# Channel Formation in Model Membranes by the Adenylate Cyclase Toxin of *Bordetella pertussis*: Effect of Calcium†

Oliver Knapp,‡ Elke Maier,‡ Georg Polleichtner,‡ Jirí Mašín,§ Peter Šebo,§ and Roland Benz*,‡

*Lehrstuhl für Biotechnologie, Theodor-Boveri-Institut (Biozentrum) der Universität Würzburg, Am Hubland, D-97074 Würzburg, Federal Republic of Germany, and Laboratory of Molecular Biology of Bacterial Pathogens, Cellular and Molecular Microbiology Division, Institute of Microbiology of the Academy of Sciences of the Czech Republic, Vídenská 1083, CZ-142 20 Prague 4, Czech Republic*

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ABSTRACT: Calmodulin-dependent adenylate cyclase toxin (ACT or CyaA) of *Bordetella pertussis* requires calcium ions for target cell binding, formation of hemolytic channels, and delivery of its enzyme component into cells. We examined the effect of calcium and calmodulin on toxin interaction with planar lipid bilayers. While calmodulin binding did not affect the properties of CyaA channels, addition of calcium ions and toxin to the same side of the membrane caused a steep increase of the channel-forming capacity of CyaA. The calcium effect was highly specific, since among other divalent cations only strontium caused some CyaA activity enhancement. The minimal stimulatory concentration of calcium ions ranged from 0.6 to 0.8 mM, depending on the ionic strength of the aqueous phase. Half-maximal channel activity of CyaA was observed at 2−4 mM, and saturation was reached at 10 mM calcium concentration, respectively. The unit size of single CyaA channels, assessed as single-channel conductance, was not affected by calcium ions, while the frequency of CyaA channel formation strongly depended on calcium concentration. The calcium effect was abrogated upon deletion of the RTX repeats of the toxin, suggesting that binding of calcium ions to the repeats modulates the propensity of CyaA to form membrane channels.

Secreted adenylate cyclase toxin (CyaA or ACT)[1] is a key virulence factor of the whooping cough agent *Bordetella pertussis* (*1*). Its purified form, endowed with cytotoxic and hemolytic activities, is a protein of 177 kDa (*2*−*7*). The toxin targets primarily myeloid phagocytic cells expressing the $\alpha_M\beta_2$ integrin receptor CD11b/CD18 (*8*). However, ACT can penetrate also a variety of cells that do not have such a receptor (*2*, *9*, *10*). The toxin molecule exhibits several unusual features dependent on different domains in its primary sequence. The first 400 amino acids from the N-terminal end comprise a calmodulin-activated and invasive adenylate cyclase (AC) enzyme that can bypass the receptor-mediated endocytosis pathway (*2*, *4*, *7*, *8*) and penetrate directly across the cytoplasmic membrane of a variety of epithelial and immune effector cells. Upon activation by the intracellular calmodulin (CaM), the enzyme catalyzes uncontrolled formation of cAMP, and as a result, the microbicidal capacities of the intoxicated cells are debilitated (*10*−*13*).

The polypeptide comprising the last 1332 residues of CyaA can function independently as a hemolysin (*14*). Thus, the hemolysin part of CyaA must contain enough information for cell targeting and pore formation. This part itself is composed of two distinct domains. It harbors several hydrophobic segments with potentially amphiphilic and hydrophobic α-helical structures (residues 500−700) that are similar to the pore-forming region of the α-hemolysin (HlyA) of *Escherichia coli* (*15*, *16*). Internal deletions around this region (aa 623−780 and 827−887) abolish the invasive activity and severely reduce the hemolytic activity of CyaA (*17*), indicating that this portion is involved in both the AC delivery and pore formation. We could show recently that a potentially α-helical segment between residues 502 and 522 of this domain plays a crucial role in membrane translocation of the AC domain, since a substitution of the glutamate residue at position 509 by a helix-breaking proline residue selectively ablated the capacity of the toxin to translocate its AC domain across plasma membrane of erythrocytes, without affecting membrane insertion and channel-forming (hemolytic) activity of the protein (*18*). Moreover, the negative charges of this putative transmembrane segment appear to modulate the frequency of formation of the CyaA channels and their cation selectivity, since lysine substitutions of glutamate residues 509 and 516 strongly enhanced the hemolytic and channel-forming activity of CyaA, while reducing up to 5-fold the cation selectivity of the formed channels, but did not affect their overall conductance (size).

The third domain, between residues 913−1612, contains about 38 glycine and aspartate-rich nonapeptide repeats,

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* Address correspondence to this author. Tel: +49-0931-888-4501. Fax: +49-0931-888-4509. E-mail: roland.benz@mail.uni-wuerzburg.de.

‡ Theodor-Boveri-Institut (Biozentrum) der Universität Würzburg.

§ Institute of Microbiology of the Academy of Sciences of the Czech Republic.

