker as compared to wild-type enzymatic components (Leuber *et al.*, 2008), those proteins interact with high affinity with the PA_{63}-channels and are accordingly transported into the cell (Mogridge *et al.*, 2002a, Elliott *et al.*, 2000, Zhang *et al.*, 2004b). Similarly, short stretches of positively charged amino acids attached to the N-terminal end of foreign proteins can lead to a PA_{63}-dependent delivery as it has been demonstrated for the amino terminus of the enzymatic A chain of diphtheria toxin (DTA; residues 1-193) or for EDIN (Blanke *et al.*, 1996, Rolando *et al.*, 2009).

2.5.3 Bound His_6-EDIN or EDIN causes a difference in voltage-dependency of PA_{63}-pores

Experiments with the epidermal cell differentiation inhibitor EDIN of *S. aureus* were performed to gain deeper insight in the binding and translocation processes through PA_{63}-channels and its His_6-tag dependence. Surprisingly, black lipid bilayer experiments displayed that not only His_6-EDIN but also EDIN itself bound to PA_{63}-channels. The affinity of EDIN and His_6-EDIN to the PA_{63}-channels was in the same range at low trans-membrane potentials because His_6-EDIN exhibited only a three times higher affinity for binding to the PA_{63}-channels than

EDIN. Under normal conditions the PA_{63}-channels only close for higher negative voltages applied to the *cis*-side (Neumeyer *et al.*, 2006a). For positive potential the channels are open and do not show a voltage-dependent closure until 100-150 mV (Neumeyer *et al.*, 2006a). However, His_6-EDIN binding to the PA_{63}-channels showed an extremely high voltage-dependence when the voltage was positive at the cis-side of the membrane indicating that the potential pulled His_6-EDIN into the channels. As a result the stability constant for binding of His_6-EDIN to the PA_{63}-channels increased at voltages of +70 mV at the cis-side (corresponding to -70 mV at the trans-side) by a factor of roughly 25 as compared to zero voltage. Bound EDIN displayed an only minor voltage-dependence. This means that the His_6-tag is responsible for the binding of all these foreign proteins to the PA_{63}-channels. Binding is presumably essential for translocation because it is the first step of the whole process (see below).

2.5.4 The PA_{63}-channel transports His_6-C2I and His_6-EDIN into the HUVECs

EDIN uptake into target cells can easily be detected because it decreases RhoA activity. Import of EDIN via PA_{63}-channels could not be observed. Import was however, possible when EDIN contained a His_6-tag. This finding demonstrated that His_6-tag itself provides the ability for proteins to be transported into cells through PA_{63}-pores. This effect was presumably promoted by the voltage-dependence of His_6-EDIN binding to the PA_{63}-channels. Biological membranes are polarized to about -60 mV to -70 mV (inside negative). This may explain the much higher effect of His_6-EDIN compared to EDIN on cells described above. In any case it clearly indicates the potentiating effect of a His_6-tag and applied voltage on binding and translocation of protein molecules to PA_{63}-channels. Summarizing the results, there definitely exists a difference in the binding and translocation mechanism between the two very homologous binding components PA_{63} and C2II of Anthrax- and C2-toxin. Obviously, this distinction is induced by unequal binding surroundings inside the head region of the two protein channels.

The amino acids responsible for binding within the N-terminal end of the enzymatic components are relatively unknown, although there is clear evidence that positively charged amino acids are involved in binding, forming salt bridges between the enzymatic components and the heptamers. The positively charged N-termini of the enzymes presumably play a crucial role, because quaternary ammonium ions and 4-aminoquinolones show PA_{63} and C2II channel block in lipid bilayer experiments (Orlik *et al.*, 2005, Bachmeyer *et al.*, 2003, Blaustein *et al.*, 1990, Finkelstein, 1994). Both channels show a high selectivity for cations, i.e. cations have a strong influence on the single channel conductance as compared to anions (Blaustein *et al.*, 1989, Schmid *et al.*, 1994). This means that negative charged amino acids play a crucial role in the

binding and constriction region of the PA_{63}-channels, where they form two rings of seven putative negatively charged amino acids in the vestibule of this pore (E398 and D426). Similarly, the channel lumen contains additional three rings of seven possibly negatively charged groups (E302, E308 and D315). Some of these charges cannot be found in the C2II-channel lumen, resulting in minor effects of His_6-tag on binding and transport. However, transport into cells seems to be possible with C2II-pores and when N-terminal parts of C2I are coupled to foreign proteins (Barth *et al.*, 2004, Barth *et al.*, 2002a, Barth *et al.*, 2002b). The most interesting result of this study was that we could use the anthrax PA_{63}-channels to deliver into cells a polypeptide completely unrelated to the AB-type toxins. In fact, we here provide evidence that the His_6-tag addition on EDIN allows its entry in target cells, in a PA-dependent manner. Thus it seems possible to design a very simple transportation system using His_6-tag on proteins unrelated to the AB_7-family and PA_{63}-channels. Further tests of His_6-tagged proteins and PA *in vitro* and *in vivo* have to proof if this potential mechanism could be used for biological purposes.

Chapter 3

Binding partners of protective antigen from Bacillus anthracis share certain common motives

This work was published in *Toxins and Ion transfers* and is used here in agreement with all authors.

General considerations regarding Anthrax toxin's PA-channel and the processes of binding and transport via this very interesting pore, lead to the assumption, that certain common motives are shared between different substrates. These range from ions in electrolytes to protein-effectors and could serve as blockers or cargo.

3.1 Summary

Binary toxins of the AB_7-type are of special interest for scientific investigations as they are among the most potent and specialized bacterial protein toxins. Initiated with the purpose to find cure against Anthrax toxin intoxication, nowadays the focus shifted to the investigation of the sophisticated transport mechanism of these molecular syringes. In the intoxication process, the B-subunits multimerize to form a pore that binds with high affinity to host cell receptors and the A-subunits. Followed by endocytosis of the complex into cells the translocation of the enzymatic component into the cytosol occurs upon acidification of the endosomes. Recent publications elucidate that the forces involved in transport include binding affinity, proton gradient and voltage across the endosomal membrane. The data presented here focus on different binding partners of *Bacillus anthracis* protective antigen (PA), which range from ions and small molecule inhibitors to effector proteins related or unrelated to the AB_7-type of toxins. Thereby, possible ways to block intoxication by anthrax toxin or to use anthrax PA as specific transportation system are discussed.

3.2 Introduction

Anthrax toxin represents one of the main virulence factors of *Bacillus anthracis*. The plasmid-encoded tripartite toxin comprises a receptor-binding moiety termed protective antigen (PA) and two intracellular active enzymes, edema factor (EF) and lethal factor (LF) (Dain *et al.,* Habki *et al.*, 2001, Mock *et al.*, 2001). EF is a calcium and calmodulin-dependent adenylate-cyclase (89 kDa) that causes a dramatic increase of intracellular cAMP level, upsetting water homeostasis and destroying the balance of intracellular signaling pathways (Mock *et al.*, 2001, Lacy *et al.*, 2002, Dixon *et al.*, 1999). LF is a highly specific zinc metalloprotease (90kDa) that removes specifically the N-terminal tail of mitogen-activated protein kinase kinases (MAPKKs) (Dain *et al.,* Turk, 2007, Abramova *et al.*). This cleavage leads to subsequent cell death by apoptosis.

Protective antigen (PA) is a cysteine-free 83 kDa protein that binds to two possible receptors, a ubiquitously expressed integral membrane receptor (ATR) and also to the LDL receptor-related protein LRP6, which can both be involved in anthrax toxin internalization (Schusdziarra *et al.*, 2011, Wei *et al.*, 2006). PA_{83} present in the serum or bound to receptors is processed by furin to a 63 kDa protein PA_{63} (Croney *et al.*, 2003, Petosa *et al.*, 1997). PA_{63} spontaneously oligomerizes in the serum and/or on the cell surface into a heptamer or octamere (Petosa *et al.*, 1997, Abramovic *et al.*) and binds EF and/or LF with very high affinity (Cunningham *et al.*, 2002, Escuyer *et al.*, 1991, Elliott *et al.*, 2000). The assembled toxic complexes are then endocytosed and directed to endosomes. There, low pH results in the translocation of EF and LF across the endosomal membrane. Combined with acidification is channel formation by PA_{63}, which could represent the mechanism for the translocation scheme of the toxins (Finkelstein, 1994, Abrami *et al.*, 2005, Zhang *et al.*, 2004b, Miller *et al.*, 1999).

3.3 Material and Methods

3.3.1 Materials

Recombinant, nicked anthrax protein PA_{63} from *B. anthracis* was obtained from List Biological Laboratories Inc., Campbell, CA. One mg of lyophilized protein was dissolved in 1 ml 5 mM HEPES, 50 mM NaCl, pH 7.5 complemented with 1.25% trehalose. Aliquots were stored at -20°C.

3.3.2 Lipid bilayer experiments

Black lipid bilayer membranes were formed as described previously (Benz *et al.*, 1978). The instrumentation consisted of a Teflon chamber with two aqueous compartments connected by a small circular hole. The hole had a surface area of about 0.4 mm^2. Membranes were formed by painting onto the hole a 1% solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) in n-decane. The aqueous salt solutions (Merck, Darmstadt, Germany) were buffered with 10 mM MES-KOH, pH 6. Control experiments revealed that the pH was stable during the time course of the experiments. PA_{63} was reconstituted into the lipid bilayer membranes by adding concentrated stock solutions to the aqueous phase to the *cis*-side of a membrane in the black state. Channel reconstitution reached its maximum between 60 to 120 minutes after addition of PA to the *cis*-side.

3.3.3 Titration experiments

Membrane conductance was measured after application of a fixed membrane potential from a battery-operated voltage source with a pair of silver/silver chloride electrodes with salt bridges inserted into the aqueous solutions on both sides of the membrane. The membrane current was measured with a home made current-to-voltage converter using a Burr Brown operational amplifier with feedback resistors between 0.1 and 10 GΩ. The potentials applied to the membranes throughout the study always refer to those applied to the *cis*-side, the side of addition of PA. Similarly, positive currents were caused by positive potentials at the *cis*-side and negative ones by negative potentials at the same side. The temperature was kept at 20°C throughout.

Titration experiments were performed with membranes containing only a few or many PA_{63}-channels. The amplified signal was recorded with a strip chart recorder to measure the absolute magnitude of the membrane current and to calculate the stability constant *K* for substrate binding to PA. The conductance data of the titration experiments were analyzed using a formalism derived earlier for the carbohydrate-induced block of the maltoporin and CymA channels (Benz *et al.*, 1987, Gao-Sheridan *et al.*, 2003, Menard *et al.*, 1996b) and the block of the PA_{63}-channels with LF and EF (Neumeyer *et al.*, 2006a, Neumeyer *et al.*, 2006b). The conductance, G(c), at a given concentration c of substrates relative to the initial conductance, G_{max} (in the absence of substrates), was analyzed using the following equation:

$$\frac{G_{\max} - G(c)}{G_{\max}} = \frac{K \cdot c}{K \cdot c + 1} \tag{3.1}$$

K is the stability constant for the binding of substrates to the PA_{63}-channel. The half saturation constant, K_s, of its binding is given by the inverse stability constant $1/K$.

3.4 Results: Known binding substrates of protective antigen

There are different substrates, which are characterized to bind to protective antigen. These are proteins, related or not related to the AB_7-type toxins, and small molecule inhibitors.

3.4.1 Native effector proteins of protective antigen

Full length EF and LF

Anthrax toxin consists of the binding and translocation component protective antigen (PA) and the two enzymatic components edema factor (EF) and lethal factor (LF). They both bind to the same motif located in domain 1 of the PA_{63}-prepore (Zhang *et al.*, 2004c, Lacy *et al.*, 2002). As two monomers of the heptameric prepore are required to bind one enzymatic component (Cunningham *et al.*, 2002) the heptameric form of the PA-channel is able to bind up to three molecules at the same time (Mogridge *et al.*, 2002a), whereas the PA octamere provides up to four binding sites (Abramovic *et al.*). Both EF and LF attach with their N-terminal end to PA. Arora and Leppla (2000b) could show that the N-terminal domain is sufficient to bind to PA and also to translocate fusion proteins. Recently, Feld and colleagues demonstrated that LF initially binds with its N-terminal domain to an amphipatic cleft on the surface of the PA_{63}-prepore, the so called α-cleft (Abramovic *et al.*).

In lipid bilayer membranes titration experiments revealed that binding only occurred when EF and LF were added to the same side as PA_{63} (the *cis*-side of the membrane), substrate given to the *trans*-side did not show any effect indicating that the PA-pore only possesses one binding site within the mushroom body (Neumeyer *et al.*, 2006b, Neumeyer *et al.*, 2006a). The conductance decreased in a dose-dependent manner. The affinity to the PA-pore is in the low nanomolar range and it could be shown that the block of PA is a single hit process. As the binding is ionic strength-dependent the K_s values increase by a factor of about 500 from 50 mM to 1000 mM KCl electrolyte concentration.

EF_N and LF_N

EF's and LF's N-terminal fragments called EF_N and LF_N as well as fusion proteins are able to bind to PA-channels, e.g. LF_N-DTA (LF fused to diphtheria toxin), and are translocated through the pore into the cytosol of target cells (Rostovtseva *et al.*, 2000). Therefore, the truncated components EF_N and LF_N were supposed to have similar binding properties as full length EF and LF. However, the affinity of the truncated proteins is tenfold weaker compared to full length

EF and LF indicating that further interactions of the C-terminal region of EF and LF are involved in the binding process (Leuber *et al.*, 2008).

His-tagged proteins

Several studies elucidated that an N-terminal His_6-tag attached to EF or LF increases the binding affinity to the PA-channel (Neumeyer *et al.*, 2006a, Rostovtseva *et al.*, 2000, Neumeyer *et al.*, 2006b). As the binding is due to interactions between negative charges of the PA-pore and positive charges of the enzymatic components, additional positive charges of the His_6-tag enhance the binding of the truncated EF_N and LF_N to the PA-channel as well by a factor of about 10 (Leuber *et al.*, 2008).

3.4.2 Cross-reactivity of anthrax- and C2-toxin

Binding of close related proteins

Another prominent member of the AB_7-toxin family is the C2-toxin of *Clostridium botulinum*. It performs a very similar mode of intoxication and the channel forming components C2II and PA exhibit about 35% amino acid homology. To test if these two toxins are also functionally interchangeable, cross-reaction experiments were performed by combining the channel forming component of one toxin with the respective enzymatic component of the other toxin (Kronhardt *et al.*, 2011). In lipid bilayer experiments binding could be observed for each combination, however, anthrax EF and LF had higher binding affinities to the C2II-channel than C2I to the PA-channel. *In vitro* experiments revealed that PA is not only able to bind but also to translocate the enzymatic component C2I of C2-toxin resulting in intoxication and cell death. The combination of C2II and EF or LF, respectively, merely led to toxic effects when exposed to HUVEC cells (Kronhardt *et al.*, 2011). Due to this high flexibility PA is an extremely interesting protein for a general transport system across membranes.

Binding of unrelated proteins is enabled by His_6-tag

It was shown before that polycationic peptides fused to diphtheria toxin (DTA) enhances the uptake of this protein *via* PA-pores (Rostovtseva *et al.*, 2000). This work focused on different, charged tags, which exhibited either no change in affinity for Glu_6-tag and random sequence (compared to untagged DTA) or increased binding for His_6-, Arg_6- and Lys-tags of different length. With the knowledge of the binding properties of his-tagged native effectors, the DTA experiments and cross-reactivity of C2I, the next step was to check for His_6-C2I. The affinity to PA *in vivo* and *in vitro* was strongly increased as expected (Abramo *et al.*). Following this set of

experiments a protein fragment of Lambda phage protein (gPJ) not related to any toxins was tested. Whereas gpJ was not able to bind to PA, its affinity towards the PA-channel was in the range of EF and LF when it was coupled to a His_6-tag (Abramo *et al.*). Finally, the authors could show similar results for EDIN of *Staphylococcus aureus*, which ADP-ribosylates and inactivates Rho GTP binding proteins. His_6-EDIN binds to the PA-channel in titration experiments and is transported through the PA-channel in intoxication assays. Additionally, it has been found that the process is highly voltage-dependent. This means that PA-pores may be used as molecular syringes, which deliver His_6-tagged target proteins into cells possessing the known receptors for PA (Knapp *et al.*, 2002).

3.4.3 Small molecule inhibitors

Chloroquine and other 4-aminoquinolones block protective antigen

Anthrax toxin is one of the most potent bacterial toxins and could be used as a biological weapon or for terroristic activities by spreading spores of multi resistant *B. anthracis* bacteria (Keim *et al.*, 2001, Inglesby *et al.*, 2002, Jernigan *et al.*, 2002). This threat could be handled by introducing small molecules which are able to block PA-pores and efficiently prohibit translocation of the effectors, therefore buying time to deal with the bacterial infection. First results were found for chloroquine and other 4-aminoquinolones formerly used as antibiotics (Lewis *et al.*, 1973, Vedy, 1975). These substrates depicted high affinity binding in titration experiments to PA (Orlik *et al.*, 2005). Additionally, it is well known that chloroquine acquires positive charges under acidic condition and accumulates in endosomes (Neumeyer *et al.*, 2008). Both effects would enhance the blockage of PA *in vivo*. Concerning the side effects of chloroquine and related substances on humans, there is the urge for blocker-molecules with homologous structure, which do not exhibit cell toxicity.

Cyclodextrin-complexes form a plug for the PA-pore

Cyclodextrins have been found to bind to CymA-porin of *Klebsiella oyxtoca* (Pajatsch *et al.*, 1999, Gao-Sheridan *et al.*, 2003). The ring-shaped complex of seven glucose units in β-cyclodextrin happens to be in a perfect size for the blockage of binary toxin channels and is itself not toxic at all. Additionally, β-cyclodextrin and PA share a sevenfold symmetry, which offers one side chain of β-cyclodextrin for each PA_{63} monomer. Therefore, β-cyclodextrin has been tested as a basis drug for PA blockage (Nestorovich *et al.*, 2010). Recently, experiments with β-cyclodextrin and C2II – a very homologous AB_7-toxin-channel as described before – were performed in a trial of

modern, literature based drug design. In this study, changes in the outward facing part of the rings functional groups led to enhanced binding stabilities. Interestingly, the introduction of a positive charge and some aromatic residues were found to be responsible for this effect (Nestorovich *et al.*, 2011). The possible seven charges in the β-cyclodextrin structure seem to match with the PA binding pocket. Even though the authors could show blockage of intoxication in cell-based assays, the seven permanent charges could avoid specificity or passage through membranes *in vivo*, which reasons in the necessity of further pharmacological studies.

3.5 Results: Binding of divalent and trivalent cations to protective antigen

The PA-channel is known to be highly cation selective (Blaustein *et al.*, 1989) and additional positive charges of His_6-tags increase the binding properties of several proteins (Rostovtseva *et al.*, 2000, Abramo *et al.,* Leuber *et al.*, 2008, Neumeyer *et al.*, 2006a, Neumeyer *et al.*, 2006b). Therefore, we addressed the question if also divalent and trivalent cations are able to bind and to block the PA_{63}-channel.

