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# Pore formation by the *Bordetella* adenylate cyclase toxin in lipid bilayer membranes: Role of voltage and pH

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**Abstract**

The bifunctional adenylate cyclase toxin (ACT or CyaA) of *Bordetella pertussis* invades target cells via transport through the cytoplasmic membrane. The membrane potential represents thereby an important factor for the uptake *in vivo*. Previous studies demonstrated that adenylate cyclase (AC) delivery into cells requires a negative membrane potential inside the cells. The results of lipid bilayer experiments with ACT presented here indicated that two different types of pore-like structures are formed by ACT dependent on the orientation of the electrical potential across the membranes. Pore formation at a positive potential at the cis side of the membranes, the side of the addition of the toxin, was fast and its conductance had a defined size, whereas at negative potential the pores were not defined, had a reduced pore-forming activity and a very short lifetime. Fluctuations inserted at positive potentials showed asymmetric current–voltage relationships for positive and negative voltages. Positive potentials at the cis side resulted in an increasing current, whereas at negative potentials the current decreased or remained at a constant level. Calcium ions enhanced the voltage dependence of the ACT pores when they were added to the cis side. The single-pore conductance was strongly affected by the variation of the pH value and increased in 1M KCl with increasing pH from about 4 pS at pH 5 to about 60 pS at pH 9. The ion selectivity remained unaffected by pH. Experiments with ACT mutants revealed, that the adenylate cyclase (AC) and repeat (RT) domains were not involved in voltage and pH sensing.


*Keywords:* Adenylate cyclase toxin; ACT; CyaA; Voltage; pH; Channel information; Calcium; *Bordetella pertussis*; Lipid bilayer

## 1. Introduction

Whooping cough is a highly contagious disease of the respiratory tract of humans caused by the gram-negative bacterium *Bordetella pertussis*. One of the main virulence factors of *B. pertussis* is the adenylate cyclase toxin (ACT or CyaA) [1]. The toxin targets primarily myeloid phagocytic cells, such as neutrophils and macrophages expressing the $\alpha_M\beta_2$ integrin receptor CD11b/CD18. By disabling their bactericidal functions, the ACT contributes to the capacity of the bacteria to survive the host immune system attacks during the early phase of colonization [1,2].

ACT is actively secreted from *B. pertussis* by a specific type I transport system, consisting of the products of the genes *cyaB* and *cyaD* and of the outer membrane protein CyaE, homologues of the proteins involved in *Escherichia coli* hemolysin export machinery [3]. Two genes, *cyaA* and *cyaC*, are required for the synthesis of the fully active toxin, where the proCyaA needs to be activated by the gene product of *cyaC*, an acyl-transferase to develop full toxin activity [4–7]. Through the enzyme action of CyaC, palmitoyl residues are covalently attached to the ε-amino groups of one or both of the two lysine residues at positions 860 and 983, which correspond to highly conserved residues among RTX-toxins. Acylation of $Lys^{860}$ seems to be dispensable for

*Abbreviations:* CyaA, ACT, adenylate cyclase toxin of *Bordetella pertussis*; HlyA, α-hemolysin of *Escherichia coli*; RTX, Repeats in Toxin; G, conductance, i.e. current divided by voltage

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the activity of ACT, while the acylation of $Lys^{983}$ plays an important role in target cell recognition and penetration by the toxin [7,8].

ACT consists of 1706 amino acids and it is a multifunctional protein belonging to the RTX-toxin (**R**epeats in **T**o**X**in) family. The 177-kDa cytotoxic form of ACT exhibits both invasive adenylate cyclase (AC) enzyme (cytotoxic) and hemolytic (pore-forming) activities [9,10]. The AC part is located within the first 400 amino-terminal residues and comprises a calmodulin-activated adenylate cyclase, which can bypass the receptor-mediated endocytosis pathway [11–15] and penetrates directly across the cytoplasmic membrane of the target cells in a calcium-dependent manner (see below). Inside cells, the AC is activated by calmodulin and catalyses uncontrolled conversion of ATP into cAMP. Formation of cAMP in the eukaryotic cells alters their phagocytic abilities, production of superoxide radicals, and it also initiates apoptosis of macrophages [4,9,16–18]. Besides that, residues 374 to 1706 of ACT act independently as a pore forming toxin and form cation-selective pore-like structures in cell membranes and black lipid bilayers, similar to α-hemolysin (HlyA) of *E. coli* but with a much smaller diameter as judged from osmotic protection experiments [19–22]. The pore-forming domain is composed of several hydrophobic segments with potentially amphipathic and hydrophobic α-helical structures (residues 500–700) [23]. Mutations within or close to this area have a major influence on membrane insertion and delivery of the AC domain into cells. Deletion of the residues 623–780 and 827–887 abolishes the invasive activity and strongly reduces the hemolytic activity of ACT [23].

The repeat domain of ACT is characterized by the presence of about 30 to 40 copies of characteristic glycine and aspartate-rich nonapeptide repeats (residues 1006–1613) of the prototype GGXG(N/D)DX(U)X (one letter code for amino acids; X represents any amino acid and U any large hydrophobic residue such as I, L, V, F, Y). Repeat domains are characteristic for all RTX toxins and play an important role in calcium-mediated target cell binding [24–29]. Intoxication of target cells by ACT occurs only in the presence of free calcium ions at millimolar concentrations [30,31]. Lipid bilayer measurements using the lipid mixture asolectin demonstrate that addition of calcium ions and toxin to the same side of the membrane caused a steep, highly specific increase of the pore-forming capacity of ACT (~50-fold) within a very narrow range (from 0.7 to 0.8 mM $Ca^{2+}$ [32]). The calcium effect clearly depends on the presence of the RTX-repeats of the toxin, suggesting that binding of calcium ions to the repeats modulates the propensity of ACT to form membrane pores [32].