The binding of $CuSO_4$, $ZnCl_2$, $NiCl_2$ and $LaCl_3$ to the PA_{63}-channel was investigated by performing titration experiments similar to those described for binding of EF and LF (Boquet *et al.*, 2003, Neumeyer *et al.*, 2006a, Neumeyer *et al.*, 2006b). After reconstitution of the PA-channels into the *cis*-side of a lipid bilayer membrane, the rate of insertions became very small. Then, concentrated solutions of divalent or trivalent cations were added to the *cis*- or the *trans*-side of the membrane, respectively, while stirring to allow equilibration. The membrane conductance decreased in a dose-dependent manner meaning that the cations bound to the PA-channel and thereby reduced the conductance. Analysis of the titration experiments by Lineweaver-Burke plots according to equation 1 indicated that the interaction between the cations and the PA-channel represents a single hit binding process. The results shown in Table 1 reveal that there are considerable differences concerning the stability constants of the respective cations to the PA-pore. Highest binding affinity was observed for Cu^{2+}, followed by La^{3+}, which was in the micromolar range, whereas the binding affinity of Ni^{2+} and Zn^{2+} were in the millimolar range. The binding constants of the divalent and trivalent cations to the PA-channel decreased in the series $K_{Cu} > K_{La} > K_{Zn} > K_{Ni}$ from about 10,000 M^{-1} to about 100 M^{-1} in 150 mM KCl (Table 3.1).

Table 3.1: Stability constants K of Cu^{2+} to PA_{63}-channels reconstituted in lipid bilayer membranes.

PA_{63} with		K [M^{-1}]	K_s [mM]
Cu^{2+}	cis +10mV	9237	0.11
	cis -10mV	7244	0.14
	trans +10mV	5254	0.19
Ni^{2+}	cis +10mV	122	8.2
	cis -10mV	47	21.3
	trans +10mV	65	15.4
Zn^{2+}	cis +10mV	1246	0.8
	cis -10mV	307	3.3
	trans +10mV	244	4.1
La^{3+}	cis +10mV	1383	0.7
	cis -10mV	654	1.5

The membranes were formed from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained four different KCl-concentrations, 10 mM MES-KOH pH 6.0; T = 20°C. The data represent means of at least three titration experiments. K_s is the half saturation constant, calculated as $1/K$. Note that the ionic strength had a considerable influence on the stability constant of binding of copper ions to the PA_{63}-channel.

Binding to the PA-channel is generally supposed to rely on ion-ion interaction. Therefore, we performed titration experiments for binding of Cu^{2+} in various electrolyte concentrations to check if this was also true for the binding of the divalent cations. The stability constants K for Cu^{2+} binding to the PA-channel decreased with increasing electrolyte concentration from about 80,000 M^{-1} at 50 mM KCl to about 1,500 mM M^{-1} at 1 M KCl (Table 3.2). That means that the stability constant of copper ion binding to the PA-channel is strongly ionic-strength dependent.

Table 3.2: Stability constants K of Cu^{2+}, Ni^{2+}, Zn^{2+} and La^{3+} to PA_{63}-channels reconstituted in lipid bilayer membranes.

PA_{63} with		K [M^{-1}]	K_s [mM]
Cu^{2+}	50 mM	87935	0.01
	150 mM	9237	0.11
	300 mM	3638	0.27
	1 M	1626	0.62

The membranes were formed from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained 150 mM KCl, 10 mM MES-KOH pH 6.0; T=20°C. The voltage was applied as indicated. The data represent the means of at least three individual experiments. K_s is the half saturation constant, calculated as $1/K$.

Interestingly, the binding of the cations was barely influenced by the side of addition. Irrespectively of the side of addition, the stability constants were nearly stable. Additionally, the binding affinities of the cations to the PA-channel were not changed when negative voltage in the

physiological range was applied. This indicates that the cations were able to equilibrate rapidly across the PA-channel irrespective of the applied voltage.

The results of the titration experiments suggested that the PA-channel either contains two different binding sites for divalent and trivalent cations, one at the *cis*- and one at the *trans*-side of the channel, or just one binding site which is accessible from both sides of the channel. This could be the case, as the small cations are able to cross the channel rapidly and equilibrate in the aqueous solution. Titration experiments with copper ions on both sides of the membrane led to subsequent decrease of PA-induced conductance. Then, 5 mM EDTA was added to the *trans*-side of the membrane. No effect on the conductance could be observed. However, addition of EDTA to the *cis*-side of the membrane resulted in increasing conductance. The copper induced blockage of the PA-channels could be fully restored meaning that the PA-pore only contains one binding site for copper ions which is localized at the *cis*-side of the channel.

3.6 Discussion

3.6.1 Binding substrates of protective antigen share common motives

Positive charges play a crucial role in binding to PA_{63}-channels

It is well known that PA-pores are strongly cation selective up to a factor of 20 p_c/p_a (Blaustein *et al.*, 1989). Additionally, recent studies found proof, that negatively charged amino acids in the vestibule of PA-channels play a crucial role in the binding of EF and LF (Leuber *et al.*, 2008, Orlik *et al.*, 2005). These findings already indicate the importance of ion-ion interaction for binding and translocation events to PA. The data presented here underline this assumption by depicting the existence of positive charges in high affinity substrates ranging from simple ions, over small inhibitor molecules and molecule complexes as well as peptides, to related proteins and finally protein effectors only containing chargeable tags.

First time evidence that different cationic electrolytes serve as a binding partner to PA-channels is presented in this work. This is of special interest, as the ions themselves seem to be too small to block the channel conductance. The sevenfold symmetry of the pore provides seven possible negative charges for each acidic amino acid facing the lumen of PA_{63}. On top of that, the constriction site of PA, the so-called Φ-clamp is surrounded by these rings of negative charges. Therefore, a plug consisting of more and more cations may form around this site explaining the results. Additional support for this theory is provided by the studies with sevenfold charged β-cyclodextrin and the length dependent binding of positive charged tags (Rostovtseva *et al.*, 2000, Nestorovich *et al.*, 2010). Binding of cations could be possible from both sides of PA-channels out of two reasons: First, multiple rings of acidic amino acids exist in the lumen of the channel, which are accessible from both sides. Second, ions might be small enough to pass the Φ-clamp and bind from the opposite side. It has been shown, that this is not the case as for all substrates starting with simple molecules only one binding site could be verified.

Aromatic residues enhance affinities towards PA-pores

Another important function is represented by aromatic ring-systems. Especially when the affinity of blocker-substrates is discussed, it becomes obvious that the existence of aromatic residues strengthens the binding to toxin channels (Nestorovich *et al.*, 2011). This could found on the existence of the Φ-clamp, too. As the on-rate derived by current noise analysis is in the range of diffusion for molecules like chloroquine, the off-rate contributes to a larger extend to the stability constant (Orlik *et al.*, 2005). That reasons that molecules, which are directed directly to the

constriction site and settle there should form the most stable block. Considering the Φ-clamps composition out of seven phenylalanine residues, it is easy to understand, that aromatic side-chains serve this purpose best (Orlik *et al.*, 2005). Taken the pharmacological use of those substrates into consideration, the aromatic residues could provide a further purpose in enabling these molecules to cross membranes and reach the endosome, where they are charged due to acidic pH. This trapping-effect known from chloroquine and other 4-aminoquinolones further increases blockage of PA-channels.

3.6.2 Binding of charged substrates is voltage-dependent

Recently a change in voltage-dependency of the PA-channel after His_6-EDIN titration has been found (Abramo *et al.*). The stability constants for binding are influenced when positive voltages are applied. It seems to be the case, that the force of the electric field pulls the tagged N-terminus of the protein deeper into the pore, thereby increasing the stability constant for binding of these His_6-tagged polypeptides. This finding partially serves as an explanation for the translocation of foreign substrates *in vivo*, which possess positively charged tags, as the acidic endosome exhibits this field direction. Further studies are necessary to fully elucidate this voltage-dependent binding and translocation process of all charged substrates to the PA-pore mentioned here.

Chapter 4

Cross-Reactivity of Anthrax and C2 Toxin: Protective Antigen Promotes the Uptake of Botulinum C2I Toxin into Human Endothelial Cells

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Large homologies between Anthrax and C2-toxin in both, B- and A-subunits open the possibility to cross-over the corresponding moieties and check with electrophysiological measurements and intoxication assays.

4.1 Summary

Binary toxins are among the most potent bacterial protein toxins performing a cooperative mode of translocation and exhibit fatal enzymatic activities in eukaryotic cells. Anthrax and C2 toxin are the most prominent examples for the $AB_{7/8}$ type of toxins. The B subunits bind both host cell receptors and the enzymatic A polypeptides to trigger their internalization and translocation into the host cell cytosol. C2 toxin is composed of an actin ADP-ribosyltransferase (C2I) and C2II binding subunits. Anthrax toxin is composed of an adenylate cyclase (EF) and a MAPKK protease (LF) enzymatic components associated to protective antigen (PA) binding subunit. The binding and translocation components anthrax protective antigen (PA_{63}) and C2II of C2 toxin share a sequence homology of about 35%, suggesting that they might substitute for each other. Here we show by conducting *in vitro* measurements that PA_{63} binds C2I and that C2II can bind both EF and LF. Anthrax edema factor (EF) and lethal factor (LF) have higher affinities to bind to channels formed by C2II than C2 toxin's C2I binds to anthrax protective antigen (PA_{63}). Furthermore, we could demonstrate that PA in high concentration has the ability to transport the enzymatic moiety C2I into target cells, causing actin modification and cell rounding. In contrast, C2II does not show significant capacity to promote cell intoxication by EF and LF. Together, our data unveiled the remarkable flexibility of PA in promoting C2I heterologous polypeptide translocation into cells.

4.2 Introduction

Binary toxins of the $AB_{7/8}$ type are highly potent and specialized bacterial protein toxins and are organized in two different polypeptide chains that are separately secreted in the external media of Gram-positive bacteria (Barth *et al.*, 2004). Component A is responsible for the intracellular enzymatic activity of the toxin, whereas heptamers or octameres, of the component B are necessary for receptor-binding and translocation of component A into target cells. Given the close homology of structure of the binding components of these two-component toxins it is of importance to decipher whether each component can functionally substitute for each other to intoxicate cells, that we termed cross-reactivity.

One of the most prominent toxins of this type of toxin is anthrax toxin from *Bacillus anthracis* (Dain *et al.*). This toxin possesses a binding and translocation component, protective antigen (PA) and two enzymatic subunits, edema factor (EF) and lethal factor (LF). Edema factor (EF) is an 89 kilo Dalton Ca^{2+}- and calmodulin-dependent adenylate cyclase which catalyzes the production of intracellular cAMP and causes severe edema. Lethal factor (LF) is a 90 kilo Dalton Zn^{2+}-binding metalloprotease that cleaves mitogen-activated protein kinase kinases (MAPK-kinases) and thereby interferes with the MAPK cascade, a major signaling pathway, triggered by surface receptors, controlling cell proliferation and survival (Young *et al.*, 2007, Turk, 2007). The binding component PA is essential for delivery of both enzymes into the target cells (Mock *et al.*, 2001, Ascenzi *et al.*, 2002, Young *et al.*, 2007). It is secreted as an 83 kilo Dalton water-soluble precursor form (PA_{83}) and needs to undergo proteolytic activation by cell-bound furin. After the activation of PA_{83}, the remaining 63 kilo Dalton PA_{63} forms an oligomeric channel responsible for the binding and translocation of EF and/or LF into the cytosol of target cells (Petosa *et al.*, 1997, Abrami *et al.*, 2004, Abrami *et al.*, 2005, Miller *et al.*, 1999).

Clostridium botulinum, well known for the production of potent neurotoxins, produces various protein toxins, such as the AB type C2 toxin (Aktories *et al.*, 2004a, Aktories *et al.*, 2004b, Boquet *et al.*, 2003). The binding component of C2 toxin, C2II (60 kilo Dalton after proteolytic cleavage with trypsin), forms heptamers that insert into biological and artificial membranes at an acidic pH and promotes the translocation of the 45 kilo Dalton enzymatic component C2I into the cytosol of the target cells upon receptor-mediated endocytosis of the complex (Barth *et al.*, 2000, Blocker *et al.*, 2000). C2I acts as an ADP-ribosyltransferase on monomeric G-actin, causing disruption of the actin cytoskeleton (Blocker *et al.*, 2003b, Considine *et al.*, 1991).

The enzymatic components of anthrax and C2 toxin differ significantly in their enzymatic activity and do not show any homology in their primary structures. However, the binding components PA and C2II share a considerable sequence homology of about 35% in two of three domains,

indicating that they are closely related in structure and hence also in function (Neumeyer *et al.*, 2006a, Petosa *et al.*, 1997, Schleberger *et al.*, 2006, Young *et al.*, 2007). In recent years, many important structural features, particularly concerning PA, have been unveiled, such as the Φ-clamp and the loop network responsible for allocation of the PA monomers (Krantz *et al.*, 2005, Melnyk *et al.*, 2006). Interesting details concerning the possible mode of translocation are known, all favoring an acid-induced disassembly of the enzymatic components to a molten globular state, followed by threading of the N-terminal part of the polypeptide chain through the pore (Krantz *et al.*, 2006, Krantz *et al.*, 2005, Krantz *et al.*, 2004, Melnyk *et al.*, 2006). However, the exact mode of transporting the enzymatic components into the cytosol of target cell is still not fully solved. The first crucial step of the translocation mechanism is the binding of the enzymatic components to the receptor-bound prepore on the cell surface (Barth *et al.*, 2004). Previous results of our and other groups evidenced that truncated forms of the enzymatic components as well as full size EF and LF block the pores formed by PA_{63}, and that an N-terminal His_6-tag strengthens their affinity (Neumeyer *et al.*, 2006a, Zhang *et al.*, 2004b, Halverson *et al.*, 2005). Binding of the N-terminal ends of EF and LF to PA_{63} is followed by endocytosis, acidification of the endosomes and finally release of the enzymatic components into the cytosol of target cells, where they exert their fatal enzymatic activities (Abrami *et al.*, 2004, Abrami *et al.*, 2005, Wei *et al.*, 2006). Interestingly, LF's amino-terminal part, LF_N (LF_{1-254}), is sufficient to confer the ability to associate with PA_{63} pores. It can even be used to drive the translocation of unrelated polypeptides into target cells via PA_{63} or C2II (Leppla *et al.*, 1999, Rolando *et al.*, 2009).

To further elucidate the mode of binary toxins' translocation into target cells and the possible cross-reactivity of the different enzymatic components via the homologous binding component of the other toxin, we performed *in vitro* and *in vivo* (i.e. cell-based assay) experiments interchanging the different A-B components of anthrax and C2 toxin. Most importantly our data show the high capacity of PA_{63} to bind C2I *in vitro* in the black lipid bilayer assay. Complementary to these findings we evidence the functionality of PA/C2I chimera toxin in cell intoxication. Further, C2II appeared more specifically involved in C2I binding and translocation. Together, our data unveiled the remarkable ability of PA to support cell intoxication by C2I, a distantly related $AB_{7/8}$ toxin component.

4.3 Material and Methods

4.3.1 Materials

PA, LF and EF genes were PCR-amplified from genomic DNA of *Bacillus anthracis* strain Sterne (a kind gift of Patrice Boquet, Nice, France) and cloned into the pQE30 (Qiagen), pET28a and pET22b (Novagen) expression plasmids, respectively. The N-terminal His_6-tag was removed from His_6-EF by incubation with thrombin and from His_6-LF with enteropeptidase, respectively. Nicked anthrax PA_{63} from *B. anthracis* was obtained from List Biological Laboratories Inc., Campbell, CA. One mg of lyophilized protein was dissolved in 1 ml 5 mM HEPES, 50 mM NaCl, pH 7.5 complemented with 1.25% trehalose. Aliquots were stored at -20°C. Channel formation by PA_{63} was stable for months under these conditions. C2I and C2II genes were PCR-amplified from genomic DNA of *Clostridium botulinum* D strain 1873 and cloned into pET22 (Novagen) and pQE30 (Qiagen) expression plasmids. All genes were cloned with *Bam*HI-*Sac*I restriction sites. Recombinant toxins containing His_6-tags were expressed in *Escherichia coli* BL21 (DE3) and purified on a Chelating Sepharose Fast Flow column previously chelated with nickel (Amersham Biosciences) as recommended by the manufacturer and described previously (Rolando *et al.*). Fractions containing toxin were pooled and dialyzed over night against 250 mM NaCl and 25 mM Tris-HCl, pH 8. Recombinant C2II and C2I proteins used for bilayer measurements were cloned in pGEX-2T vector in *E. coli* BL21 cells and expressed as glutathione *S*-transferase (GST) fusion proteins with the glutathione *S*-transferase-fusion Gene Fusion System from Amersham Pharmacia Biotech (Blocker *et al.*, 2003b, Blocker *et al.*, 2000). The proteins were purified as described previously (Blocker *et al.*, 2003b) and incubated with thrombin (3.25 NIH units/ml bead suspension) for cleavage of the GST-tag (Blocker *et al.*, 2003a). C2II was activated with 0.2 µg of trypsin per microgram of protein for 30 min at 37°C (Blocker *et al.*, 2003a).

4.3.2 Western blot, cell culture, adenylate cyclase activity and ADP-ribosylation

Western blot

The polyclonal antibodies against the N-terminal part of MEK2 (N20) were purchased from Santa Cruz Biotechnology; monoclonal antibodies against ß-actin were obtained from Sigma-Aldrich (clone AC-74). Primary antibodies were visualized using goat anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (DakoCytomation), followed by chemiluminescence detection ECL (GE Healthcare).

Cell culture

Human umbilical vein endothelial cells (HUVECs, a human primary cell line obtained from PromoCell) were grown in serum-free medium (SFM) supplemented with 20% FBS (Invitrogen), 20 ng/ml basic ßFGF (Invitrogen), 10 ng/ml EGF (Invitrogen) and 1 µg/ml heparin (Sigma-Aldrich) as described previously (Doye *et al.*, 2006).

Adenylate cyclase activity

Intracellular concentration of cyclic AMP (cAMP) was determined using the Cyclic AMP Assay (R&D Systems).

ADP-ribosylation

Control cells or intoxicated HUVECs (10^5 cells/conditions) were homogenized in 0.25 ml cold BSI buffer (3 mM imidazole pH 7.4, 250 mM sucrose) supplemented extemporaneously with 1 mM phenylmethylsulfonyl fluoride. Cells were lysed by passing through a 1 ml syringe equipped with a 25G x 5/8"-needle (U-100 Insulin, Terumo) 40 times. Nuclei were removed by centrifugation for 10 minutes at 4°C. Protein concentrations of the post-nuclear supernatants were determined using Dc protein assay (Bio-Rad). ADP-ribosylation was performed for 90 minutes at 37°C on 5 µg of intoxicated cell lysates, supplemented with 0.5 µCi [^{32}P]-NAD (800 Ci/mmol) and 1 µg of C2I. Proteins were resolved on 12% SDS-PAGE and *in vitro* ADP-ribosylated actin was revealed using a phosphor-imaging system.