Membrane potentials may represent another important factor for the biological activity of ACT *in vivo*. Previous patch clamp studies showed that AC delivery into certain cell types appears to require a negative membrane potential. It was demonstrated that the delivery of the enzymatic component is driven and controlled by the electric field across the plasma membrane [16]. A rapid intoxication of atrial cells occurred only when the cells exposed to ACT were held at negative potentials. Toxin action was totally abrogated when the membrane potential was positive [17]. However, ACT is also able to provoke colloid-osmotic cell lysis or delivery of its AC domain efficiently even into cells with low membrane potential, such as sheep erythrocytes [4,18]. It is also able to release marker substances from multilamellar liposomes devoid of membrane potential [12]. In this study we investigated in detail the effect of membrane potential on pore formation by ACT in black lipid bilayer membranes. We demonstrate that the sign of the voltage with respect to the addition of ACT determines the type of pores formed and their voltage dependence. Moreover, calcium ions in millimolar concentration were found to enhance the voltage dependence of ACT-pores.

## 2. Materials and methods

### 2.1. Bacterial strains, growth conditions and plasmids

The *E. coli* K12 strain XL1-Blue (Stratagene) was used throughout this work for DNA manipulation and for expression of ACT and its mutants. Bacteria were grown at 37 °C in LB medium supplemented with 150 μg/mL ampicillin. pCACT3 is a construct for coexpression of *cyaC* and *cyaA* [33], and it allows production of recombinant CyaC-activated ACT in *E. coli* under control of the IPTG-inducible *lac*Z promoter.

### 2.2. Production and purification of the ACT-derived proteins

Full-length recombinant ACT protein and the ACT mutants ΔAC-ACT (lacks the residues 1 to 373 comprising the AC-domain of ACT) and ACT1008 (devoid of residues 1009 to 1706 containing the RTX repeat domain) were produced with CyaC co-expression [33] from plasmids pCACT3, pCACTΔ1–373 and pCACTΔC699, respectively using the *E. coli* strain XL1-Blue (Stratagene), transformed by the respective plasmid(s). The proteins were extracted with 8 M urea in 50 mM Tris–HCl, pH 8.0, and 0.2 mM $CaCl_2$ (buffer A) from cell debris after sonication. ACT and ΔAC-ACT were purified by a two-step chromatographic procedure on DEAE- and Phenyl-Sepharose columns [8], while ACT 1008 was purified by a single step affinity chromatography on calmodulin agarose, as described previously [33]. Purified ACT-derived proteins were eluted in 8 M urea, 50 mM Tris–HCl, pH 8.0, and 2 mM EDTA (buffer B). They were essentially free of contaminating proteins as shown previously [34].

### 2.3. Lipid bilayer experiments

Black lipid bilayer membranes were formed as described previously [35]. The instrumentation consisted of a Teflon chamber with two aqueous compartments connected by a small circular hole with a surface area of about 0.4 $mm^2$. Membranes were formed across the hole by painting on a 1% solution of asolectin (lecithin type IIIs from soy beans from Sigma Chemical Co., St. Louis, MO) in n-decane. The aqueous salt solutions (Merck, Darmstadt, FRG) were buffered with 10 mM HEPES–KOH and had a pH around 7 if not stated otherwise. The temperature was kept at 20 °C throughout. The potentials applied to the membranes throughout the study refer always to those applied to the cis side, the side of addition of ACT. Similarly, positive currents were caused by positive potentials at the cis side and negative ones by negative potentials at the same side. The membrane current was measured with a pair of silver/silver chloride electrodes with salt bridges switched in series with a voltage source and an electrometer (Keithley 617). In the case of the current recordings the electrometer was replaced by a home-made current amplifier with a band-width of either 100 or 300 Hz dependent on the membrane conductance. The amplified signal was monitored and recorded with a personal computer or a strip chart recorder.

For the selectivity measurements, the membranes were formed in a 100-mM KCl solution. ACT was added to both sides of the membrane, and the increase of the membrane conductance due to insertion of pores was observed with an electrometer. After incorporation of 100–1000 pores into a membrane the instrumentation was switched to the measurement of the zero-current potential, and a KCl gradient was established by adding 3 M KCl solution to one side of

the membrane. Analysis of the zero current membrane potential was performed using the Goldman–Hodgkin–Katz equation [36]. For activity measurements ACT wild type or mutants were added at given concentrations to the membrane and the membrane conductance was taken 30 min after the addition, when further conductance increase in time was small [34]. The applied membrane potential was 50 mV.

## 3. Results

### *3.1. Positive membrane potentials are necessary for proper pore formation by ACT*

The influence of polarity of the membrane potential on pores formed by ACT could be analyzed upon addition of small amounts of ACT to one side of a black asolectin/n-decane membrane. Fig. 1A (upper trace) depicts the result of a typical experiment, in which the applied potential was positive on the cis side of the membrane, the side at which toxin was added, with the negative voltage being applied at the trans side of the membrane, thus mimicking the situation of a cell membrane exposed to the toxin *in vivo*. Upon ACT addition (160 ng/ml), the membrane current started to increase in discrete steps until resolution of single pores became impossible due to incorporation of a high number of pores that exhibited a limited lifetime (about 2 s) and thus opened and closed frequently [34]. It was, nevertheless, possible to analyze individual conductance of pores formed at lower toxin concentrations or at the starts of the recordings [34] (data not shown).

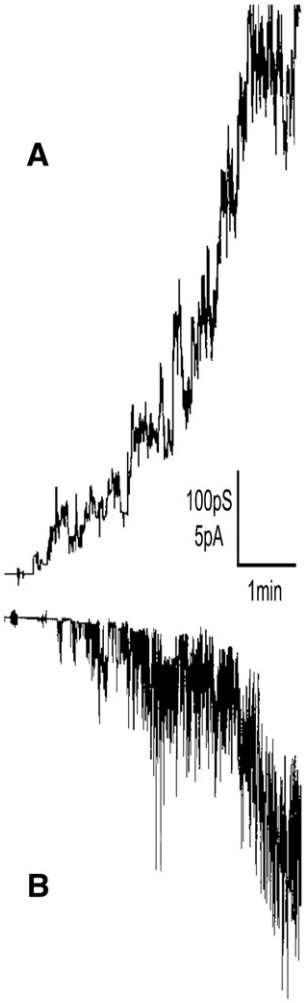


Fig. 1. Current recordings of asolectin membranes in the presence of adenylate cyclase toxin. The aqueous phase contained 1 M KCl and 10 mM HEPES-KOH pH 7. The applied membrane potential was 50 mV at the cis side (A) or at the trans side (B); the temperature was 20 °C. The aqueous phase contained either 160 ng/ml ACT at the cis side (A) or 320 ng/ml at the cis side (B).