4.3.3 Lipid bilayer experiments

Black lipid bilayer measurements were performed as described previously (Benz *et al.*, 1978). The instrumentation consisted of a Teflon chamber with two aqueous compartments connected by a small circular hole. The hole had a surface area of about 0.4 mm^2. Membranes were formed by painting a 1% solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) in n-decane onto the hole. The aqueous salt solutions (Merck, Darmstadt, Germany) were buffered with 10 mM MES to pH 5.5 to pH 6. Control experiments revealed that the pH was stable during the time course of the experiments. The binding components of the binary toxins were reconstituted into the lipid bilayer membranes by adding concentrated solutions to the aqueous phase to one side (the *cis*-side) of a black membrane. The temperature was kept at 20°C throughout. Membrane conductance was measured after application of a fixed membrane potential with a pair of silver/silver chloride electrodes inserted into the aqueous solutions on both sides of the membrane. Membrane current was measured using a homemade current-to-

voltage converter made with a Burr Brown operational amplifier. The amplified signal was monitored on a storage oscilloscope and recorded on a strip chart recorder.

4.3.4 Binding experiments

The binding of EF and LF to the C2II channel and the binding of C2I to the PA_{63} and to the C2II channel was investigated performing titration experiments similar to those used previously to study the binding of 4-aminoquinolones to the PA_{63} and C2II channels and LF to the PA_{63} channel in single- or multi-channel experiments (Bachmeyer *et al.*, 2003, Neumeyer *et al.*, 2006b, Orlik *et al.*, 2005). The PA_{63} and C2II channels were reconstituted into lipid bilayers. About 60 minutes after the addition of either activated PA_{63} or C2II to the *cis*-side of the membrane, the rate of channel insertion in the membranes was very small. Then concentrated solutions of EF, LF or C2I were added to the *cis*-side of the membranes while stirring to allow equilibration. The results of the titration experiments, i.e. the blockage of the channels, were analyzed using Langmuir adsorption isotherms (equation 4.1) (Benz *et al.*, 1987, Neumeyer *et al.*, 2006a).

$$G(c) = G_{\max} \frac{1}{(K \cdot c + 1)} \qquad [4.1]$$

$G(c)$ is the conductance of the channels at a given concentration c of the enzymatic components and G_{max} is their conductance before the start of the titration experiment (at $c = 0$). K is the stability constant for binding of the enzymatic components of the binary toxins to the PA_{63} or C2II channels. The half saturation constant K_s of binding is given by the inverse stability constant $1/K$. The percentage of blocked channels is given by:

$$\% \text{ closed channels } = \frac{100 \cdot K \cdot c}{K \cdot c + 1} \qquad [4.2]$$

Statistics

Unpaired, two-sided Student's t-test was used to analyze biological data with * $p<0.05$. The statistical software used was Prism 5.0b. The fit of the data from the titration experiments with lipid bilayers was performed using Fig.P. For most of the fits of the titration data with equation 4.2 we obtained $r^2 > 0.99$.

4.4 Results

4.4.1 Interaction of PA_{63} with C2I *in vitro*

The stability constant *K* for the binding of C2I to the PA_{63} channel was measured in multi-channel experiments, performed as described previously (Neumeyer *et al.*, 2006b). About 60 minutes after addition of the protein, the rate of conductance increase had slowed down considerably. At that time, small amounts of a concentrated enzyme solution were added to the *cis*-side of the membrane and the PA_{63}-induced membrane conductance decreased in a dose-dependent manner. Figure 4.1A shows an example for a titration experiment with an applied voltage of 20 mV in 150 mM KCl in which increasing concentrations of C2I (arrows) were added to the *cis*-side of a membrane containing about 5500 PA_{63} channels. The membrane conductance decreased as a function of the C2I concentration within a few minutes after addition of C2I (Fig. 4.1A). The data of figure 4.1A and of similar experiments were analyzed using equation 4.2 as described previously (Neumeyer *et al.*, 2006b, Benz *et al.*, 1986). The plots of the percentage of closed channels as a function of the enzyme concentrations were used to calculate the stability constants *K* for binding as it is shown in figure 4.1B for the data of Figure 4.1A. The fit curve (solid line in figure 4.1A) corresponds to a stability constant *K* of $(3.98 \pm 0.063) \times 10^6$ M^{-1} for C2I binding to PA_{63} (half saturation constant $K_S = 251$ nM). The stability constant *K* of the binding of C2I to the PA_{63} channels was averaged out of at least five individual experiments resulting in *K* $(5.1 \pm 1.5) \times 10^6$ M^{-1} (half-saturation constant $K_s = 196$ nM) (Table 4.1). Measurements with artificial bilayer membranes of the wild-type AB components C2II and C2I revealed a stability constant *K* of $(3.7 \pm 0.4) \times 10^7$ M^{-1} with a half saturation constant K_S of 27.2 nM.

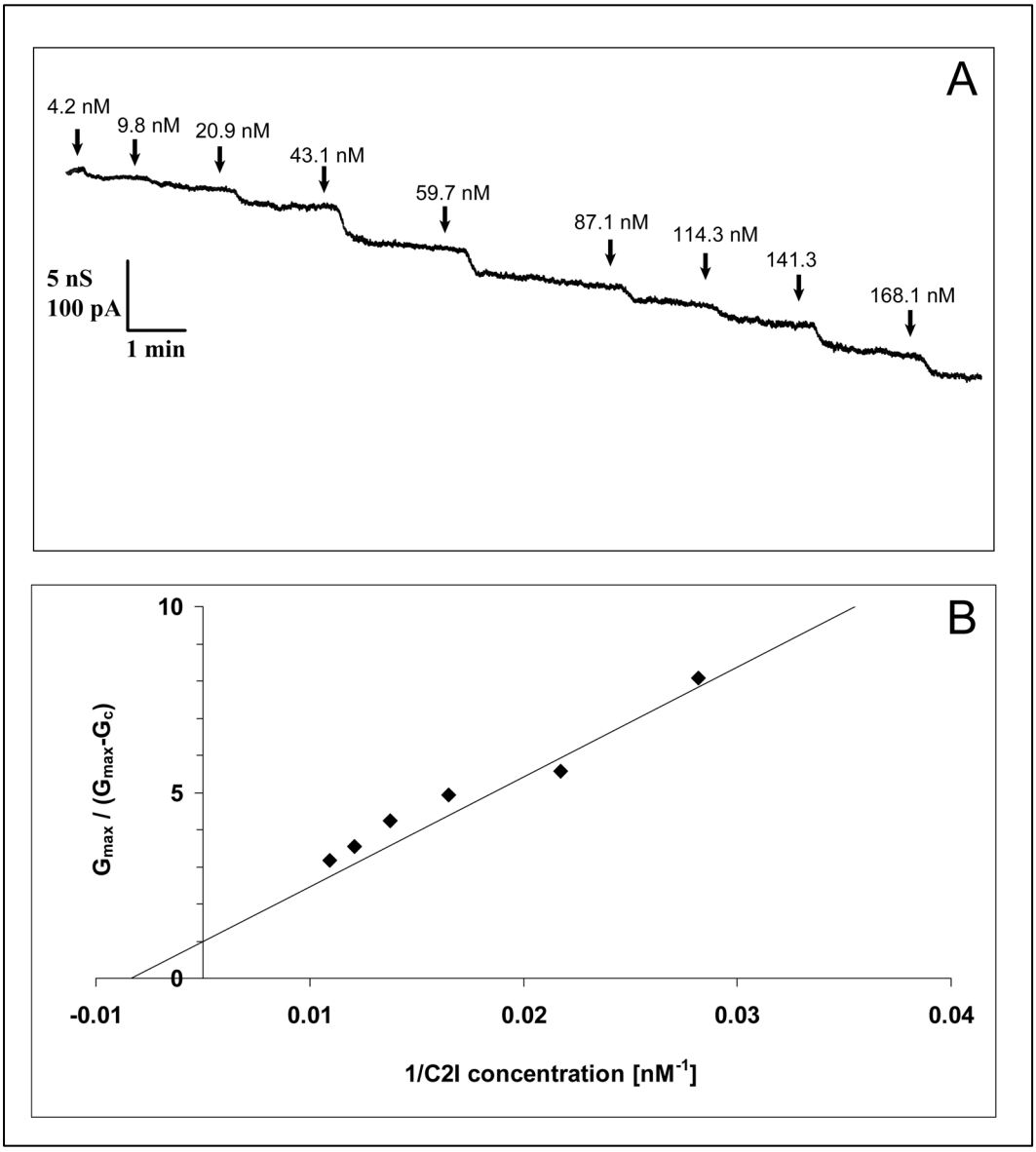


Fig. 4.1:
A: Titration of PA_{63} induced membrane conductance with C2I. The membrane was formed from diphytanoyl phosphatidylcholine/n-decane, containing about 5500 channels. C2I was added at the concentrations shown at the top of the panel. Finally, about 40% of the PA_{63} channels were blocked. The aqueous phase contained 1 ng/ml activated PA_{63} protein (added only to the *cis*-side of the membrane), 150 mM KCl, 10 mM MES pH 6. The temperature was 20°C and the applied voltage was 20 mV. Note that C2I only blocks PA_{63} channels when they are added to the *cis*-side of the membrane (data not shown).
B: Lineweaver-Burke plot of the inhibition of the PA_{63}-induced membrane conductance by C2I. The fit was obtained by linear regression of the data points taken from Figure 1A (r^2=0.996654) and corresponds to a stability constant *K* for C2I binding to PA_{63} of (3.98 ± 0.063) × 10^6 M^{-1} for C2I binding to PA_{63} (half saturation constant K_S = 251 nM).

Table 4.1: Stability constants *K* for the binding of C2I, EF or LF to PA_{63} or C2II channels reconstituted in lipid bilayer membranes.

Toxin combination		K [M^{-1}]	K_s [nM]
PA with			
	C2I	$(0.51 \pm 0.15) \times 10^7$	196
	EF	$\mathbf{14.5 \times 10^7}$	**6.9**
	LF	$\mathbf{36.2 \times 10^7}$	**2.8**
C2II with			
	EF	$(7.7 \pm 4.8) \times 10^7$	13
	LF	$(2.0 \pm 0.3) \times 10^7$	49.9
	C2I	$(3.7 \pm 0.4) \times 10^7$	27.2

The membranes were formed from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained 150 mM KCl, buffered to pH 5.5 to 6 using 10 mM MES-KOH; T = 20°C. Measurements were performed at a membrane potential of 20 mV. The data represent the means of at least three individual titration experiments. K_S is the half saturation constant, i.e. $1/K$. Some of the wild-type toxin combinations (given in bold) were taken from reference (Neumeyer *et al.*, 2006a).

4.4.2 Binding of C2II with EF and LF *in vitro*

As demonstrated in recent studies, EF and LF of anthrax toxin are able to block the PA_{63} pore in artificial bilayer membranes at nanomolar concentrations (Neumeyer *et al.*, 2006a) and C2II channels can be blocked by their enzymatic counterpart C2I (Blocker *et al.*, 2003b). The possible binding of EF and LF to the C2II channels was studied using titration experiments as described above for PA_{63} and C2I shown in figure 4.1. These measurements allowed the calculation of the stability constants *K* of EF and LF binding to the C2II channels, resulting in $(7.7 \pm 4.8) \times 10^7$ M^{-1} and $(2.0 \pm 0.3) \times 10^7$ M^{-1}, respectively (Table 4.1). The data indicated that EF and LF have a high affinity for the C2II channels *in vitro*, as the half saturation constants K_S for EF and LF binding to the C2II channels were 13.0 nM and 49.9 nM, respectively.

4.4.3 PA_{63} translocates C2I in HUVECs

C2I acts as an ADP-ribosyltransferase targeting cellular G-actin. Therefore, successful delivery of this enzymatic component into target cells can be detected by disruption of the cytoskeleton followed by rounding up of target cells and detachment of target cells from the extracellular matrix, defined as intoxicated cells (Blocker *et al.*, 2003b) or by direct measurement of the modified G-actin as described in *Materials and Methods*. C2I, added to different concentrations of its native binding component C2II, led to increasing numbers of round cells after 24 hours of intoxication (data not shown). Figure 4.2A shows the direct measurement of cellular ADP-ribosylated actin (ADPr-actin) in HUVECs after treatment with different PA-C2I and C2II-C2I

combinations. The cells were intoxicated with different concentrations of binding components and effectors as indicated. Levels of ADP-ribosylated actin (ADPr-actin) were determined by *in vitro* ADP-ribosylation of cell lysates with C2I and radiolabeled [^{32}P]-NAD. Under these conditions ADP-ribosylated actin produced by the intoxication process is no longer labeled by *in vitro* ADP-ribosylation. The results clearly demonstrated that the radioactivity combined with labeled ADPr-actin decreased for the combinations PA-C2I, when PA was applied in high concentration, and C2II-C2I suggesting that both channels were able to transport C2I into HUVECs. Controls did not reveal any change of the labeling of actin, which means that neither PA nor C2I alone, respectively, modified intracellular actin (data not shown).

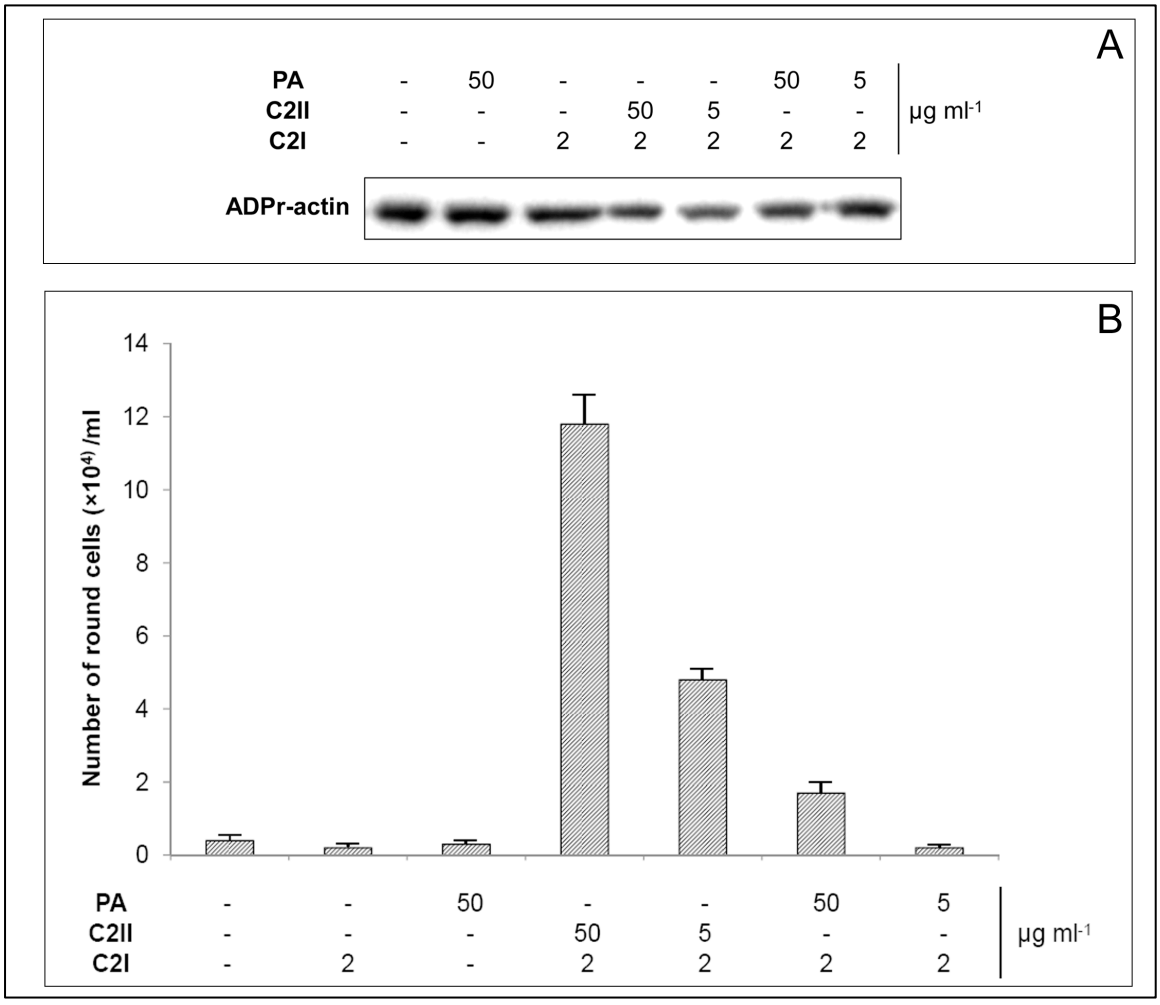


Fig. 4.2:
HUVECs (5 × 10^5 cells/100 mm well) were intoxicated with the indicated concentration of polypeptides during 48 (A) or 24 hours (B).
A: Cells were intoxicated as indicated and levels of cellular ADP-ribosylated actin (ADPr-actin) were determined by *in vitro* ADP-ribosylation of cell lysates with C2I and radiolabeled [^{32}P]-NAD. Under these conditions ADP-ribosylated actin formed during the intoxication process is no longer labeled by *in vitro* ADP-ribosylation. Immuno-blotting anti-beta-actin was performed in parallel on cell lysates to show actin protein levels engaged in the ADP-ribosylation experiments. ADP-ribosylation signals were normalized to actin immuno-blot signals and expressed as fold, as compared to PA-treated cells.
B: Efficiency of cell intoxication. Cells were intoxicated and the number of round cells was directly assessed by counting floating cells. The columns show mean values of 5 independent counting for the individual conditions ± SEM (ns: non significant; * $p<0.05$ versus control).

Similar results were obtained when the number of intoxicated HUVECs was determined by counting round cells as a result of C2I activity on actin. Figure 4.2B shows the efficiency of cell intoxication under different experimental conditions. HUVECs were incubated with different combinations of PA-C2I and C2II-C2I as indicated, and the number of intoxicated cells was directly assessed. The results shown in Figure 2B revealed that the number of intoxicated cells was highest for the native combination C2II-C2I. However, when HUVECs were incubated with C2I and different quantities of PA, rounding of cells was detected even at lower probability (Fig. 4.2B). This demonstrated that C2I was transported by PA channels into HUVECs. The results of figure 4.2B indicated a dose-dependent process as some combinations failed to induce any significant effect compared to the controls.

4.4.4 Interaction of C2II with LF and EF in vivo (cell-based assay)

The enzymatic activity of the lethal factor (LF) of anthrax toxin can be measured by monitoring the cleavage of MAPKK, e.g. with MEK2 amino-terminal antibodies (anti-MEK2) (Turk, 2007). HUVEC monolayers were intoxicated overnight with different combinations of PA-LF or C2II-LF and the activity of LF was analyzed on cell lysates by anti-MEK2 immuno-blotting. Control experiments were performed in the absence of binding components. Whereas the wild-type lethal toxin (PA-LF) did not give any MEK2 signal after blotting, the combination of LF with different quantities of C2II revealed a defined signal of intact MEK2 (Fig. 4.3A). Considering our findings that C2II mediated an efficient translocation of C2I into cells under these conditions (Fig. 4.2A and 4.2B) we can present evidence that C2II has a dramatically lower capacity to promote translocation of LF into target cells.

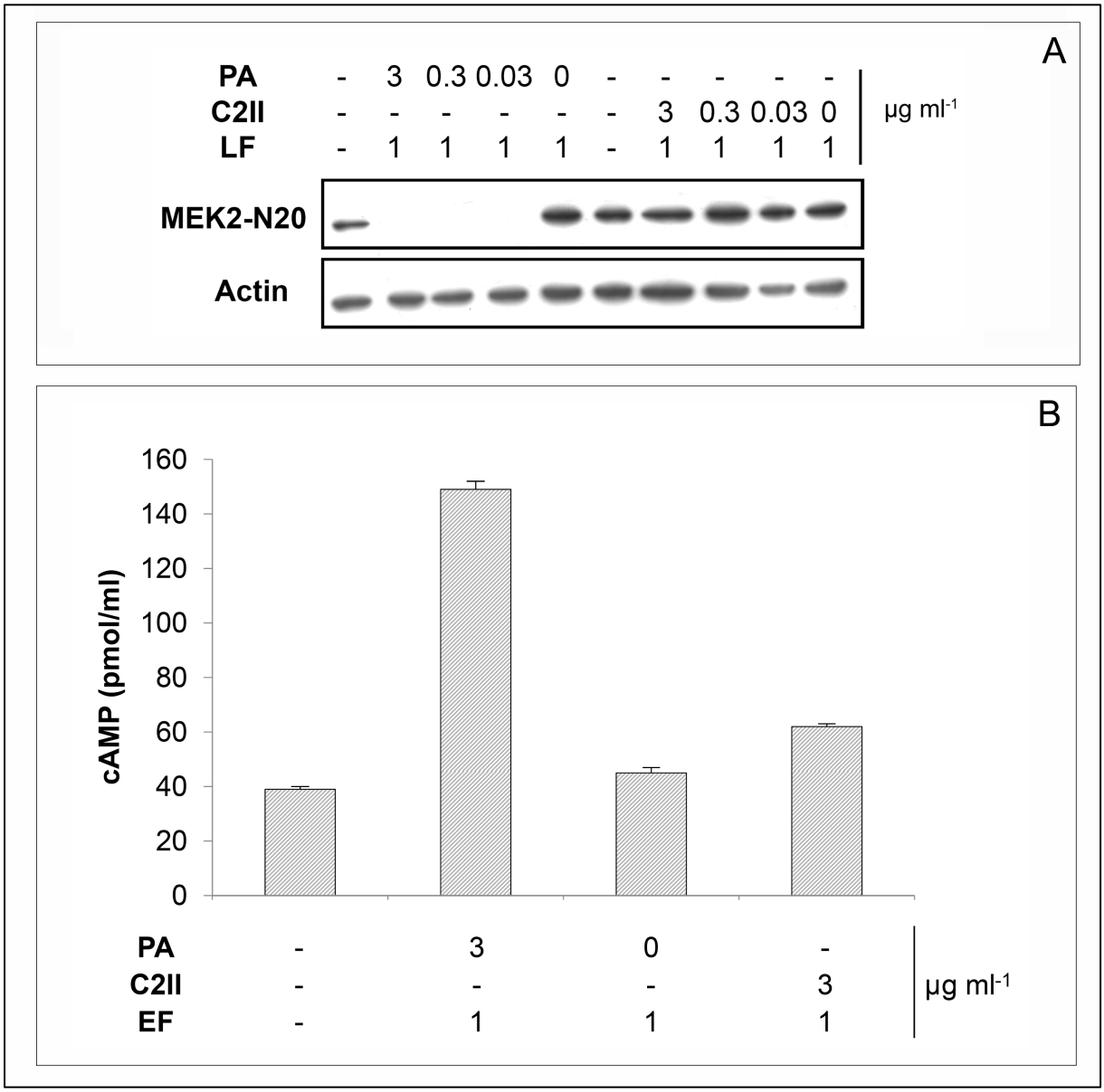


Fig. 4.3:

All intoxication experiments were performed on HUVEC monolayers. Cells were treated overnight with either 1µg/ml of LF or EF in the presence or absence of different amounts of PA or C2II, as indicated.
A: Immuno-blot anti-MEK2 showing the effect of MEK2 proteolysis by LF. 30 µg of total protein lysate were resolved on 12% SDS-PAGE. Anti-beta-actin immuno-blot shows protein loading. MEK2 signals were normalized to actin and expressed as fold, as compared to untreated control condition.
B: Graph shows measure of cyclic AMP (cAMP) cellular concentrations, expressed as pmol/ml. Mean values of two independent experiments ± SEM (ns: non significant and * $p<0.05$).

Anthrax edema factor (EF) is known to increase cAMP in target cells when applied with its native binding partner PA_{63} (Dal Molin *et al.*, 2006). We next tested whether C2II is able to promote translocation of EF by measure of the intracellular concentration of cAMP after overnight incubation of HUVEC monolayers. The combination of PA with EF led to a significant increase of intracellular cAMP level as a function of PA concentration. In contrast, he application of C2II-EF did not increase cAMP cellular levels significantly (Fig. 4.3B). We next verified that addition of EF was able to compete with C2I binding to C2II. The results are

summarized in figure 4.4. At a concentration of 50 µg/ml EF could significantly block C2II-mediated transport of C2I into HUVECs. These data further suggest that EF binds to the C2II channel *in vivo* (cell-based assay). Together, these findings show that C2II does not promote cell intoxication by EF and LF efficiently.

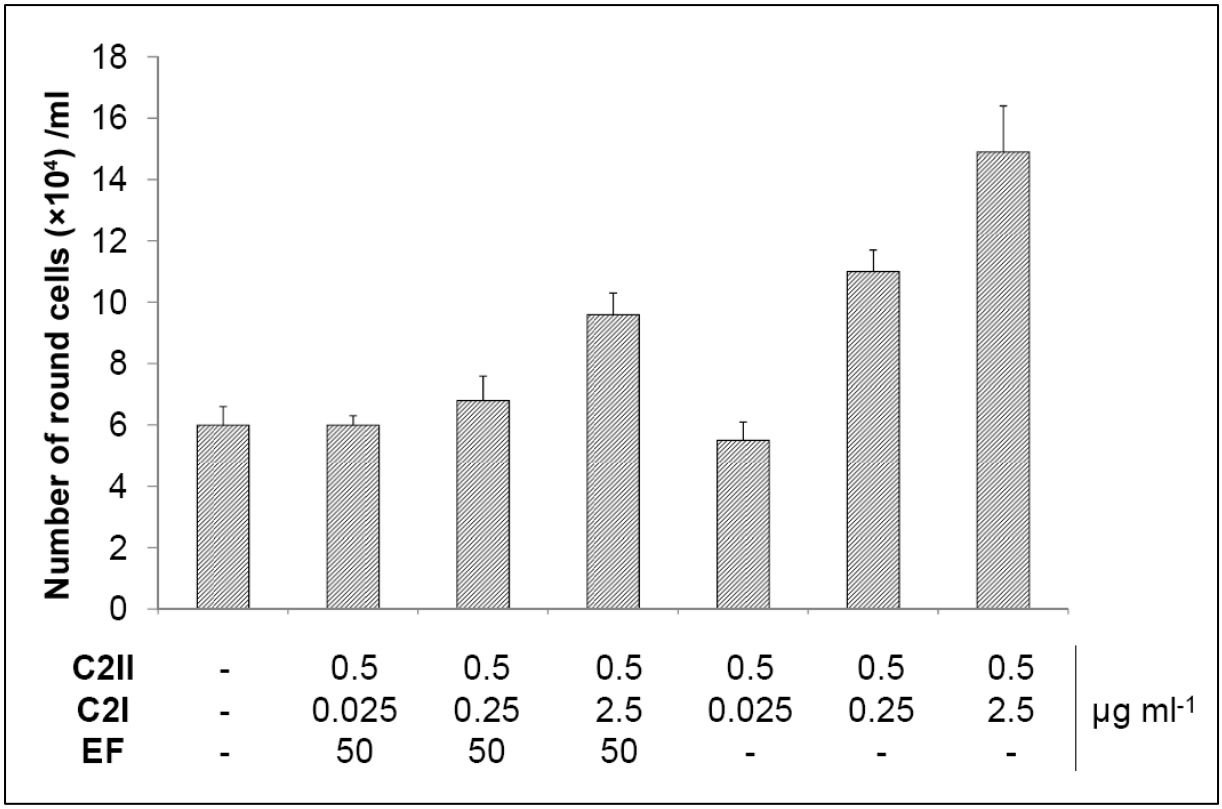


Fig. 4.4:
EF-mediated inhibition of C2II-promoted C2I uptake into HUVEC cells. Cells were intoxicated with different concentrations of the binding component C2II and the effectors EF and C2I, as indicated, and the number of intoxicated cells was directly assessed by counting round cells. One representative experiment showing mean values of 5 independent counting for each condition.

4.5 Discussion

In previous studies we already demonstrated that the enzymatic components EF and LF of anthrax toxin bind to their B component protective antigen (PA_{63}) and C2I of C2 toxin binds to its B component C2II *in vitro* (Bachmeyer *et al.*, 2003, Blocker *et al.*, 2003b, Neumeyer *et al.*, 2006a). PA shares significant sequence homology (35%) with C2II, indicating that the two proteins have similar modes of action. PA_{63} has been crystallized in its monomeric and heptameric prepore form (Petosa *et al.*, 1997) and a model of the C2II prepore structure has been constructed based on the corresponding assembly of the protective antigen prepore (Schleberger *et al.*, 2006). The similarity of both structures supports the view of a common mode of action, including the assumption that the enzymes bind in the vestibule of the channels of the corresponding binding component.

The results presented here suggest an interesting cross-over reactivity of anthrax and C2 toxins, despite a completely different primary and 3D-structure of the enzymatic compounds EF, LF and C2I (Han *et al.*, 1999, Pannifer *et al.*, 2001, Shen *et al.*, 2005). The stability constants *K* for binding in the cross-over experiments *in vitro* were generally smaller than those for the native combinations, except the combination of C2II-EF. However, EF, LF and C2I show a high stability constant *K* for binding to PA_{63} and C2II heptamers in the cross-over experiments as the half saturation constants K_s are between 2 and about 200 nM (Table 4.1). These results refer to a common mechanisms and binding motifs within the enzymes' primary structures, in particular within the first three hundred amino acids of EF, LF and C2I. Truncated forms of EF and LF, called EF_N and LF_N, bind with high affinity to the PA_{63} channels and support the transport of other polypeptides into target cells (Elliott *et al.*, 2000, Mogridge *et al.*, 2002a, Zhang *et al.*, 2004b, Rolando *et al.*, 2009), although the binding affinity of EF_N and LF_N for the PA_{63} channel is substantially reduced as compared to wild-type EF and LF (Leuber *et al.*, 2008). Similarly, the N-terminal part of C2I is sufficient for transport of truncated forms of C2I and chimera proteins between the N-terminal end of C2I and other proteins into target cells (Barth *et al.*, 2002a, Barth *et al.*, 2002b). This means that the N-terminal ends of all enzymatic compounds interact with the PA_{63} and the C2II pores. Some of the amino acids responsible for these interactions are well known within the primary sequence of PA_{63} and its water-soluble prepore, e.g. amino acids E398, D425 and F427 (also known as the **Φ**-clamp) (Cunningham *et al.*, 2002, Krantz *et al.*, 2005, Lacy *et al.*, 2005), but relatively unknown for C2II, although there exist some indications that the corresponding amino acids E399, D426 and F428 may play a similar role for C2I binding (Neumeyer *et al.*, 2008). However, further amino acids responsible for this interaction still need to be identified.

The amino acids responsible for binding within the N-terminal end of the enzymatic components are relatively unknown, although there is clear evidence that positively charged amino acids are involved as they form salt bridges between the enzymatic components and the channels. The positively charged N-termini of the enzymes is presumably decisive as quaternary ammonium ions and 4-aminoquinolones show a blockage of PA_{63} and C2II channels in lipid bilayer experiments (Bachmeyer *et al.*, 2003, Blaustein *et al.*, 1990, Finkelstein, 1994, Orlik *et al.*, 2005). The selectivity of the two channels for cations, which is at least partially due to the charged amino acids in the β-barrel, may also play a significant role. Both channels are known to prefer cations over anions in zero-current membrane-potentials, the P_{cation} over P_{anion}, as described by the Goldman-Hodgkin-Katz equation (Benz *et al.*, 1979), are 20 for PA_{63} channels and 10 for C2II channels, respectively (Blaustein *et al.*, 1989, Schmid *et al.*, 1994). Therefore cations have a strong effect on the single channel conductance as compared to anions (Blaustein *et al.*, 1989, Schmid *et al.*, 1994). It may be possible that the differently charged channel interiors of PA and C2II have a decisive influence on binding and transport of the enzymatic components (see below). Altogether there exist strong indications that binding to the different channels follows different mechanisms.

Another conceivable possibility is that the structure of the channel itself is important for translocation. The extended channel-forming β-sheets of the PA_{63} monomers contain three glutamic acids and three aspartic acids (E302, E308, E343 and D276, D315, D335), so the extended β-barrel could contain up to 48 negatively charged groups, which probably cannot be counterbalanced by the at least partially positively charged histidines H304 and H310 (Nassi *et al.*, 2002, Nguyen, 2004, Santelli *et al.*, 2004). However, the C2II channel contains 7 glutamic acids (E307) and 14 histidines (H296 and H332), indicating that it has a much smaller overall charge (Blocker *et al.*, 2003a). The interaction of the charged groups of the channel interior and the bound enzymatic components could be different for channels leading to divergent uptake efficiency. Considering the fact that the charges in the vestibule domain are quite balanced in both PA_{63} and C2II, i.e. both have 14 acidic amino acids facing the interior of the vestibule domain, the effect of the charges in the water filled β-barrels should be striking. As mentioned beforehand, the C2II channel is missing most of them.

The most interesting result of this study was that the combination of PA with C2I showed HUVEC toxicity. This appeared specific of PA considering the rather poor capacity of C2I to bind and to trigger cell intoxication by LF. This clearly reveals that PA has the remarkable ability to bind and to translocate an enzymatic component of another $AB_{7/8}$ type toxin into cells. The level of cell intoxication with C2I via PA, however, was approximately 5-fold lower than with the

wild-type combination C2II-C2I. We can only speculate about the reasons of this higher flexibility of PA as compared to C2II. One possibility is that a different driving force is required to translocate EF and LF through the C2II channel because EF and LF are released at the state of the late endosome (Abrami *et al.*, 2004), whereas C2I leaves the early endosome following acidification and, in addition, depends on the help of the cytosolic chaperon Hsp90 (Barth *et al.*, 2000, Haug *et al.*, 2003). A similar requirement is not known for the translocation of LF, EF or LF's N-terminal domain (LF_N) through the PA_{63} channel, where a pH-gradient across the membrane creates a sufficient driving force for translocation of the proteins (Krantz *et al.*, 2006). With the evidence presented here, i.e. that some components of the highly specialized binary toxins can be interchanged without loss of toxicity, further work with mutated binding components, enzymatic moieties and chimeras seems to be necessary to understand the different translocation capacities of PA_{63} and C2II channels.

Chapter 5

Interaction of Chloroquine-like Blocker-substrates with Anthrax toxins Protective Antigen (PA) *in vitro* and *in vivo*

Due to the late onset of symptoms during an Anthrax infection and the possible use of multi-resistant strains for bioterrorism, it is of great importance to find ways to fight the intoxication. A clever option is to block the pore with high-affinity blocker-molecules, which are non-toxic themselves.

5.1 Summary

The Anthrax toxin from *Bacillus anthracis* consists of three different molecules; Protective antigen (PA) forms heptameric channels binding to host cell´s receptors and mediates the translocation of the enzymatic components lethal factor (LF) and edema factor (EF) into the cytosol. The cation-selective PA_{63}-channel is able to reconstitute in lipid bilayer membranes and can be blocked by chloroquine and related compounds as it contains a binding site for 4-aminoquinolines. In the recent study we present new substrate structures allowing a detailed investigation of ligand binding. Titration experiments revealed the changes in binding affinity for different specially designed blocker-substrates to PA, which could be related to the functional groups of these molecules. Noise-analysis reasoned a very stable plug being formed in the lumen of the channel. The results directly lead to the urge, to further investigate the blocker-abilities via cell-based assays. Additional information about cytotoxicity and possible usage for medical treatment has still to be found.

5.2 Introduction

The Anthrax toxin is one of the main virulence factors of *Bacillus anthracis*. It represents a plasmid-encoded tripartite toxin composed of a receptor-binding moiety termed protective antigen (PA) and two different enzymatically active components, lethal factor (LF) and edema factor (EF) (Friedlander, 1986, Mock *et al.*, 2001, Collier *et al.*, 2003). The binding component PA transports both (EF and LF) into the cytosol of target cells where they exert their enzymatic activity. The 90 kDa highly specific zinc-dependent metalloprotease lethal factor targets mitogen-activated protein kinase kinases (MAPKKs), e.g. MEK2. Interference with this important pathway leads to subsequent death by apoptosis for some types of macrophages. Furthermore, the release of pro-inflammatory mediators like nitric oxide, tumour necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1 β) from macrophages is inhibited (Hanna *et al.*, 1993, Menard *et al.*, 1996a, Pellizzari *et al.*, 1999). The second factor, termed edema factor (89 kDa), is a calmodulin- and Ca^{2+}- dependent adenylate cyclase. By an increase of the cytosolic cAMP level, EF interferes with cell signalling. Altered processes including water homeostasis cause the cells do die finally (Mock *et al.*, 2001, Lacy *et al.*, 2002, Dixon *et al.*, 1999).

B. anthracis secrets monomeric protective antigen (83 kDa) as a water-soluble precursor form (PA_{83}). PA oligomerizes into heptamers after proteolytic cleavage of a 20 kDa N-terminal fragment (PA_{20}) by a furin-like proteases, which leaves an activated PA_{63} monomer (Young *et al.*, 2007, Petosa *et al.*, 1997). Afterwards, up to three molecules of EF and/or LF can bind with high affinity to this prepore (Cunningham *et al.*, 2002, Escuyer *et al.*, 1991, Elliott *et al.*, 2000). Binding to a cell-surface exposed receptor (Bradley *et al.*, 2001) is followed by endocytosis of the complex. The acidification of the endosome results in translocation of the enzymatic components into the target cell's cytosol (Miller *et al.*, 1999, Nassi *et al.*, 2002). C2 toxin from *Clostridium botulinum* and iota toxin from *Clostridium perfringens* share this translocation mechanism (Barth *et al.*, 2002a).

Although the crystal structure of the prepore and a hypothetical model deliver a useful idea of the membrane-spanning functional pore, it would be helpful to know the crystal structure of the membrane-associated PA_{63}-channel (Petosa *et al.*, 1997, Nguyen, 2004). Due to the high toxicity of Anthrax toxin, channel-forming properties, as well as prominent structural and biophysical features of the PA_{63} channel were investigated (Finkelstein, 1994, Blaustein *et al.*, 1989). The Φ-clamp, including the loop network for stabilization of Phe427, was characterized as an important structure in recent studies (Krantz *et al.*, 2005, Krantz *et al.*, 2006, Melnyk *et al.*, 2006). It has been proven to steer a potential translocation mechanism (Krantz *et al.*, 2004, Zhang *et al.*, 2004a, Zhang *et al.*, 2004b, Zhang *et al.*, 2004d). *In vitro* as well as *in vivo* PA- and C2II-channels

can be blocked by Chloroquine and related compounds (Orlik *et al.*, 2005). The binding site was identified inside the lumen of the C2II channel (Neumeyer *et al.*, 2008).