In contrast, as shown in Fig. 1B (lower trace), upon addition of the toxin to a membrane with a negative potential at the cis side an increase of membrane current was also observed, most of the conductance fluctuations were, however, poorly defined and exhibited a very short lifetime smaller than 10 ms as judged from the time resolution of the current measuring device, which was 100 to 300 Hz. The interaction of ACT with membranes kept at negative potential resulted rather often in a burst-like induction of membrane conductance, as also documented in Fig. 1B. A defined pore size could not be observed under these conditions, which indicated that the pores formed by ACT were dependent on the sign of the voltage at the cis side, although other parameters such as temperature, pH or salt solution were the same as in the measurements at positive voltage. In particular, at negative potentials the membrane conductance increased gradually, but slower than for membranes under positive potentials and in a more flickering way. The same behavior was also observed in other salt solutions such as LiCl or KAc. Thus, the toxin exhibited a more “detergent-like” mode of action when the voltage at the cis side had a negative sign.

At positive membrane potentials, however, the conductance increments observed due to single pore incorporation and opening were found to be fairly homogeneous. The mean single-pore conductance observed under the conditions of the experiment shown in Fig. 1A (performed at pH 7 in 1 M KCl with +50 mV applied membrane potential) was found to be about (45±7) pS (mean±SD of 235 single events). Interestingly, this conductance value was about two times higher than that found previously by measurements performed at pH 6 [34], suggesting a pH effect on pore properties (see below). Single-pore experiments were also performed with salts other than KCl to check if different salts could influence the pore characteristics. However, no difference other than a changed single-pore conductance could be observed in these experiments.

To investigate the effect of voltage polarity on the properties of the ACT-pores in more detail, experiments were performed at different concentrations of ACT, always added to the cis side of black membranes being bathed in 1 M KCl solutions, as documented in Fig. 1. The membrane potential was kept either positive (+50 mV) or negative (−50 mV) on the cis side throughout the period of 30 min, over which time the conductance increased by several orders of magnitude and reached a plateau [34,37]. A substantially higher conductance was reached upon 30 min of toxin action on membranes with positive potential at the cis side, especially at low toxin concentration (Fig. 2). Collectively, the above outlined results demonstrate the importance of the orientation of the membrane potential for optimal ACT pore formation, with a clear requirement for a positive potential at the cis side of the target

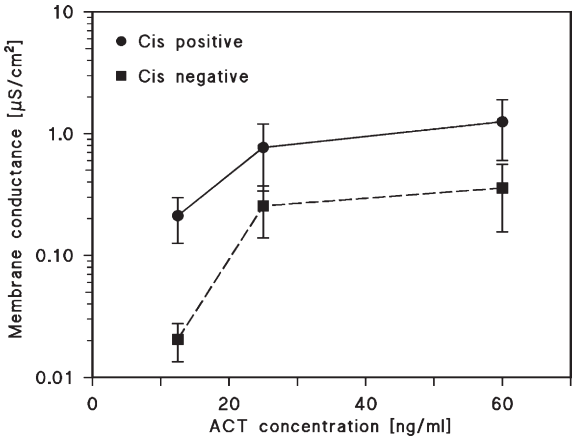


Fig. 2. Dependence of membrane conductance on ACT concentration observed at positive (+50 mV) or negative potential (−50 mV) at the cis side. The membrane conductance was measured 30 min after the addition of the protein. The aqueous phase contained 1 M KCl, 10 mM HEPES-KOH pH 7 and ACT at the indicated concentrations. The temperature was 20 °C. The conductance–concentration relationship was plotted on a double-logarithmic scale. The steep conductance–concentration relationship at low toxin concentration indicates that the pores are formed by ACT oligomers [37].

membrane. Interestingly, the results shown in Fig. 2 further suggest that the increase of toxin-mediated membrane conductance was not a linear function of ACT concentration in the aqueous phase. This indicates formation of conductive ACT oligomers from non-conductive monomers as discussed previously [34,37]. Moreover, if formation of higher order oligomers of ACT were a prerequisite for formation of ACT pores, the considerable variation of data points apparent in Fig. 2 would be explained, since a small variation in protein concentration translates into a large effect on the resulting membrane conductance, because of a high cooperativity of the membrane conductance in function of toxin concentration.

### 3.2. ACT pores show an asymmetric voltage-dependence

The experiments described above suggested an asymmetric effect of voltage on the ACT pores. Therefore, to characterize this phenomenon in more detail, multi-pore experiments with progressively increasing potential of an alternating polarity were performed. The toxin was added in concentrations ranging from 20 to 320 ng/ml to the cis side of the membranes and pore formation was initiated by applying a positive potential of +50 mV at the cis side. Upon about 15 to 20 min, once the conductance had increased considerably and reached a nearly constant level increasing voltages of both polarities were now applied to the ACT-membrane pores, and the membrane current was measured as a function of time. As shown for typical experiments in Fig. 3, potentials of alternating polarity ranging from ±10 mV to ±100 mV could be applied before the membrane was destroyed. Almost no voltage-dependence was observed when ±10 mV was applied to the asolectin membrane (Fig. 3A). However, starting with potentials higher than +20 mV the current increased after a step increase of voltage. The increase was in some experiments linear with time and in others it looked like an exponential increase. At high negative potentials, it decreased in most cases by about 30 to 50% or it remained constant at lower potentials. Experiments at different voltages suggested that the increase of current due to pore-forming activity during application of positive voltages was fully reversible upon switching to negative voltage. Fig. 3B shows an