Here we studied the binding properties of chloroquine-based blocker-substrates to the protective antigen channel in artificial membranes leading to a dose-dependent decrease of membrane conductance in titration experiments. On- and off-rate constants of *in vitro*-binding to the PA-channels were determined by the current noise analysis indicating a strong relationship between compound structure and binding kinetic to the PA channels.

5.3 Material and Methods

5.3.1 Materials

Recombinant, nicked PA_{63} from *B. anthracis* was obtained from List Biological Laboratories Inc., Campbell, CA. One mg of lyophilized protein was dissolved in 1 ml 5 mM HEPES, 50 mM NaCl, pH 7.5 complemented with 1.25% trehalose. Aliquots were stored at -20°C.

5.3.2 Lipid bilayer experiments

Black lipid bilayer experiments were performed as described previously (Benz *et al.*, 1978) using a 1% solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster AL) in n-decane as membrane forming lipid. The instrumentation consisted of a Teflon chamber with two aqueous compartments separated by a thin wall. The small circular whole between the two compartments had a surface area of about 0.4 mm^2. The aqueous salt solutions were buffered with 10mM MES, pH 6. All salts were obtained from Merck (Darmstadt, Germany). PA_{63} was added from concentrated stock solutions after the membrane had turned black, to the aqueous phase to one side (the *cis*-side) of the membrane. The PA-induced membrane conductance was measured after application of a fixed membrane potential with a pair of silver/silver chloride electrodes with salt bridges inserted into the aqueous phase on both sides of the membrane. The electrodes were connected in series to a voltage source and a home made current-to-voltage converter made with a Burr Brown operational amplifier. The amplified signal was monitored on a storage oscilloscope (Tektronix 7633) and recorded on a strip chart recorder (Rikandenki, Freiburg, Germany). The temperature was kept at 20°C throughout.

5.3.3 Titration experiments

The binding of the blocker-substrates to PA_{63}-channels was studied by titration experiments similar to those used previously to investigate binding of carbohydrates to the LamB-channel of *E. coli* or binding of chloroquine or EF and LF, respectively, to C2II- and PA_{63}-channels in single- or multi-channel experiments (Orlik *et al.*, 2005, Neumeyer *et al.*, 2006a, Neumeyer *et al.*, 2006b, Benz *et al.*, 1987, Bachmeyer *et al.*, 2003). PA_{63}-channels were reconstituted into lipid bilayer membranes from the *cis*-side of the artificial membrane and about 30-60 minutes after addition the rate of channel insertion decreased rapidly. Subsequently, concentrated solution of one blocker-compound was added to the same side of the membrane while stirring to allow equilibration. Figure 5.1A shows an example of a titration experiment of substance HA1383. The

membrane conductance decreased as a function of concentration of the added substance. The data of the channel blockage were analysed similar as performed previously (Benz *et al.*, 1978). The conductance, *G(c)* , of a PA-channel in the present of HA1383 or a related compound with the stability constant, *K*, and the ligand concentration, *c*, is given by the maximum conductance (without ligand), G_{max}, times the probability that the binding site is free.

$$G(c) = \frac{G_{\max}}{(1 + K \cdot c)} \qquad [5.1]$$

This equation may also be written as follows,

$$\frac{(G_{\max} - G(c))}{G_{\max}} = \frac{K \cdot c}{1 + K \cdot c} \qquad [5.2]$$

which means that the conductance as a function of the ligand concentration can be analysed using Lineweaver-Burk plots. *K* is the stability constant for the ligand binding to the PA-channel. The half saturation constant, K_s, of its binding is given by the inverse stability constant $K_s=K^{-1}$.

5.3.4 Current-noise-analysis

The membrane current was measured by a pair of silver/silver chloride electrodes switched in series with a battery operated voltage source and a current amplifier (Keithley 427 with a four pole filter or a home-made operational amplifier with a tree pole filter). Feedback resistors between 0.01 and 10 GΩ were used. The membrane current increased as a result of insertion of reconstituted PA channels. The amplified signal was simultaneously monitored by a strip chart recorder and fed through a low pass filter (4 Pole Butterworth Low-Pass Filter) into an AD-converting card of an IBM-compatible PC. The digitized data were analysed with a homemade fast Fourier transformation program, which yielded identical results as compared to a commercial digital signal analyser (Ono Sokki CF210, Addison, IL). The spectra were composed of 400 points and averaged either 128 or 256 times. To analyse the data commercial graphic programs were used. For the derivation of the rate constants of ligand binding they were fitted to equations described in previously performed studies (Benz *et al.*, 1987, Andersen *et al.*, 1995, Orlik *et al.*, 2002b, Orlik *et al.*, 2002a).

5.4 Results

5.4.1 Binding of blocker-substrates to the PA_{63}-channel

The PA_{63}-channel is fully oriented in artificial membranes when it is added to only one side of the membrane (Blaustein *et al.*, 1989). In previous studies we demonstrated that reconstituted PA-channels as well as C2II-channels can be blocked in lipid bilayer membranes by the addition of 4-aminoquinolines (Bachmeyer *et al.*, 2001, Bachmeyer *et al.*, 2003, Orlik *et al.*, 2005) and identified the binding site for chloroquine to C2II-channels (Neumeyer *et al.*, 2008). The binding affinity strongly depends on negatively charged amino acids near the vestibule of PA-channels. The stability constants *K* for substrate binding to the PA-channels were calculated by multi-channel titration experiments. Activated PA_{63} was added to the *cis*-side (the side of the applied potential) of an artificial bilayer membrane while stirring. This lead to an increase of conductivity caused by channel insertion in the membrane, which was monitored by a strip chart recorder. After about one to three hours, when the conductance was virtually stationary the titration with blocker-substrates was started. Small amounts of concentrated solution of one compound were added to the aqueous phase on the *cis*-side of the membrane while stirring to allow equilibration. Subsequently, the PA-channels were blocked and a dose-dependent decrease of conductance was measured as a function of time.

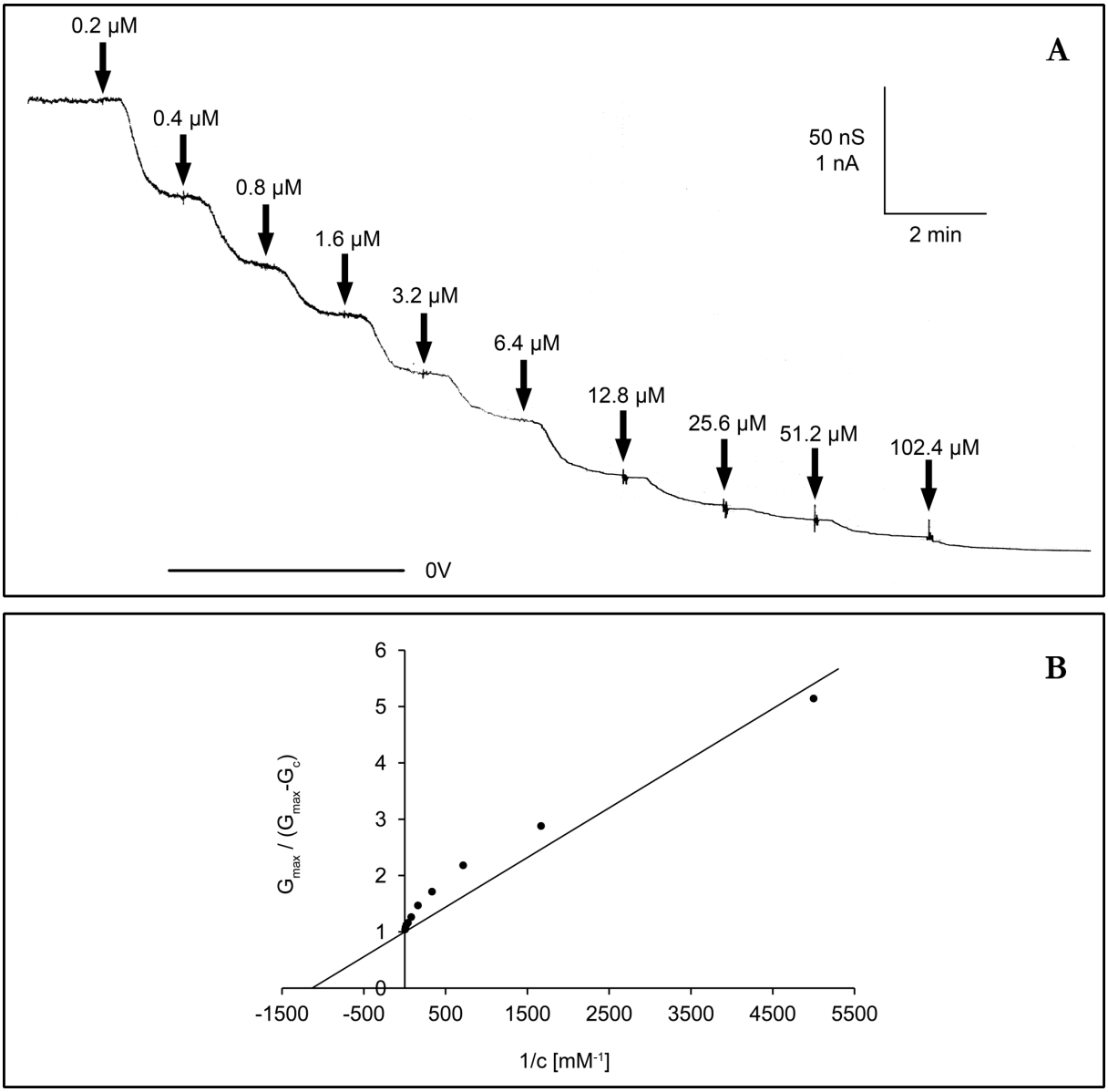


Fig. 5.1:
A: Titration experiment of PA_{63} induced membrane conductance with HA1383. The membrane was formed from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained 1 ng/ml PA_{63} protein (added to the *cis*-side of the membrane), 150 mM KCl, 10 mM MES, pH 6.0. The temperature was constantly 20°C and the applied voltage was 20 mV. The membrane contained about 2000 PA_{63}-channels. The bottom line represents zero level of conductance.
B: Lineweaver-Burke plot of the inhibition of PA_{63} induced membrane conductance by chloroquine and related blocker-substrates. The straight lines correspond to the data points taken from titration experiments (Fig. 1A).

The titration curve shown in figure 5.1A was analysed by Lineweaver-Burk plot (Fig. 5.1B), which yielded a binding constant K of 749 x 10^3 M^{-1} (half saturation constant K_s of 1.34 μM) for the binding of HA1383 to the PA-channel. This was considerably the lowest measured half saturation concentration, which is comparable to that of chloroquine (K_s = 1.43 μM). The half saturation constants of the other blocker-substrates were higher than for chloroquine. While HA1568, which possesses high homologies to HA1383 and reached nearly the same half saturation concentration (K_s = 2.65 μM), HA1196 and HA1371 revealed considerably higher half saturation constants (K_s = 368 μM and K_s = 320 μM respectively). In total we tested 17 substrates (Table 5.1 and Fig. 5.2). The determined half saturation constants range from K_s = 1.34 μM for HA1383 to K_s = 368 μM for HA1196.

Table 5.1: Stability constants K and half saturation constants K_s for chloroquine and related blocker-substrates to PA-channels, when added to the *cis*-side of lipid bilayer membranes.

	K [M^{-1}]	K_s [mM]
HA 42	1456	0.69
HA 47	503	1.99
HA 112	18445	0.057
HA 486	37840	0.027
HA 708	11308	0.089
HA 728	11755	0.085
HA 766	29368	0.035
HA 1196	3750	0.37
HA 1212	7028	0.17
HA 1221	5967	0.17
HA 1371	3156	0.32
HA 1383	749020	0.0014
HA 1495	274457	0.0042
HA 1504	82911	0.012
HA 1568	477501	0.0027
HA 1882	7900	0.13
HA 1885	10829	0.11
Chloroquin	861620	0.0014
Primaquine	51086	0.020

The data represent means of several individual titration experiments. K_s values from chloroquine are given for comparison and are taken from (Orlik *et al.*, 2005). Membranes were formed from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained 150 mM KCl, 10 mM MES, pH 6.0, and about 1 ng/ml PA_{63}; T=20 °C.

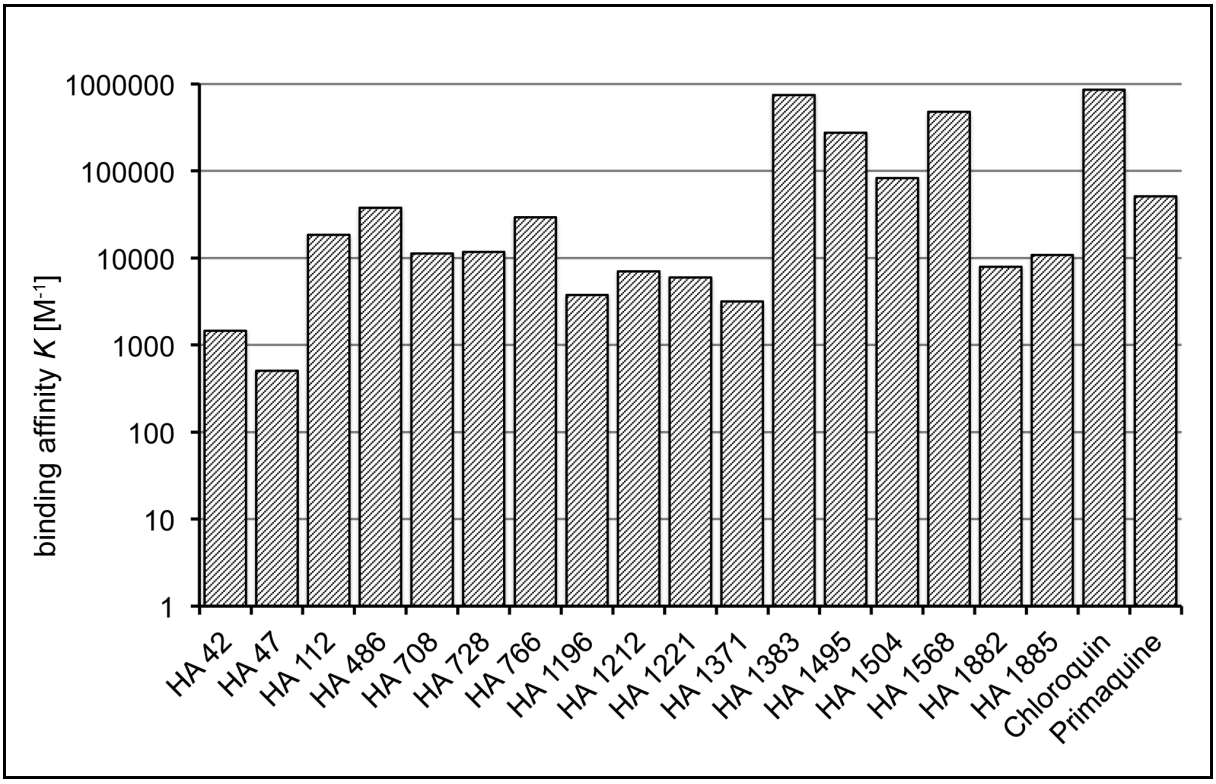


Fig. 5.2:
Diagram of different *K* values calculated from titration experiments to the PA-channels compared to that of Chloroquine taken from (Orlik *et al.*, 2005).

5.4.2 Substrate-induced current-noise-analysis of the PA_{63}-channel

The frequency-dependence of the spectral density values were measured using Fast-Fourier transformation of the current noise parallel to the titration experiments. The measurement of the current noise requires absolutely stationary conditions (Nekolla *et al.*, 1994, Andersen *et al.*, 1995). Therefore, the time between membrane formation and the start of the measurement was extended compared to standard titration experiments. Afterwards, recording of a reference spectrum with only PA present in the aqueous phase, exhibits $1/f$ noise in the frequency range between 1 and 50 Hz (Wohnsland *et al.*, 1997, Nekolla *et al.*, 1994). Figure 5.3 depicts an example of a current noise measurement from PA_{63}-channels before adding any substrate (trace 0). At small frequencies up to ~100 Hz the spectral density was dependent on $1/f$, which is typical for several open bacterial porin channels (Bezrukov *et al.*, 2000a, Jordy *et al.*, 1996, Nekolla *et al.*, 1994, Wohnsland *et al.*, 1997).

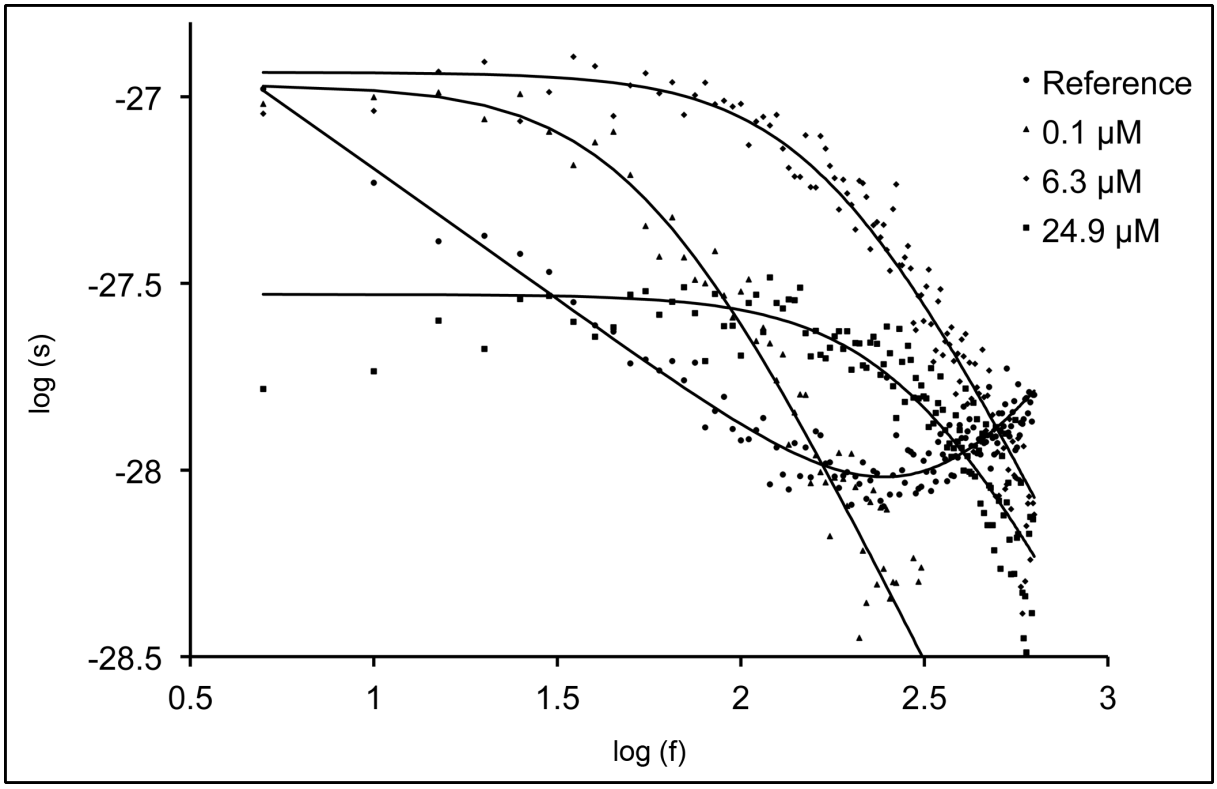


Fig. 5.3:
Power density spectra of HA1383 induced current noise of ~450 PA_{63}-channels (added to the *cis*-side). Trace 0 shows the control (PA without substrate). Trace 1-3: The aqueous phase contained 0.1, 6.3 and 24.9 µM HA1383 and the power density spectrum of trace 0 was subtracted.

The intrinsic noise of the preamplifier that produces a frequency-dependent current noise through the membrane capacity C_m led to an increase of the spectral density at frequencies above ~200 Hz. In further experiments the concentration of the prevailing substrate was increased in defined steps. The power density spectra of the current noise in figure 5.3 corresponded to that of Lorentzian type and could be fitted to a single Lorentzian after the subtraction of the reference spectrum. The power density spectrum, *S(f)*, is given by a Lorentzian function:

$$S(f) = \frac{S_0}{1 + (f/f_c)^2} \qquad [5.3]$$

Table 5.2: Parameters of substrate-induced current noise in PA_{63}.

	K [M^{-1}]	K_s [mM]	k_{on} [10^3 M^{-1} s^{-1}]	k_{off} [s^{-1}]
HA 1383	443493	0.0024	245025	516

k_{on} and k_{off} were derived from a fit of the corner frequencies as a function of the ligand concentration. K is the stability constant for ligand binding derived from the ratio k_{on}/k_{off}. The data represent means of multiple individual experiments with the same substrate.
Membranes were formed from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained 150 mM KCl, 10 mM MES, pH 6.0, and about 1 ng/ml PA_{63} on the cis-side; T=20 °C.

Such a type of noise is expected for a random switch with different on- and off-probabilities (Verveen *et al.*, 1974, Lindemann *et al.*, 1981, Conti *et al.*, 1975). The corner frequencies, f_c, of the Lorentzians are dependent on the on- and off-rate constants, k_1 and k_{-1}. This means that the corner frequencies f_c, should increase with increasing substrate concentration.

Assuming small perturbations in the number of closed channels due to microscopic variations in the number of bound ligand molecules, the reaction rate of the second order reaction is given by equation 4:

$$\frac{1}{\tau} = 2\pi \times f\mathrm{c} = k_1 \times c + k_{-1} \qquad [5.4]$$

As k_1 and k_{-1} stand for the on- and off-rate of binding-events, they are also called k_{on} and k_{off}, respectively. Figure 5.4 depicts the linear fit over the corner frequencies derived over the time course of a complete current-noise-analysis measurement of HA1383 and PA. The according k_{on}-value is calculated to 120 x 10^6 $M^{-1}s^{-1}$, which is in the range of diffusion limited processes. The off-rate for HA1383 is very slow (k_{off} = 352 s^{-1}), reasoning a stable plug being formed in the PA-channel. The corresponding constants K and K_s are in good agreement with the binding parameters derived from titration experiments.

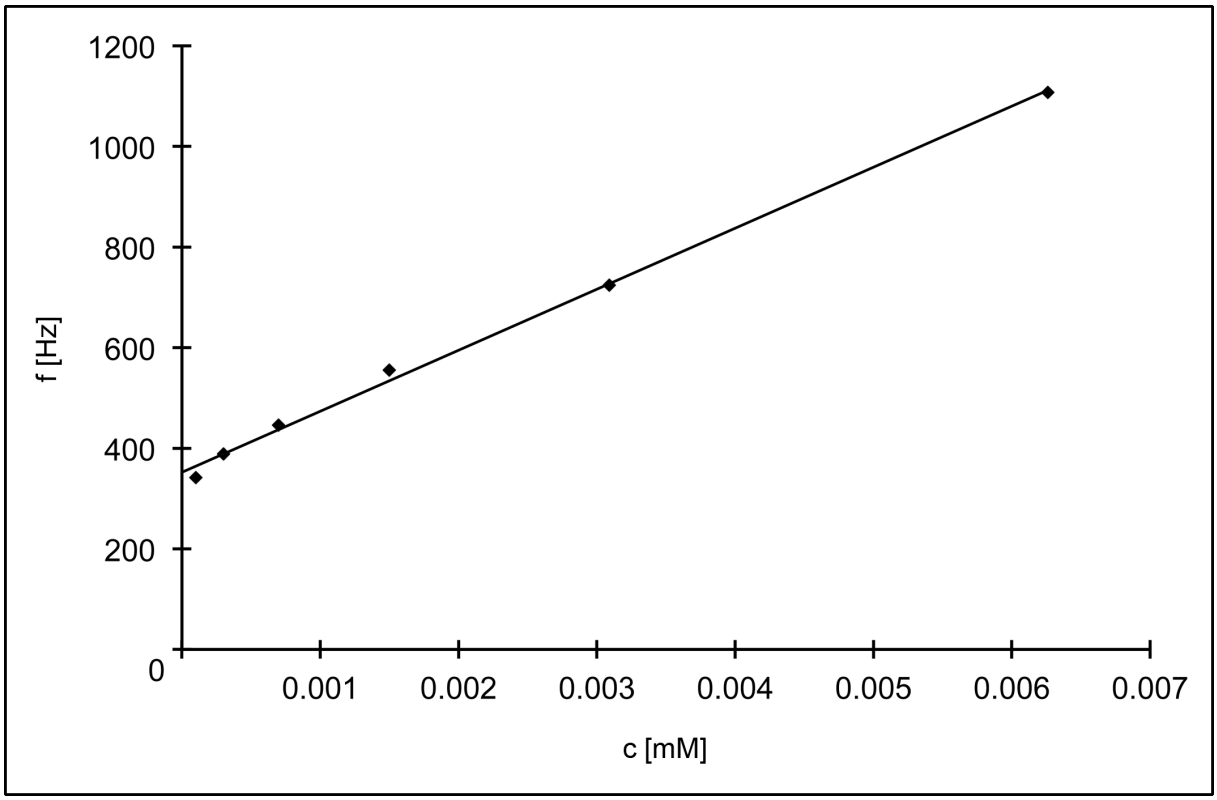


Fig. 5.4:
Tau-diagram of current noise analysis for the interaction of HA1383 and PA

5.5 Discussion

5.5.1 Half saturation constants emphasize the possibility to block PA_{63}-channels

Comparison of the half saturation constants derived from titration experiments (Table 5.1 and Fig. 5.2) lead to the conclusion that changes in functional groups of the designed blocker-substrates result in large differences of binding affinity. One could easily determine substances with lower and more or less equal affinities to PA_{63}-pores compared with chloroquine, which is well known as a strong binding reagent towards that channel (Neumeyer *et al.*, 2008). Thereby, substrates with similar affinity are of special interest regarding a good possibility to rival binding of LF and EF, in order to inhibit intoxication. The high *K*-values of HA1383 and HA1568 are in an interesting range to presumably block the uptake of enzymatic domains. These results have to be proven right by cell-based assays.

5.5.2 Titration and noise data allow deeper insight in the chemical-group-dependent binding constants

Regarding the results of the noise-analysis (Table 5.2) one has to recognise that k_{on} is merely in the range of a diffusion controlled process, while the k_{off} values determine the time the substrate stays bound to the pore and therefore the stability of the blockage. This has been found for LF and EF in former measurements, too (Neumeyer *et al.*, 2006a, Neumeyer *et al.*, 2006b). Concerning merely equal k_{on}-values The stability constant *K*, being calculated by a quotient of k_{on} over k_{off}, is mainly depending on k_{off}.

Presumably, the plug, formed by HA1383, surrounds the ϕ–clamp and is very stable under the acidic conditions in the endosome. Additionally, the aromatic core combined with positive charges should lead to assimilation in this compartment. These results imply the assumption that the blockage of PA-channels for the transport of the native effectors could be achieved *in vivo* as well.

A closer look to the results reveals, that positive charges in the substrates have the strongest influence on the binding affinity. The role of positive charges in binding or transport through PA-channels has been analysed in many different publications (Blanke *et al.*, 1996, Beitzinger *et al.*, 2011a, Beitzinger *et al.*, 2011c). There have been speculations about a profound binding which is related to two negative charged rings of amino acids surrounding the constriction site of the ϕ–clamp. On the on hand, this would represent a motive, which could introduce the unfolding and therefore trafficking via PA. On the other hand, substrates, which bind tightly in that region of

the pore, would form a block that hinders the entrance of effectors even if they are bound to the channel before.

The ionic interaction of the blocker-substrates with the channel is contradictory to the effect of accumulation in the endosomes. Only substrates that have a change in overall charge from neutral in the cytosol or cell exterior to positive under acidic conditions in the endosome posses this desired feature. Therefore, the tested substrates are designed to have the charge dispersed over a large aromatic system, which should render them possible to cross membranes. In the endosome, the charges stabilize by acidic pH and the functional groups of the molecules. Additionally, there could be hydrophobic interactions between the aromatic ring system of the substrate and those of the ϕ–clamp. In former studies the enhanced affinity of cyclodextrins, which inherit aromatic residues was found, but could not be explained (Nestorovich *et al.*, 2010, Nestorovich *et al.*, 2011). In the duality of the aromatic group described above, we now present a hypothesis that has to be proven right by cell-based assays.

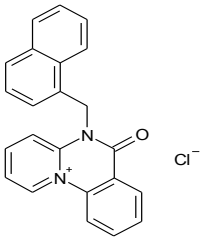
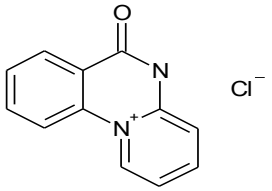
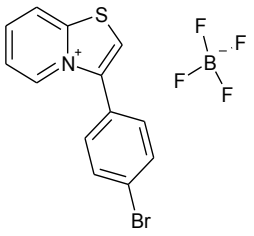
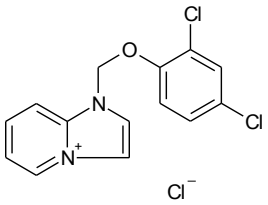
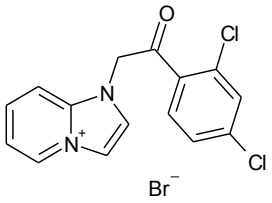
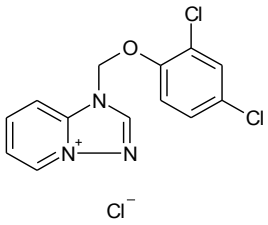
The most interesting results could be achieved by comparing the binding of two very homologue substrates: HA1383 and HA 1568. They just differ in two functional groups. While HA1383 has an OH- and a NO_2-group, HA1568 possesses chloride and bromide residues in the correlating positions (Table 5.3). All four administrate electron-pulling forces to the structure, thereby stabilizing the positive charge over the aromatic system. Nevertheless, a significant difference in K_s-values of HA1383 (1.34 μM) and HA1568 (2.65 μM) was observed, implementing the important feature of those residues. Especially for the pharmacological characteristics and further design of blocker-substrates the possibility to alter certain qualities and at the same time residing in the desired range of affinity is crucial.

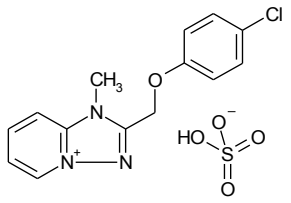
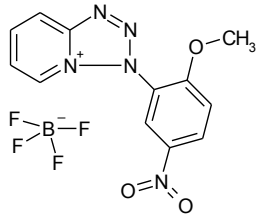
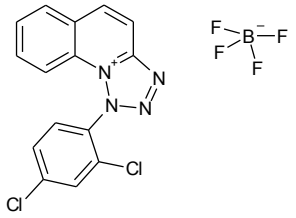
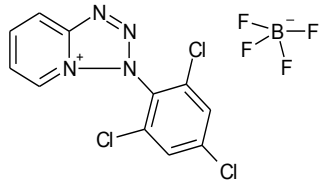
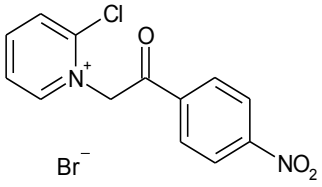
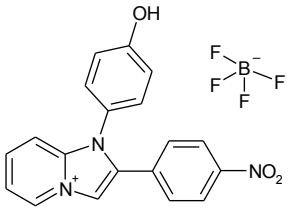
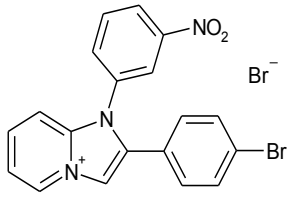
5.5.3 Out view

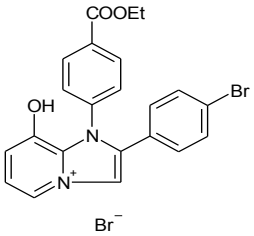
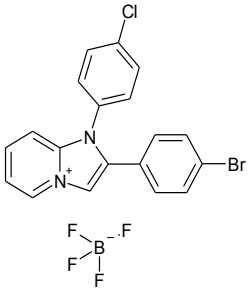
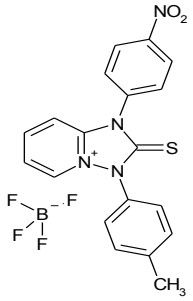
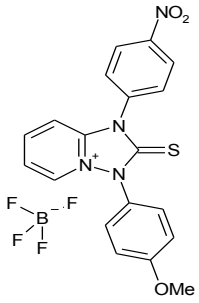
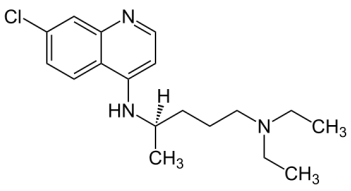
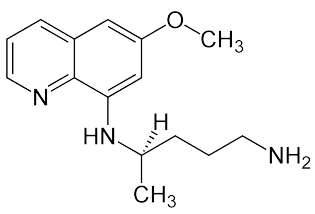
The results presented in this work were acquired in an *in vitro* system. Nevertheless, they strongly suggest a possible use of blocker-substrates tested for further experiments. These should include a cytotoxicity assay for the substances to get more information about critical concentrations in living organisms. Afterwards, cell-based assays should be used to determine whether the assumption of a stable block for the transport of the toxic-moieties of Anthrax is formed. The experiments with cell-cultures could further proof if an assimilation in the endosomal compartments is taking place, which would allow the use of lower overall concentrations of the substrates. In the end, *in vivo* studies could lead to the development of a new sort of drugs, which prevent infected persons to suffer from the symptoms of Anthrax intoxication. This would buy

time to invent agents to deal with the infection and therefore could be a "live safer" after bio-terroristic assaults with multi-resistant strains.

Table 5.3: Structures of different blocker-substrates

	Structure	Formula	Mass [g/mol]
HA 42		C23H17ClN2O	372.86
HA 47		C12H9ClN2O	232.67
HA 112		C13H9BBrF4NS	378.00
HA 486		C14H11Cl3N2O	329.62
HA 708		C15H11BrCl2N2O	386.08
HA 728		C13H10Cl3N3O	330.60

	Structure	Formula	Mass [g/mol]
HA 766		C14H14ClN3O5S	371.80
HA 1196		C12H10BF4N5O3	359.05
HA 1212		C15H9BCl2F4N4	402.98
HA 1221		C11H6BCl3F4N4	387.36
HA 1371		C13H10BrClN2O3	357.59
HA 1383		C19H14BF4N3O3	419.15
HA 1495		C19H13Br2N3O2	475.14

	Structure	Formula	Mass [g/mol]
HA 1504		C22H18Br2N2O3	518.21
HA 1568		C19H13BBrClF4N2	471.49
HA 1882		C19H15BF4N4O2S	450.23
HA 1885		C19H15BF4N4O3S	466.23
Chloroquine		C18H26ClN3	319.87
Primaquine		C15H21N3O	259.35

Chapter 6

Anti-His Antibody is Able to Block PA-Pores in an Ion Dependent Manner

One of the possible ways to fight an Anthrax intoxication is to develop antibodies against it's compounds. Normally, these are designed to target the enzymatic moieties EF and LF. Up to now there is no known way to block transport via PA with antibodies. In this chapter, first-time evidence of anti-His antibody binding to PA is presented, opening a wide field of new and exciting experiments for future projects.

6.1 Summary

The important role of charges inside and facing the ends of the PA_{63}-channels is of special interest in the field of research concerned with transport via binary toxins (Beitzinger *et al.*, 2011a, Beitzinger *et al.*, 2011b, Beitzinger *et al.*, 2011c, Fischer *et al.*, 2011). The occurrence of a histidine on the proposed opening, on the *trans*-side of the heptameric pores, led to the estimation that anti-His antibody should be able to bind to the channel from this side. We could demonstrate *in vitro* that small concentrations of anti-His antibody are sufficient to block ion-current through the pores up to 60 - 80%. This blockage was found to be electrolyte-concentration-dependent and mainly took place if the antibody was added to the *trans*-side of PA_{63}-saturated membranes.
Binding to the *cis*-side of the PA_{63}-channels (the so-called mushroom head) did only occur in low concentrated salt solutions (50 mM KCl) and led to a fast decrease in conductance. The remaining conductivity could be further reduced afterwards by adding antibody to the *trans*-side, which results in the estimation, that there should be another binding position. This position has to be revealed by structural changes that take place under these conditions. Even if the existing model does not provide any histidines on the opening of the channel, the structural approach using electron microscopy has shown some differences to this model in the appearance of the mushroom body (Katayama *et al.*, 2010) which could include the existence of another binding site for the antibody.

6.2 Introduction

One of the main virulence factors of *Bacillus anthracis* is Anthrax toxin. The plasmid encoded tripartite toxin comprises a receptor-binding moiety termed protective antigen (PA) and two different enzymatically active components, lethal factor (LF) and edema factor (EF) (Collier *et al.*, 2003, Friedlander, 1986, Mock *et al.*, 2001). Both EF and LF require the binding component PA for delivery into the cytosol of target cells where they exhibit enzymatic activity. Lethal factor (90 kDa) is a highly specific zinc-dependent metalloprotease targeting mitogen-activated protein kinase kinases (MAPKKs), e.g. MEK2, thereby initiating a still poorly understood mechanism, which leads to subsequent death by apoptosis of some types of macrophages and to the inhibition of the release of proinflammatory mediators like nitric oxide, tumour necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1 β) from macrophages (Hanna *et al.*, 1993, Menard *et al.*, 1996a, Pellizzari *et al.*, 1999). Edema factor (89 kDa) is a calmodulin- and Ca^{2+}-dependent adenylate cyclase, which interferes with cell signalling by increasing the cytosolic cAMP level. Thereby it alters water homeostasis and destroys the balance of intracellular signalling pathways (Dixon *et al.*, 1999, Lacy *et al.*, 2002, Mock *et al.*, 2001).