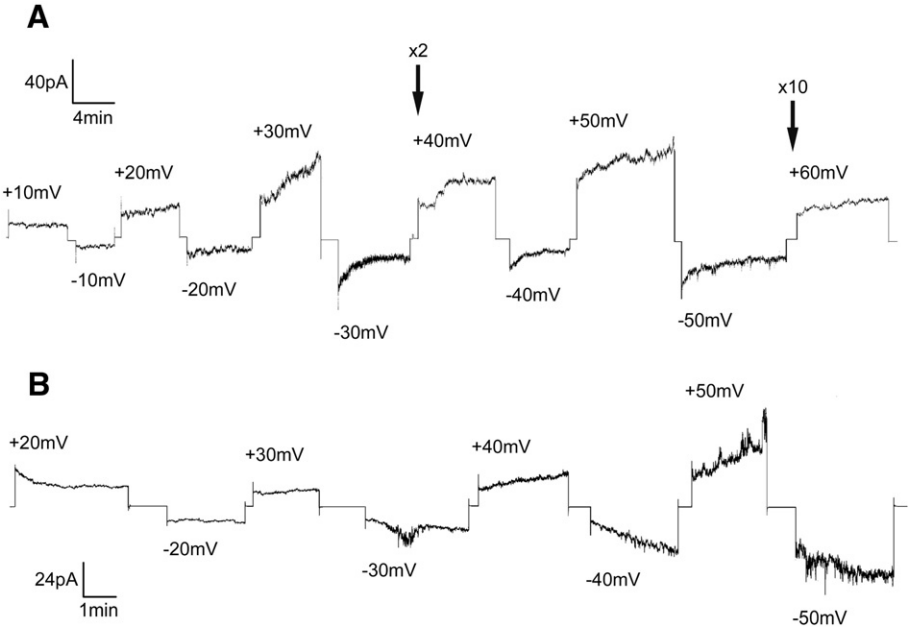


Fig. 3. (A) Current response of wildtype ACT upon application of positive and negative potentials to the cis side of an asolectin/n-decane membrane bathed in 150 mM KCl, 10 mM HEPES-KOH pH 7. The cis side contained 160 ng/ml ACT and pore formation occurred at positive potential at the cis side (+50 mV); $T$=20 °C. Note that the resolution of the current amplifier was decreased two times (indicated by arrows). (B) Current response of wildtype ACT upon application of positive and negative potentials to the cis side of an asolectin/n-decane membrane bathed in 150 mM KCl, 10 mM HEPES-KOH pH 7. The cis side contained 400 ng/ml ACT and pore formation occurred at negative potential at the cis side (−50 mV); $T$=20 °C.

experiment, where pore formation occurred at negative potential at the cis side. The increase of conductance following a voltage step was by far smaller as in the case of initiation of ACT-mediated conductance by positive potentials. In addition, the voltage dependence was approximately symmetrical with respect to the applied membrane potential and increased approximately linearly with time.

The polarity of the membrane potential present during insertion of ACT pores into the membrane had a major impact on voltage dependence. Fig. 4A summarizes the effects of positive and negative potentials on conductance of pores formed by intact and truncated ACT variants in experiments where the formation of pore-like structures in the membrane was initiated by a positive potential at the cis side of the membrane (the *in vivo* situation). Indeed, pores formed by ACT wild type and by its two different truncated variants (see below) exhibited asymmetric current–voltage characteristics, similar to an outward rectifier. A high membrane conductance mediated by ACT was observed when the voltage was positive on the cis side, presumably resulting either by voltage effects on the CyaA reconstitution in the lipid bilayer and/or an effect of membrane potential on the oligomerization of CyaA in the membrane, which both result in formation of new pores. In contrast, application of a negative potential at the cis side during toxin insertion into membrane led to a very small negative current, which did not increase with increasing negative membrane potential, thereby suggesting that the conductance was more or less independent of the applied negative voltage. Furthermore, the ionic strength of the aqueous phase (150 mM or 1 M KCl) had no effect on these current–voltage curves (data not shown).

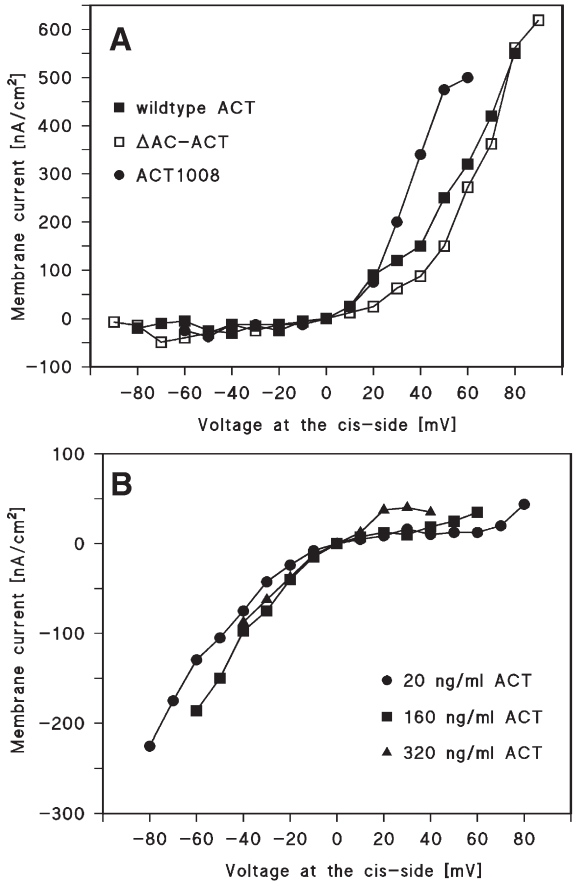


Fig. 4. (A) Current–voltage relationships of ACT-pores initiated by positive potential (+50 mV) applied to the cis side. The membranes were formed from asolectin/n-decane. The aqueous phase contained 150 mM KCl, 10 mM HEPES-KOH pH 7; the temperature was 20 °C. The cis side contained in addition 320 ng/ml wildtype ACT (full squares), 320 ng/ml ΔAC-ACT (open squares) or 320 ng/ml ACT 1008 (full circles). (B) Current–voltage relationships of "false" ACT-pores initiated by negative potential (−50 mV) applied to the cis side. The membranes were formed from asolectin/n-decane. The aqueous phase contained 150 mM KCl, 10 mM HEPES-KOH pH 7; the temperature was 20 °C. The cis side contained in addition 20 ng/ml wildtype ACT (full circles), 160 ng/ml wildtype ACT (full squares) or 320 ng/ml wildtype ACT (full triangles).