Monomeric protective antigen (83 kDa) is secreted by *B. anthracis* as a water-soluble precursor form (PA_{83}). Proteolytic cleavage of a 20 kDa N-terminal fragment (PA_{20}) by a furin-like proteases leads to the activated PA_{63}-monomer, which is able to oligomerize into heptamers (Petosa *et al.*, 1997, Young *et al.*, 2007). This prepore can bind up to three molecules of EF and/or LF with high affinity (Cunningham *et al.*, 2002, Elliott *et al.*, 2000, Escuyer *et al.*, 1991). Bound to a cell-surface receptor (Bradley *et al.*, 2001) the complex is endocytosed and acidification of the endosome results in translocation of the enzymatic components into the target cell's cytosol (Miller *et al.*, 1999, Nassi *et al.*, 2002). This translocation mechanism is common among several other A-B type toxins, such as C2 toxin from *Clostridium botulinum* or iota toxin from *Clostridium perfringens* (Barth *et al.*, 2002a).

Prominent structural and biophysical features of the PA_{63}-channel were investigated, e.g. channel-forming properties of the PA-heptamers (Blaustein *et al.*, 1989, Finkelstein, 1994). Recent studies underline the importance of the Φ–clamp including the loop network for stabilization of Phe427 (Krantz *et al.*, 2006, Krantz *et al.*, 2005, Melnyk *et al.*, 2006) and a potential translocation mechanism (Krantz *et al.*, 2004, Zhang *et al.*, 2004a, Zhang *et al.*, 2004b, Zhang *et al.*, 2004d). Chloroquine and related compounds block PA_{63}- as well as C2II-channels *in vitro* as well as *in vivo* (Orlik *et al.*, 2005). The binding site was identified inside the lumen of the C2II-channel (Neumeyer *et al.*, 2008). Although the crystal structure of the membrane-associated PA_{63}-channel is not solved yet, both the crystal structure of the prepore and a hypothetical model deliver a

rough idea of the membrane-spanning functional pore (Nguyen, 2004, Petosa *et al.*, 1997). Lately, electron microscopy has been used as an approach to the structure of the pores. These results differ slightly from the proposed model (Katayama *et al.*, 2010).

We studied the binding properties of anti-His antibody to the PA_{63}-channel in artificial membranes leading to a dose-dependent decrease of membrane conductance when added to the *trans*-side of the pore. The over all closure of the channel was measured by *in vitro*-binding experiments with anti-His antibody and PA_{63}-pores. The percentages of conductance decrease after addition of small concentrations of antibody to PA_{63}-saturated membranes were determined by single- and multichannel titration analysis indicating a strong relationship between electrolyte concentration and binding kinetic of the antibody to PA_{63}-channels. These findings provide a hint towards ion-dependent changes in structure of PA_{63}-pores.

6.3 Material and Methods

6.3.1 Materials

Nicked Anthrax PA_{63} from *B. anthracis* was obtained from List Biological Laboratories Inc., Campbell, CA. One mg of lyophilized protein was dissolved in 1 ml 5 mM HEPES, 50 mM NaCl, pH 7.5 complemented with 1.25% trehalose. Aliquots were stored at -20°C. Channel formation by PA_{63} was stable for months under these conditions.
Anti-His antibody (Mouse) was obtained from GE-Healthcare (20 - 40 µg/µl).

6.3.2 Mutation of Histidine 310 to Glycine

Quick-exchange PCR was conducted with pet19b vectors containing the wildtype sequence of PA and mutagenesis primers designed with the Glycine-triplet in the corresponding locus. Annealing took place at 55°C and prolongation was achieved with a high performance *pfu*-polymerase from Fermentas at 68°C. Afterwards, PCR-products were transferred by heat shock at 42°C for 1 minute in Neb5α-cells from NewEnglandBiolabs. Plasmids were prepared using a miniprep-Kit (NucleoSpin© Plasmid Kit) from MacheryNagel and afterwards transformation into omp8 BL21 DE3 cell-line took place in an Eppendorf Electroporator 2510 with 2500 V and < 5 ms application time. All exchanges have been justified by sequencing of single colonies from transformed cultures performed by GATC-biotech.

6.3.3 Lipid bilayer experiments

Black lipid bilayer measurements were performed as described previously (Benz *et al.*, 1978). The instrumentation consisted of a Teflon chamber with two aqueous compartments connected by a small circular hole. The hole had a surface area of about 0.4 mm^2. Membranes were formed by painting a 1% solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) in n-decane onto the hole. The aqueous salt solutions (Merck, Darmstadt, Germany) were buffered with 10 mM MES to pH 5.5 to pH 6. Control experiments revealed that the pH was stable during the time course of the experiments. The binding components of the binary toxins were reconstituted into the lipid bilayer membranes by adding concentrated solutions to the aqueous phase on one side (the *cis*-side) of a black membrane. The temperature was kept at 20°C throughout. Membrane conductance was measured after application of a fixed membrane potential with a pair of silver/silver chloride electrodes inserted into the aqueous solutions on both sides of the membrane. Membrane current was measured using a homemade current-to-

voltage converter combined with a Burr Brown operational amplifier. The amplified signal was monitored on a storage oscilloscope and recorded on a strip chart recorder.

6.3.4 Binding experiments

The binding of the anti-His antibody to the binding component PA_{63} was investigated with titration experiments similar to those used previously to study the binding of 4-aminoquinolones to the C2II- and PA_{63}-channels and EF and LF to the PA_{63}-channel in single- or multi-channel experiments (Bachmeyer *et al.*, 2003, Neumeyer *et al.*, 2006b, Orlik *et al.*, 2005). The C2II- and PA_{63}-channels were reconstituted into lipid bilayers. About 60 minutes after the addition of activated PA_{63} to the *cis*-side of the membrane, the rate of channel insertion in the membranes was very small. Then small amounts of anti-His antibody were added to the *cis*-side of the membranes while stirring to allow equilibration. The results of the titration experiments, i.e. the blockage of the channels, were analyzed in a quantitative correlation of the initial conductivity and the conductivity reached after addition of the antibody.

6.4 Results

6.4.1 The binding of anti-His antibody is site-dependent

Titration experiments were performed as described above. After saturation of the membrane has been reached, 20-40 µg of the anti-His antibody were added to the *cis*-side first. Figure 6.1 shows a stable conductivity after this addition. The second addition of 20-40 µg of the antibody to the *trans*-compartment is followed by a steep and fast decrease of 77.2% of the conductivity. This behavior could be reproduced in several measurements.

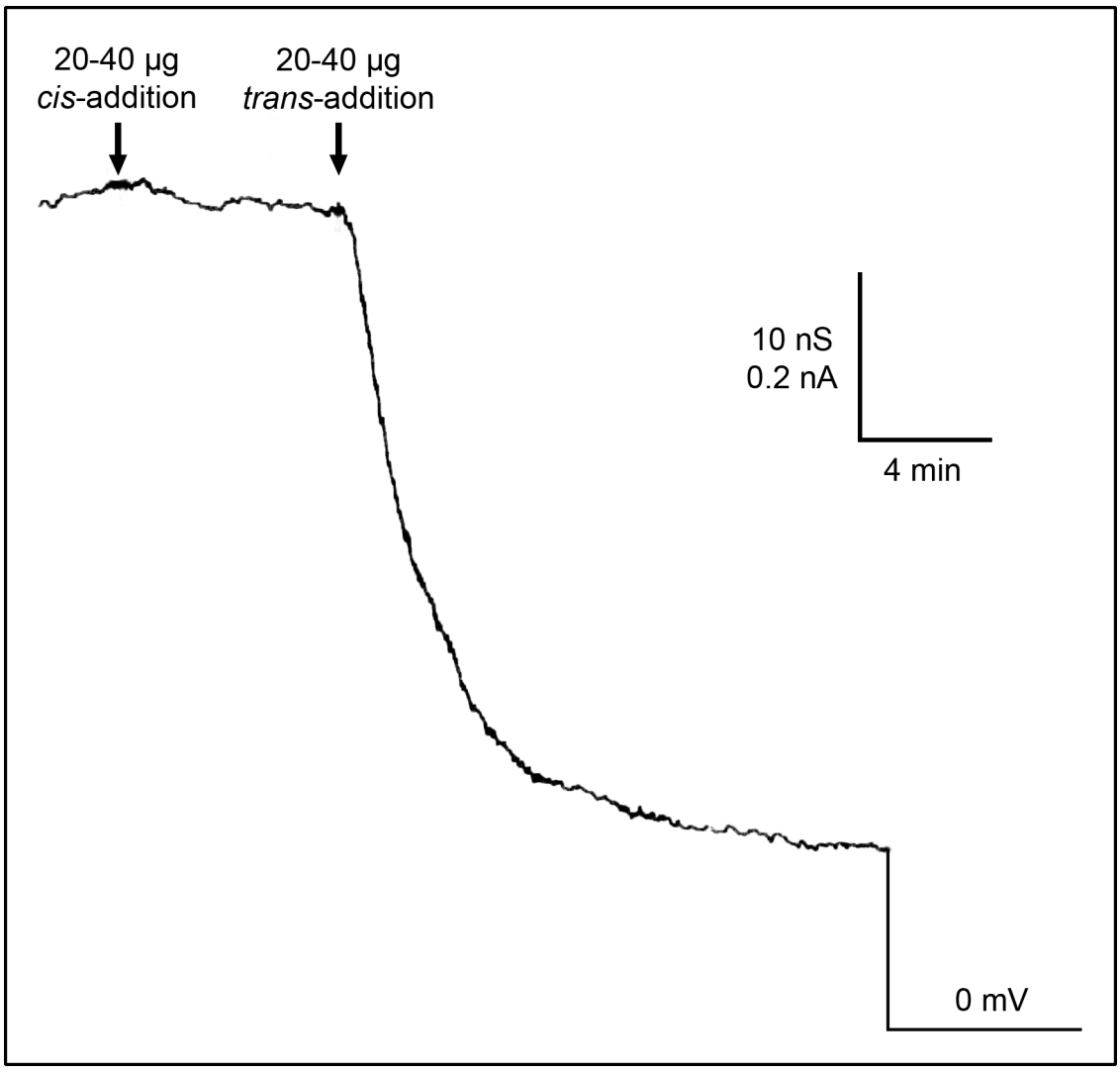


Fig. 6.1: Titration experiment of anti-His antibody and PA-pores in 150 mM KCl. The membrane was formed from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained 1 ng/ml PA_{63} protein (added to the *cis*-side of the membrane), 10 mM MES, pH 6.0. The temperature was constantly 20°C and the applied voltage was 20 mV. The membrane contained about 600 PA_{63}-channels. The bottom line represents zero level of conductance.

6.4.2 Binding of anti-His antibody to PA_{63}-pores depends on ion concentration

A second set of experiments was performed with varying electrolyte concentrations. While the graphs of measurements in 150 mM, 1 M and 3 M KCl looked like the one presented in figure 6.1 and only led to small differences in conductivity decrease (Table 6.1), the measurement in 50 mM depicted a huge change in this behavior. In figure 6.2A an initial decrease of 80.9% of conductivity could be observed, when the antibody is added to the *cis*-side. The second addition to the *trans*-compartment led to a further step, which is 93.9% of the remaining conductance. When the antibody was added to the *trans*-side first, the decreases represented 68.5% of conductance after the first and 85.0% of remain conductance after the second addition (*cis*-compartment) (Fig. 6.2B).

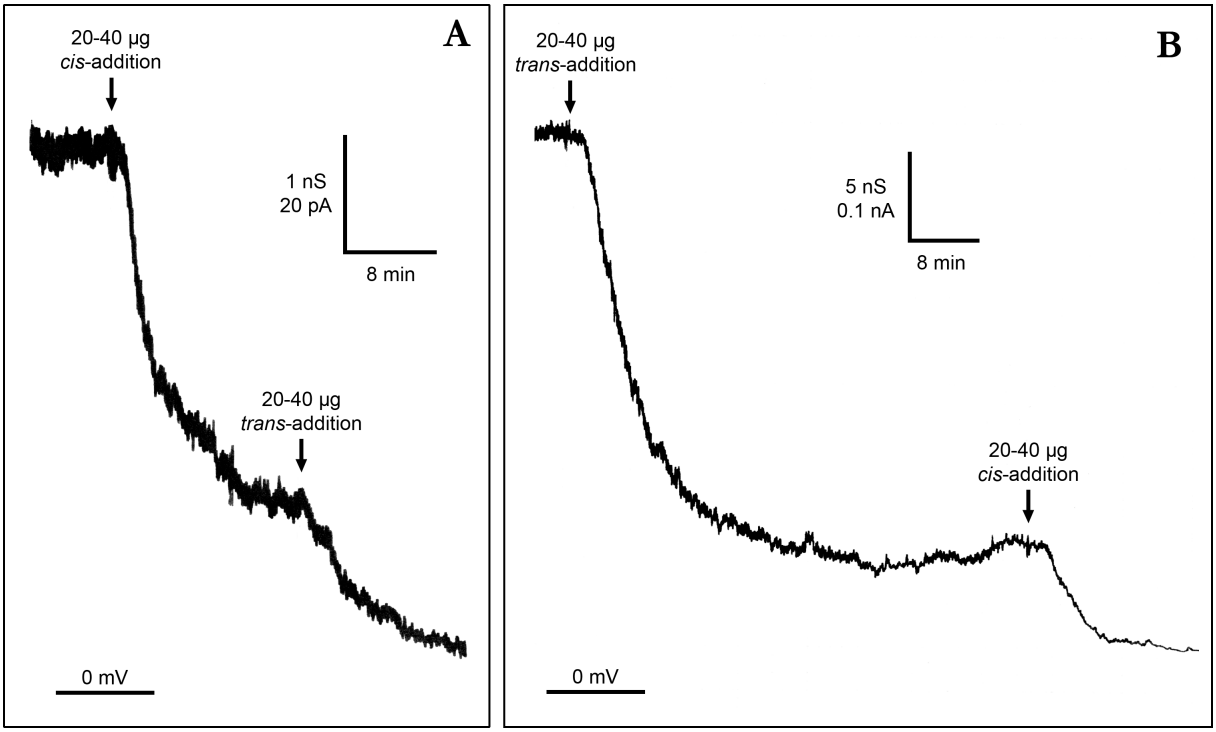


Fig. 6.2: Titration experiment of anti-His antibody and PA-pores in 50 mM KCl. The membrane was formed from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained 1 ng/ml PA_{63} protein (added to the *cis*-side of the membrane), 10 mM MES, pH 6.0. The temperature was constantly 20°C and the applied voltage was 20 mV. The membrane contained about A:70, B:350 PA_{63}-channels. The bottom line represents zero level of conductance.
A: Addition *cis*-side first, than *trans*-side
B: Addition *trans*-side first, than *cis*-side

Table 6.1: Decreased conductance [%] by anti-His antibody in different electrolyte concentrations from *cis*- and *trans*-side of PA-channels

		***cis*-side addition**		***trans*-side addition**	
$c_{(KCl)}$ [M]	**order**	**ΔG [%]**	**SD**	**ΔG [%]**	**SD**
3	cis - trans	0	0	62.5	0.04
1	cis - trans	0	0	77.7	0.11
0.15	cis - trans	0	0	77.2	0.02
0.05	cis - trans	80.9	0.15	93.9	0.04
0.05	trans - cis	68.5	0.07	85.0	0.06

The data represent means of at least three individual titration experiments.
Membranes were formed from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained 150 mM KCl, 10 mM MES, pH 6.0, and about 1 ng/ml PA_{63}; T=20 °C.

6.4.3 Closure of PA_{63}-channels by anti-His antibody is very stable

Single-channel titration experiments were performed to proof to what extend each channel is blocked by the antibody. PA wt was added to the *cis*-side of the cuvette in a 1:1000000 dilution (from a 1 mg/ml stock solution). After a single pore formed and stayed stable in the membrane for some minutes, 20-40 µg anti-His antibody were added to the *trans*-side. Figure 6.3 depicts the initial stepwise increase of conductivity due to the insertion of the pore, which produces a typical noise, known for PA-channels. Shortly after the second addition, the conductivity dropped back to zero and remained there for the rest of the measurement (several minutes).

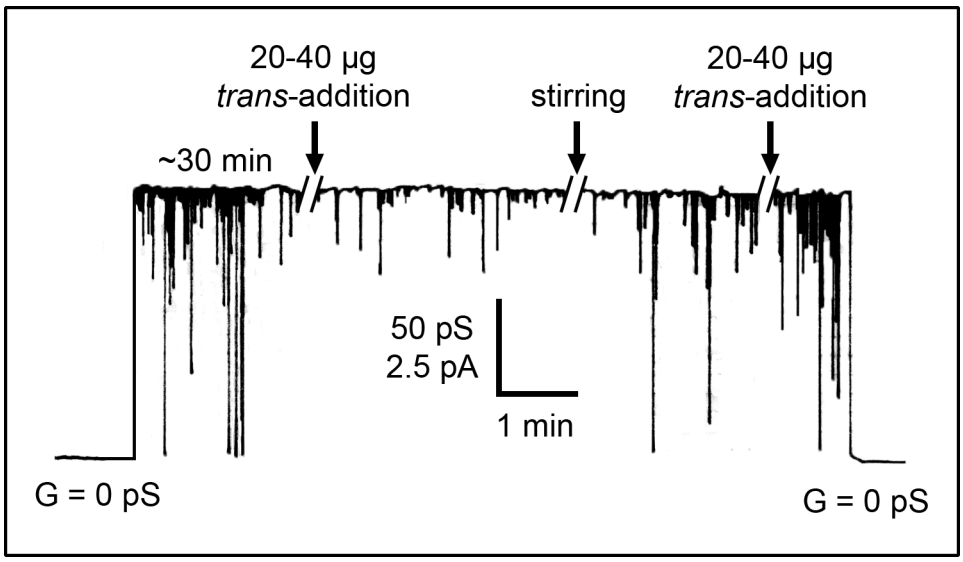


Fig. 6.3: Single-channel closure of one PA-pore after anti-His antibody addition. The membrane was formed from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained 10 mM MES, pH 6.0. The temperature was constantly 20°C and the applied voltage was 20 mV. Addition of 1 ng anti-His antibody occurred two times.

6.4.4 Anti-His antibody changes voltage-dependency of PA_{63}-pores

PA-channels possess a well-described voltage-dependency, which reasons in a decrease in conductivity over time when voltages over +50 mV or in the negative range are applied to the saturated membrane. Voltage-dependency experiments, which were performed after the titration of anti-His antibody, exhibit a significant change in this behavior (Fig. 6.4). The graph shows a faster onset of the voltage-induced closure of pores in the negative range. On the positive side one could determine an increase at voltages between 20-30 mV, while the decrease afterwards is steeper than without the antibody. This effect is similar to the voltage-induced closure of PA-channels caused by His-tagged proteins (Beitzinger *et al.*, 2011c).