To investigate whether the different domains of the ACT molecule interfere with the voltage dependence of the formed pores, the effect of voltage on pore-like structures formed by two ACT mutants (ΔAC-ACT and ACT1008) was also assessed. This was done to check if the AC domain or the RTX repeat region of ACT had a negative control effect on voltage sensing and pore formation. Therefore, the ΔAC-ACT protein lacking the AC domain (residues 1–373) and the ACT1008 mutant, lacking the entire RTX domain (residues 1009–1706) and not responding to calcium concentration [32], respectively, were used in these experiments. Membrane conductance was induced by intact or mutant toxins at a positive potential (+50 mV) at the cis side of a membrane to which the protein was added. When stationary conditions were obtained, potentials of positive and negative polarity were applied to the membranes, similar as shown in Fig. 3. The results (Fig. 4A) showed that the loss of the RTX domain had virtually no influence on the voltage response of ACT in the absence of calcium. Similarly, as observed for wild-type ACT, the current was very low for applied negative potentials and increased steeply for positive potentials (Fig. 4A). High currents were also observed in experiments performed with the ΔAC-ACT mutant, which was lacking most of the adenylate cyclase domain and positive potentials (Fig. 4A). Again, there was no difference to the behavior of intact ACT. These results suggested that the AC and repeat domains are not involved in voltage sensing by ACT. The hydrophobic domain between the amino acid residues 374 and 1005 may be responsible for voltage sensing, caused probably by the negatively charged groups in or near the cation selective pore formed by ACT [34,37].

It is noteworthy that the current–voltage curves showed completely different characteristics when pore formation occurred at negative (−50 mV, see Fig. 4B) or positive (+50 mV) potentials at the cis side. Similarly to the experiments described above, no voltage dependence was observed at small transmembrane potentials. When higher potential were applied the current increased in about 40% of the current–voltage curves for negative and positive potentials after the initial step increase. However, in contrast to the experiments described above for initiation of ACT-pores with positive potential at the cis side, the conductance increased only slightly for negative potentials (see Fig. 4B), whereas it remained more or less constant for positive voltages or increased less than for negative voltages resulting in an almost symmetrical current–voltage relationship. The current–voltage relationships were independent from the

concentration of wild type ACT in the aqueous phase as Fig. 4B clearly indicates. In addition, the conductance saturated at very low ACT concentration as Figs. 3 and 4B showed. The results of the measurements where potentials of opposite polarity were applied to the asolectin membranes indicated, again, that different pores may be formed by ACT dependent on the orientation of the electric field with respect to cis side of the membrane, where ACT was added. The ionic strength of the aqueous phase (150 mM or 1 M KCl) had no effect on the current–voltage curves.

### 3.3. Effect of calcium on voltage-dependence of the ACT pores

In a previous paper we showed that calcium has a considerable effect on ACT-mediated membrane conductance. Starting with about 0.7 to 0.8 mM calcium, slightly dependent on ionic strength in the aqueous phase, the membrane conductance increased by orders of magnitude [32]. Smaller amounts of calcium in the micromolar range that could be present as impurities in 150 mM KCl or in asolectin failed to create any effect on ACT in lipid bilayer membranes. Therefore, we also studied here the effect of calcium on the current–voltage characteristics of the ACT pores. In contrast to the experiments described above 1–2 mM $CaCl_2$ was added to the cis side of the membrane. Fig. 5 shows current recordings of ACT pores with 1 mM calcium (Fig. 5A, upper trace) and without calcium (Fig. 5B, lower trace) for voltages between ±10 and ±30 mV. The results demonstrated that the ACT pores showed the same behavior as described above at negative potential in the presence of calcium. However, at a positive membrane potential the conductance increase was much stronger and in particular much faster than compared to measurements in the absence of calcium (see Fig. 5A). These results demonstrated that calcium bound to ACT facilitates the voltage dependent formation of ACT pores as is also shown in Fig. 6. Similarly, calcium is also able to promote voltage-dependent formation of the ΔAC-ACT mutant.

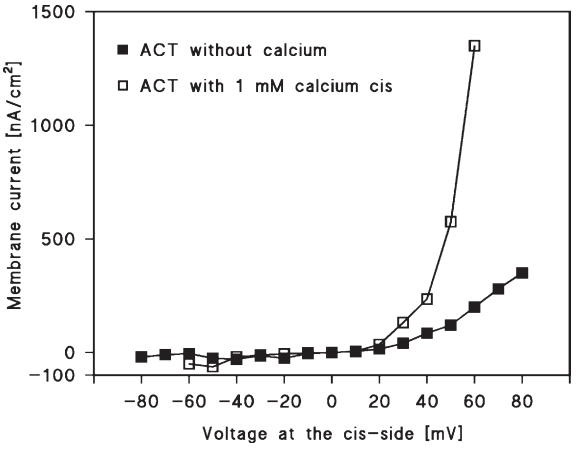


Fig. 6. Current–voltage relationships of ACT-pores initiated by positive potential (+50 mV) applied to the cis side in the presence or absence of calcium ions. The membranes were formed from asolectin/n-decane. The aqueous phase contained 150 mM KCl, 10 mM HEPES-KOH pH 7; the temperature was 20 °C. In addition, the cis side contained 160 ng/ml wildtype ACT (full squares), or 160 ng/ml wildtype ACT and 1 mM $Ca^{2+}$ (open squares).

However, no calcium effect was observed when the ACT 1008 mutant was used in similar experiments (data not shown) [32].