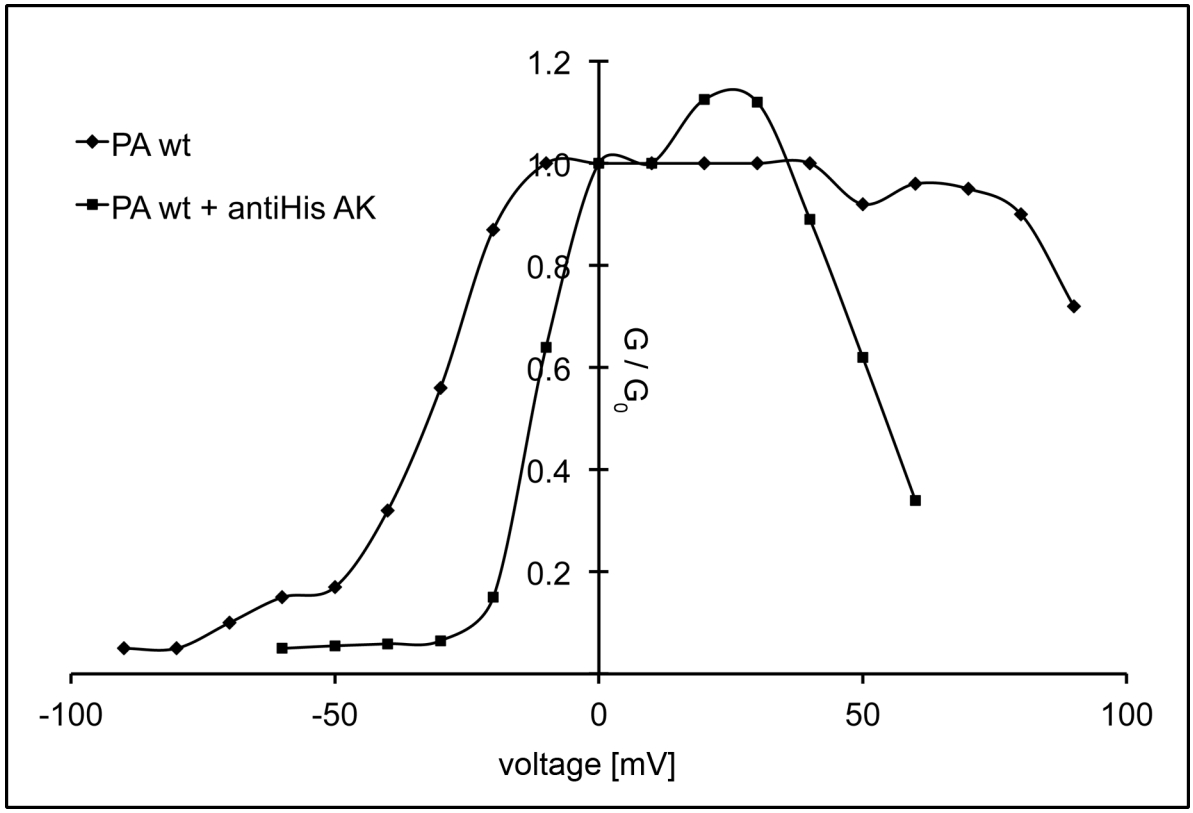


Fig. 6.4: Diagram of voltage-dependent closure of PA-channels before and after interaction with anti-His antibody.

6.4.5 Mutation of Histidine 310 to Glycine does neither affect PA's biophysical properties nor the binding of anti-His antibody

Titration measurements of a histidine mutant of PA should elucidate the binding site. This mutant has a single channel conductance of 70 pS and single steps look similar to wildtype PA-channels (Fig. 6.5). Selectivity measurements disclose a slightly reduced quotient of cations over anions, that is still in the range of high cation-selectivity (data not shown). The voltage-dependency is comparable to that of the wildtype (data not shown).

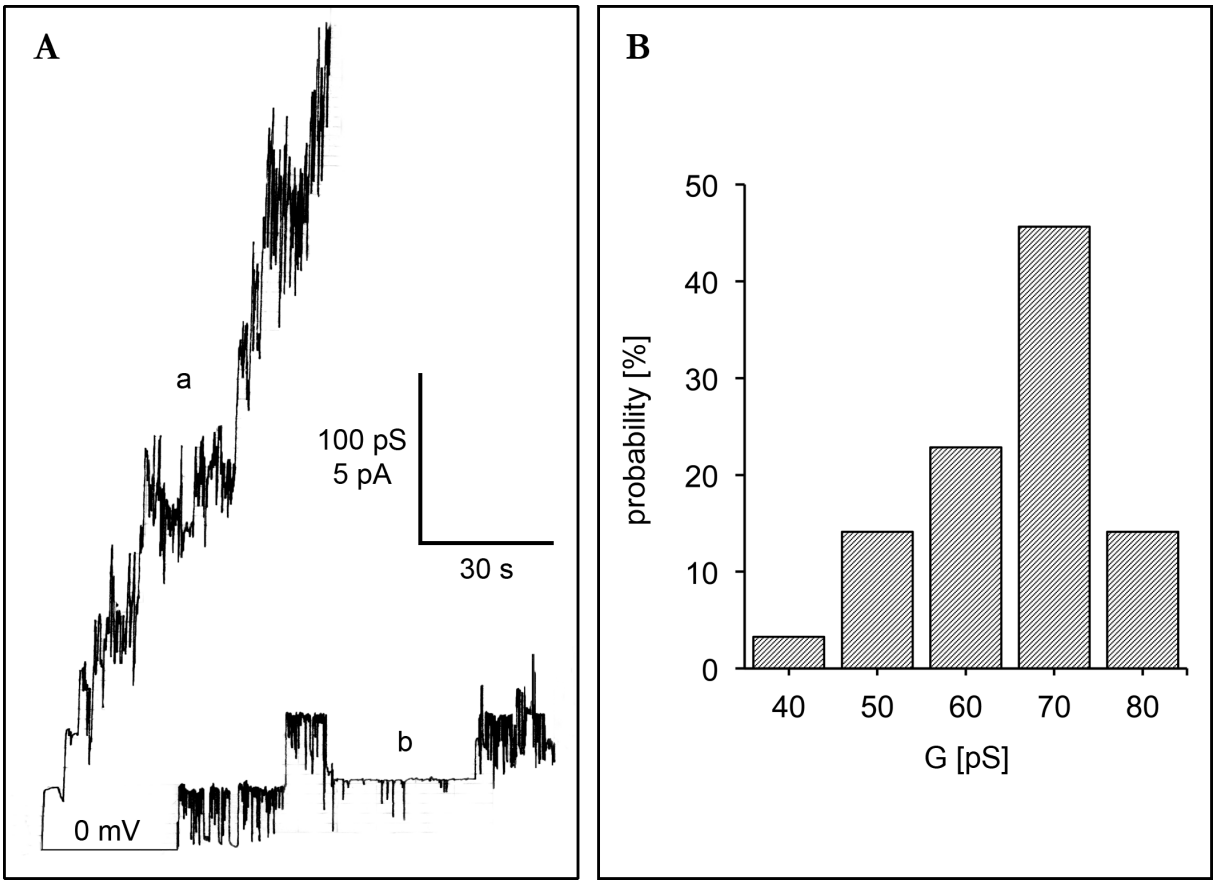


Fig. 6.5: Single-channel conductance of PA H310G
A: Stepwise increase of conductance after addition of PA H310G to the *cis*-side of the membrane. Membranes were formed from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained 150 mM KCl, and 10 mM MES, pH 6.0; T=20 °C.
B: Histogram for probability of occurrence for single-channel events

The titration experiments were performed as with the PA wt in 150 mM KCl. Unexpectedly, they demonstrated the same curves and values as determined before. Figure 6.6 depicts an experiment, where no reduction after an addition of 20-40 μg antibody to the *cis*-side is followed of an decrease of conductivity by 81.1% on the *trans*-side. The comparison of the mean values of experiments in different electrolyte concentrations in table 6.2 presents identical data for wildtype and mutant.

Table 6.2: Comparison between PA wt and the mutant PA H310G

		cis-side addition		*trans*-side addition	
PA	**$c_{(KCl)}$ [M]**	**ΔG [%]**	**SD**	**ΔG [%]**	**SD**
wt	0.15	0	0	77.2	0.02
H310G	0.15	0	0	81.1	0.04
wt	0.05	80.9	0.15	93.9	0.04
H310G	0.05	65.9	0.10	81.6	0.02

The data represent means of at least three individual titration experiments.
Membranes were formed from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained 150 mM KCl, 10 mM MES, pH 6.0, and about 1 ng/ml PA_{63}; T=20 °C.

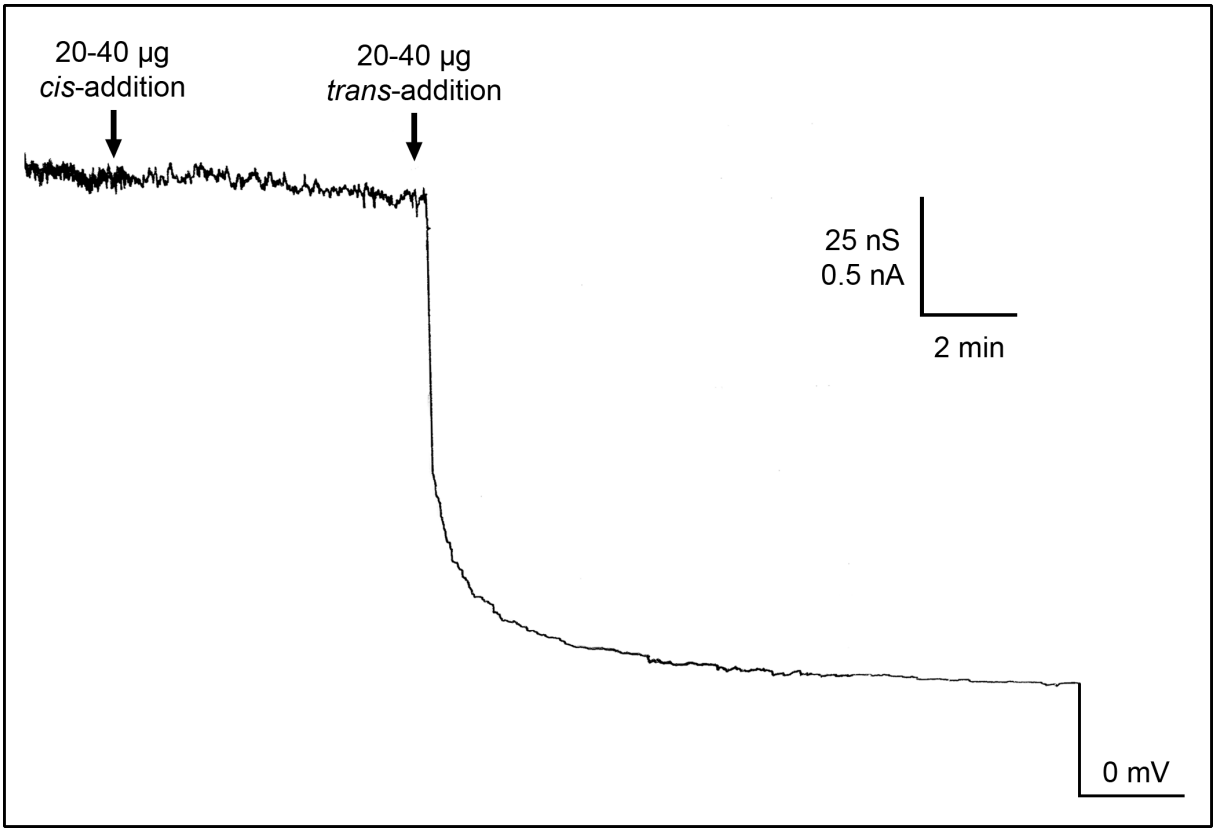


Fig. 6.6: Titration experiment of anti-His antibody and PA H310G-pores in 150 mM KCl. The membrane was formed from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained 1 ng/ml PA_{63} protein (added to the *cis*-side of the membrane), 10 mM MES, pH 6.0. The temperature was constantly 20°C and the applied voltage was 20 mV. The membrane contained about 1500 PA_{63}-channels. The bottom line represents zero level of conductance.

6.5 Discussion

6.5.1 Anti-His antibody is able to block PA-channels from the *trans*-side only

The titration experiments provide first time evidence of the possibility to block ion current through the PA-pore with an antibody. Similar results were found earlier for Epsilon-toxin of *Clostridium perfringens* (Knapp *et al.*, 2009). The conductivity was only reduced when the antibody was present on the *trans*-side of the channel, which leads to the assumption, that there should be a structure, which serves as a target for the antibody. Anti-His antibody is able to detect six or more histidines, which normally form a curved shape. It has been speculated, that a ring of histidines, formed by the seven monomers of PA, is the binding site on the exit of the pore. Concerning the model of Nguyen (Nguyen, 2004), this ring should consist of seven His310 residues, which are closest to the *trans*-end of the channel.
Additionally, single-channel titration proofed, that the antibody blocks the current through the pore completely and that this block is stable for at least several minutes, which could be expected for the binding of an antibody. A change in the voltage-dependency of PA after titration with a binding-partner has lately been describe by our group (Beitzinger *et al.*, 2011c). Similar results were found now for the antibody to pore interaction. The binding tightens when voltages in the physiological range were applied to the system from both sides. While this effect is clearly involved in transport through PA-channels for His-tagged proteins, it is not known so far what causes the further closure of pores after the binding of the antibody.

6.5.2 The binding-site on the *trans*-side of PA_{63}-channels is not formed by a ring of Histidine 310

The electrophysiological characterization of the PA H310G mutant did not show considerable changes in the pore. Single-channel conductance, selectivity and voltage-dependency were in the same range and illustrated, that no structural changes occurred. Nevertheless, there was no change in the binding properties of the antibody to the pore. This unexpected result represents a clear proof, that His310-residues do not represent the target for the interaction of the antibody with the pore. The similar changes in voltage-dependency are further hints for a different binding site. This site could either be formed by another histidine residue or by the occurrence of structural closely related phenylalanine (P313, P314) residue in combination with charges of a nearby-located aspartic acid (D315). The main binding properties of the antibody are described as ionic interactions and formation of hydrogen bounds between aromatic residues.

The closest histidine in the proposed β-barrel is residue His304, which is hidden inside the pore and should not be reachable by the antibody. Further experiments with a single mutation of this amino acid and a double mutant of both histidines should proof, if the model has to be changed.

6.5.3 Binding in low salt concentrations reveals a possible second binding-site on the *cis*-side

Binding affinities of effector molecules to PA-channels are strongly influenced by electrolyte concentration in the surrounding media (Neumeyer *et al.*, 2006a, Neumeyer *et al.*, 2006b). This is due to the strong ionic interactions of the binding-partners. The experiments in 150 mM, 1 M and 3 M KCl did not show significant differences in conductivity reduction, leading to the assumption, that ionic interactions do not influence the binding of the antibody to a huge extend. A result that should be discussed in more detail is the unexpected change of binding-behavior in low salt concentrations. At 50 mM KCl the antibody was able to block the ion current from the *cis*-side as well. This decrease in conductance was around 80% and could additionally be achieved before or after the 70% decrease from the *trans*-side. As the antibody, due to it's size, is not supposed to cross the pore, the only explanation for this effect is the existence of a second binding site on the *cis*-side. This location is only revealed in low salt concentrations and therefore could be related to a structural change in the mushroom-body of PA. Lately, cryo-electron-microscopy images of PA-channels depicted, that the model is not completely correct for this region of the pore (Katayama *et al.*, 2010). Therefore, additional mutants and binding experiments are necessary to identify the amino acid responsible for this interaction.

Chapter 7

Conclusions

The data presented in this work imply some general features, that could be relevant for further studies on AB-toxins. On the one hand, some of the important steps during the transport via PA could be further elucidated and may be of great help to understand this complex mechanism of intoxication or to use it as a molecular tool. On the other hand, some of the results provide interesting possibilities to treat the severe consequences of Anthrax intoxication and therefore, buy some time to handle the infection.

7.1 General conclusions of this work

One of the main issues of this thesis was the determination of details of the transport processes through PA-pores. The translocation represents the crucial step of the intoxication pathway and additionally offers the possibility to use PA-channels as molecular syringes. His-tag was proven to play the role of a general transport motive by enhancing the affinity of several protein effectors to PA-channels. Additionally, this feature has been shown to be highly voltage-dependent. Thereby, a trigger for the transport out of the endosomes, where a positive potential coupled to a proton gradient exists, could be elucidated. Furthermore, PA-pores seem to offer a biochemical tool, which can be used as a molecular syringe to inject desired cargo proteins containing His-tag into certain cell-types. Evidence of this feature could be attained by experiments on HUVECs, which have been able to transport His_6-EDIN dependent on PA concentration.

The common motives shared by binding partners of PA found before and recently were summed up in this work. Included in this overview were results from such different binding partners as complex effector molecules and ions, sharing features like positive charges or aromatic residues. The similarities found could help to develop of drugs or/and provide further insight in translocation.

Information about homologies and differences between PA and C2II are of special interest, as they enlighten, that closely related AB_7-type toxins share huge homologies in amino acid sequence, but despite that fact differ in translocation and substrate specificity. Especially the possibility to transport C2I via PA-pores into HUVECs helped in investigation of factors needed for transport. C2II does not share the affinity increase of PA for positive charged substrates and

is dependent on HSPs. Therefore, different approaches seem to be necessary for medication of intoxication caused by these two members of the AB_7-family.

The pharmacology of PA and C2II was further clarified by binding studies with blocker-substrates. On top of offering a chance to form a plug in the lumen of the pore and therefore hindering the effectors to pass, some of these substrates should be able to accumulate in the endosome. This effect could be useful in reducing the concentration for application of those drugs, thereby circumventing unwanted side effects. Nevertheless, the promising data, which was attained for some of the blockers electrophysiogically, has to be proven harmless in cell-based and *in vivo* studies in the future.

In the end, this thesis provides first-time evidence of an antibody blocking the ion current from the *trans*-side of the PA-pore. This binding was analysed *in vitro* and led to interesting questions about the structural details of the channel. The finding, that histidine at position H310 does not represent the target for the antibody opens the field for speculations about the possible different composition of the β-barrel. Additionally, the second binding site on the *cis*-side's mushroom body of PA reveals flexibility in this part of the channel under low-salt concentrations and delivers further evidence for differences to the proposed model.

7.2 Outlook

Binary toxins of the AB_7-type represent not only some of the most potent toxins in the world, they also exhibit very interesting and important features in the context of molecular transport. The work presented here elucidates some parts of the intoxication processes, but on the way opening a lot of questions, which could only be answered in future research. Why is C2II not specific for positive charges? Can PA really be used as a molecular syringe? And, if the answer is "yes", for which substrates? Does a thread of combined toxicity from different binary toxins exist? Is the model of PA correct or do we have to change our minds about it? Is it possible to treat the intoxication with chemically produced blocker-substrates *in vivo*?

Even if the data provided and summed up here about binding and translocation concerning PA could give some interesting information, there is need for more investigation. Not only to understand some of the most potent bacterial toxins in more detail, but in order to cure their intoxication in the context of biological terrorism and their potential usage as a versatile molecular syringe for various purposes, further work has to be done in this vital field of studies.

Chapter 8

Appendix

8.1 References

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