### 3.4. ACT pore formation occurs also in the absence of membrane potential

The results described above suggested that the membrane potential has an influence on the orientation and the typical behavior of the ACT pores. On the other hand, ACT was also shown to interact with and penetrate into cells that lack the specific CD11b/CD18 receptor for ACT and bear a very low membrane potential such as red blood cells [4,18]. To study this situation, experiments were performed, experiments in which ACT was allowed to interact for some time with membranes without membrane potential. Asolectin membranes were formed

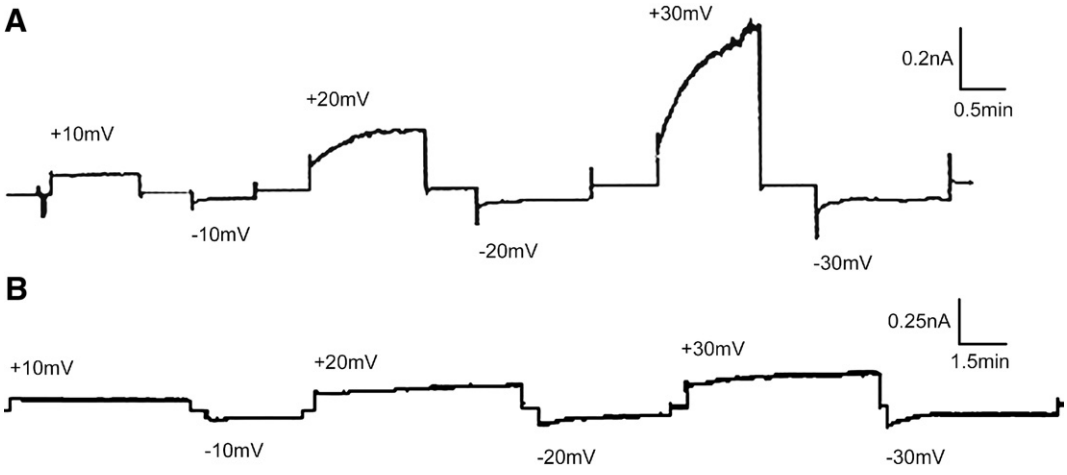


Fig. 5. (A) Current response of wildtype ACT upon application of positive and negative potentials to the cis side of an asolectin/n-decane membrane bathed in 150 mM KCl, 10 mM HEPES-KOH pH 7. The cis side contained 160 ng/ml ACT and 1 mM $Ca^{2+}$. Pore formation occurred at positive potential at the cis side (+50 mV); $T$=20 °C. (B) Current response of wildtype ACT upon application of positive and negative potentials to the cis side of an asolectin/n-decane membrane bathed in 150 mM KCl, 10 mM HEPES-KOH pH 7. The cis side contained only 160 ng/ml ACT and no calcium. Pore formation occurred at positive potential at the cis side (+50 mV); $T$=20 °C. Note that the control experiment (B) showed a much lower increase of the current as compared to the presence of 1 mM $Ca^{2+}$ (A).

in 1 M KCl, pH 7, and when the membranes were in the black state, the voltage was switched off and the toxin was added to the cis side of the membrane. After 15 min a voltage of +50 mV was applied to the cis side of the membrane (see Fig. 7A). At the application of the voltage the membrane had already a considerable conductance, which exponentially increased further in a similar way as in Fig. 3 for the initiation of membrane conductance by a positive potential. In a similar experiment voltage pulses of ±50 mV were applied to the cis side – the side of toxin addition – of another membrane, where the membrane conductance was also initiated without voltage (see Fig. 7B). The ACT pores showed an asymmetric voltage response similar to the results of Figs. 3 and 5. For positive voltage at the cis side the current started to increase in an exponential fashion. For negative potentials at the cis side, the current amplitude remained more or less constant at a much lower level. This result clearly indicated that a membrane potential is not necessary for pore formation by ACT. Moreover, the pores inserted into the membranes at zero voltage had an orientation in the membranes and voltage–current characteristics similar to those of pores initiated by application of positive potentials to the cis side.

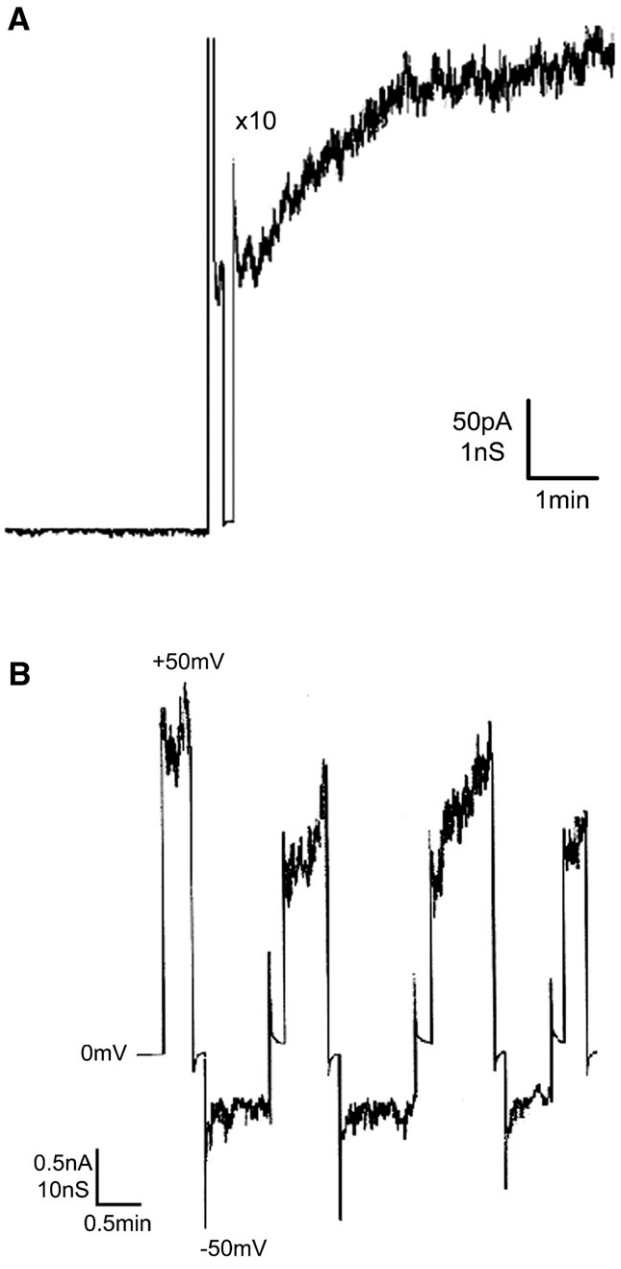


Fig. 7. (A) Demonstration of the conductance increase caused through pore formation in the absence of a membrane potential. 160 ng/ml ACT were added to the 1 M KCl, 10 mM HEPES-KOH pH 7 solution on the cis side of a black asolectin membrane without voltage. The experiment started 15 min after addition of ACT, when 50 mV were applied to the cis side of the membrane. (B) Current responses of wildtype ACT pores formed the absence of a membrane potential in upon application of positive and negative potentials to the cis side of a membrane made of asolectin/n-decane. The cis side contained 160 ng/ml ACT. The aqueous phase contained 1 M KCl, 10 mM HEPES-KOH pH 7. The applied membrane potential was ±50 mV at the cis side; the temperature was 20 °C.

### *3.5. Effect of pH on pore properties of the adenylate cyclase toxin*

Comparison of the single-pore conductance derived here for pH 7 and former results at pH 6 [34] suggested that the ACT pores show a strong dependence on pH of the aqueous salt solution. This was, therefore, examined here in more depth. As documented in Table 1, below pH 5 the conductance of the ACT-pores was so small that it could not be resolved. The smallest single pore conductance was obtained at pH 5 and it increased until pH 8 by a factor of more than 10 (Table 1). Also the pore-forming activity was slightly reduced for pH values below 6. The amount of toxin, which caused a strong increase of membrane conductance at pH 6 or 7, formed only a small number of pores at pH 5 and 5.6.

Additional experiments with the two ACT-mutants ACT1008 and ΔAC-ACT suggested that neither the repeat domain nor the adenylate cyclase part of the toxin is responsible for the pH effect on the single-pore conductance. The two mutants and wild-type ACT had all a single-pore conductance of around 15 to 18 pS at pH 5.6. It is noteworthy that the conductance of other RTX-toxins such as EHEC-hemolysin of from enterohemorrhagic (EHEC-HlyA) *E. coli* is also pH dependent [38] (see Table 1), but to a much smaller extent, which suggests that ACT may undergo major structural changes at lower pH.

To obtain more information about the influence of pH on the adenylate cyclase toxin, selectivity measurements were performed. The asolectin membranes were formed in 0.1 M KCl and the toxin was added to black membranes. After the incorporation of a sufficient number of pores, a fivefold salt gradient was established through the addition of small amounts of concentrated KCl solution to one side of the membrane. In all experiments, the more diluted side became positive, as has previously been described for wild type ACT. This result indicated that the pore-like structures are cation selective [34]. The analysis of the zero-current membrane potentials at pH 5.6, 6 and 8 did not show any significant difference (see Table 1). In all three cases the ratios of permeability $P_{cation}/P_{anion}$ had values around 10. The mutations did not change the ionic selectivity of ACT at pH 5.6. Only the minimum calcium concentration required to cause the steep conductance increase when calcium

Table 1
Effect of pH on pore conductance and permeability ratio $P_{cation}/P_{anion}$ of the adenylate cyclase toxin (ACT) pores of *B. pertussis* and the single-pore conductance of hemolysin (EHEC-HlyA) of enterohemorrhagic *E. coli*

| pH | $P_{cation}/P_{anion}$ | Single-pore conductance G [pS] | |
|---|---|---|---|
| | | ACT (*B. pertussis*) | EHEC-HlyA (enterohemorrhagic *E. coli*) |
| | | (1 M KCl) | (150 mM KCl) |
| 5 | n.m. | 4.8±1.2 (56) | 380 |
| 5.6 | 10±0.8 (4) | 18±3 (129) | n.m. |
| 6 | 10±1.2 (5) | 29±5 (78) | 450 |
| 7 | n.m. | 45±7 (235) | 500 |
| 8 | 11±0.9 (6) | 48±6 (85) | 570 |
| 9 | n.m. | 53±5 (114) | n.m. |

Membranes were formed of asolectin/n-decane. The aqueous solution contained 1 M KCl in the case of the single-channel measurements. The membrane potential was 50 mV; the temperature was 20 °C. The average single pore conductance was calculated from the number of single events given in brackets after mean value±SD. The average single-pore conductance of EHEC-HlyA of *E. coli* in 150 mM KCl is given for comparison [38]. The permeability ratio $P_{cation}/P_{anion}$ was calculated using the Goldman–Hodgkin–Katz equation [36] as the average of a number (given in brackets) of individual experiments for a fivefold KCl-gradient (100 mM versus 500 mM KCl). n.m. means not measured.

is added to the cis side of the membrane increased somewhat from 0.7 to 0.8 mM at pH 7 to 1.0 to 1.2 mM at pH 5.6, suggesting some minor effect on the repeat domain. This is presumably caused by protonation of some of the negatively charged amino acids of the many acidic residues within about 30 to 40 nonapeptide repeats.

## 4. Discussion

### *4.1. Orientation of the electrical potential influences pore formation by ACT*

The results presented here indicate that two different types of pores are formed by ACT, dependent on the orientation of the electrical potential across the membranes. When the voltage is positive by 50 mV at the cis side, the side of ACT addition, regular pores with an average single-pore conductance of about 45 pS in 1 M KCl (pH 7) are formed. These pore-like structures have a lifetime of about 2 s and are obviously formed by ACT oligomers. The characteristics of pores change completely when the cis side is set to negative potential. Under these conditions irregular current fluctuations with a burst-like appearance and a lifetime shorter than 10 ms are formed. These observations probably have something to do with the pore-forming domain of ACT that is localized within several hydrophobic segments with potentially amphipathic and hydrophobic α-helical structures (residues 500–700) [37]. The other domains of ACT, the repeats and the AC-domain, have a very small if not negligible influence on the pore characteristics observed under the different orientation of the electrical field. The formation of two types of different pores dependent on the direction of the electric field is an intrinsic property of the hydrophobic domain of ACT. The most likely explanation for this is an asymmetric distribution of charges or an intrinsic dipole within the hydrophobic domain of ACT, which leads to its asymmetric orientation within the membrane dependent on the direction of the electric field.

The absence of an electric field across membranes results in an orientation of the ACT-pores which is the same as when positive potentials are applied to the cis side. This corresponds to the *in vivo* situation of most target cells. It has been suggested that polarization of the target cells is needed for invasion of the AC-domain [16]. However, also in the *in vivo* situation it is also possible for ACT to invade cells, which have a low membrane potential and do not contain the αMβ2 integrin receptor for ACT such as erythrocytes [4,18]. This is not a contradiction to the situation in experiments with lipid bilayer membranes, as these interact with most membrane active toxins without receptor probably because of their smooth surfaces, which allow easy access of the toxins [32,34,37,39]. On the other hand, it is clear that a positive potential at the cis side facilitates the pore formation. If this is a prerequisite for transport of the AC-domain across membranes, a polarized cell membrane also promotes the invasion of this domain in the cell. On the other hand, it is not clear if pore formation facilitate import of the AC-domain into the target cells, because there exists some evidence that pore formation and translocation of the AC domain are unrelated events [19–21,37].

### *4.2. The two forms of the ACT-pores show different voltage dependences*

The two pores, formed at different polarity, show highly asymmetric voltage dependences similar to that of a diode (see Fig. 4A). For positive potential at the cis side (corresponding to negative potential at the trans side—the *in vivo* situation), the current shows some increase following the initial step. For negative potential (corresponding to positive potential at the trans side) the current decreases slightly or remains constant after the onset of the voltage. These results suggest that at positive potential new conductive oligomers were created probably because additional ACT molecules become inserted into the membrane through the action of the electric field acting on the hydrophobic domain of ACT. This means that the time constant of the exponential conductance increase reflects either the insertion of ACT-monomers in the membrane or the formation of new oligomers if the latter process is rate-limiting. Because of the decrease of the time constant with increasing voltage, it is more likely that the relaxation reflects the insertion of the ACT molecules into the membranes followed by fast formation of new pores. It may represent a similar process as the voltage-dependent formation of pores of the alamethicin or melittin type, where the membrane potentials act on dipoles within the peptides [40].

The voltage-induced formation of ACT-pores was highly reversible. Removal of the voltage resulted in a rapid switching off of the additional pores as Fig. 3 clearly indicates. This suggests largely different reaction rates for the on- and off-process of voltage-induced pore formation by positive potentials at the cis side. The observation of an asymmetric current–voltage curve agrees to some extent with the previous results of Szabo et al. [39]. In this study the conductance was lost when the

potential at the cis side was switched to negative polarity indicating complete closure of the ACT-pores. From the results presented here it is clear that at negative potential not all pores are closed. The voltage-independent part of the pores remains open and conducts both at negative and positive potentials. These pores are presumably also responsible for the current steps that are observed when both negative and positive voltages are switched on (see Fig. 3).

### 4.3. Calcium enhances the voltage dependence of the ACT-pores

Comparison of the two current traces in Fig. 5 recorded with 1 mM (upper trace) and without calcium (lower trace) indicates a substantial effect of calcium on the voltage dependence of ACT. This result suggested a facilitated membrane insertion of ACT when calcium was bound to the repeat domain. Different studies have shown that ACT is a calcium binding protein that undergoes conformational changes upon binding of calcium [24,31,41]. Furthermore, the entry/translocation of ACT into target cells is strictly dependent upon the presence of calcium ions in the millimolar range, and the RTX domain is supposed to be directly involved in this process as it harbors many low affinity $Ca^{2+}$-binding sites [31]. The mechanism of calcium-mediated translocation of ACT into cells remains open but it may have something to do with the formation of the special calcium binding structure of the RTX-domain, which forms a parallel β-roll motif upon calcium binding [24,41]. This structure is not only involved in transport of the AC-domain into target cells, but also facilitates the formation of ACT pores in artificial lipid bilayers and erythrocytes [32,42]. It is possible that calcium binding lowers the net charge of the RTX-domain thus facilitating transport of the AC-domain across the hydrophobic interior of membranes. Positive transmembrane potentials at the cis side enhance this process and lead to fast pore formation in lipid bilayer membranes. Translocation of the AC-domain through target cell membranes may something have to do with pore formation as is the case for transport of A–B type of toxins [12,43,44]. This could mean that calcium-dependent pore formation plays an important role in ACT function as an intracellular toxin although there exists also evidence that both processes represent unrelated events [19–21,37].

### 4.4. The aqueous pH influences the properties of the ACT-pore

The single-pore experiments at different pH demonstrate that the conductance of the ACT-pore is highly pH-dependent. One possible reason for this observation could be the protonation–deprotonation reaction of one or several charged amino acids in a strategic position within the pore. Charges should also influence ion selectivity in such a case. However, a pH-sensitive selectivity was not observed in the experiments. An explanation for this could be that the real permeability ratio $P_{cation}/P_{anion}$ is much higher than measured because of charge effects in or near the pore, which change the cation concentration on both sides of the pore. Protonation of one or two of the many negatively charged groups within the hydrophobic domain would not influence the selectivity to any appreciable amount but also not the single-pore conductance. Examples for this could be the glutamates 509 and 516, which are crucial for membrane translocation of adenylate cyclase toxin [37]. Thus, it seems to be more reasonable to assume that a structural change within the ACT-pore is responsible for the change of single-pore conductance. It is only possible to speculate about such a change because the structure of the ACT-pore is not known. In any case it is clear that neither the repeat nor the AC-domain are involved in such a change because experiments with the mutants ACT 1008 and ΔAC-ACT show the same results as with ACT wild type. It is noteworthy that the related EHEC-hemolysin (EHEC-HlyA) of from enterohemorrhagic (EHEC) *E. coli* exhibited also some pH-dependence [38]. However, in contrast to ACT the conductance increased from pH 5 to 8 only by a factor of 2 and not of 10. This means that both types of pores are probably not very similar, which make conclusions about their structure difficult.

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