[1] Abbreviations: CyaA, 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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(L/I/F)-X-G-G-X-G-(N/D)-D-X, characteristic of all RTX toxins, that are involved in calcium and target cell binding (*19*−*24*). The mechanism of pore formation by CyaA is unknown. Intoxication of target cells by CyaA occurs only in the presence of free calcium ions, at concentrations above 0.1 mM $Ca^{2+}$ (*25*, *26*), that induce important conformational changes of CyaA leading to a burst in toxin activity (*27*). Two types of calcium binding sites have been postulated to exist in CyaA: a few high-affinity binding sites and many low-affinity ones, with half-saturation constants between 0.5 and 0.8 mM (*26*). Rogel and Hanski (*28*) showed that after a single exposure to calcium the toxin becomes competent for membrane insertion and hemolytic activity, even without free calcium ions, due to calcium loading of the "high-affinity" sites (*26*). They also showed that translocation of the AC domain can be uncoupled from membrane insertion of the toxin at low temperature and can be restored by raising the temperature or adding free calcium. The internalization of AC seems to be a monomolecular process, while more than one molecule of CyaA appears to be involved in the formation of a CyaA channel in vivo and in vitro (*18*, *29*−*32*). However, it has been shown recently that mutant ACT defective in adenylate cyclase activity is able to accelerate invasion of wild-type ACT into cells (*33*). Hence, oligomer formation could even support CyaA translocation into target cells.

Here, we characterized the effect of calcium and calmodulin binding on the pore-forming properties of CyaA and of its derivatives in artificial planar lipid bilayer membranes. We show that binding of calmodulin, which is an important cofactor for AC activity, has no influence on channel formation by CyaA, while binding of calcium ions to low-affinity sites induces a steep increase of channel-forming activity of CyaA.

## EXPERIMENTAL PROCEDURES

*Bacterial Strains, Growth Conditions, and Plasmids.* The *Escherichia 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 $\mu$g/mL ampicillin. pCACT3 is a construct for coexpression of *cyaC* and *cyaA* (*30*), and it allows production of recombinant CyaC-activated ACT in *E. coli* (r-*Ec*-ACT) under control of the IPTG-inducible $lacZ_p$ promoter.

*Production and Purification of the CyaA-Derived Proteins.* Full-length recombinant CyaA protein or the different truncated variants (ACT1008 and ACTΔ1−373) were produced with or without CyaC coexpression (*29*) 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 and purified by single-step affinity chromatography on calmodulin−agarose (Sigma) as described previously (*29*). Purified proteins were eluted in 8 M Urea, 50 mM Tris-HCl, pH 8.0, and 2 mM EDTA (buffer B) and stored at −20 °C. CyaA and the CyaA mutants were essentially free of contaminant proteins as shown previously (*32*).

*Loading the High-Affinity Binding Sites with Calcium.* CyaA isolated and purified by the method described above is essentially calcium free and has no biological activity when calcium is not added (*8*, *26*). To load the high-affinity binding sites of CyaA, the toxin was treated with 1 mM $CaCl_2$ and 10 mM HEPES−KOH, pH 7, for 2 h in the cold. During this process 8 M urea was highly diluted to avoid any interference between urea and the calcium binding sites. Afterward, free and loosely bound calcium was removed by dilution of the 1 mM $CaCl_2$ solution containing concentrated CyaA (1:1) with 4 mM EDTA and 10 mM HEPES−KOH, pH 7, for another 2 h in the cold. Thus, only the high-affinity binding sites were saturated with calcium ions (*26*).

*Membrane Experiments.* Black lipid bilayer membranes were formed as described previously (*34*). 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 across the hole by painting onto a 1% solution of asolectin (lecithin type IIIs from soybeans 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. The temperature was kept at 20 °C throughout. 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 channel recordings the electrometer was replaced by a homemade current amplifier. The amplified signal was monitored with a storage oscilloscope and recorded with a tape or a strip chart recorder.

## RESULTS

*Effect of Calcium Ion Concentration on the Channel-Forming Activity of CyaA.* The presence of free calcium ions at concentrations above 0.1 mM was repeatedly shown to be essential for target cell binding and penetration of CyaA, as well as for its capacity to mediate ion conductance across planar lipid bilayer membranes made of pure lipids (*8*, *26*, *31*). However, pure lipid bilayers appear to be poor targets for formation of the CyaA channels (*32*) and do not allow characterizing the calcium activation of toxin channels in sufficient detail. In contrast, lipid bilayers made of the lipid mixture asolectin (black lipid membranes) support formation of small cation-permeable toxin channels at reasonable frequency, even when calcium-depleted CyaA is used within solutions containing only trace amounts of free calcium ions (*32*). Therefore, we took advantage of the exquisitely sensitive black lipid membrane system for analyzing the calcium requirements and activation of the CyaA channels.

Calcium dependency of the steady-state membrane conductance induced by CyaA in black lipid bilayers was determined first (Figure 1A). In agreement with our previous results (*32*), the calcium-depleted CyaA (300 ng/mL) was able to form channels and to increase the steady-state conductance of asolectin bilayers to about 100-fold of the control membranes level, when the bilayers were bathed in 1 M KCl solution containing only trace amounts of the calcium ions. It is noteworthy that channel formation was also observed when 2 mM EDTA was added to the aqueous phase to remove all free calcium that could be present as impurities in the salt solutions and/or in the lipid asolectin (calculated free calcium concentration below 10 nM under

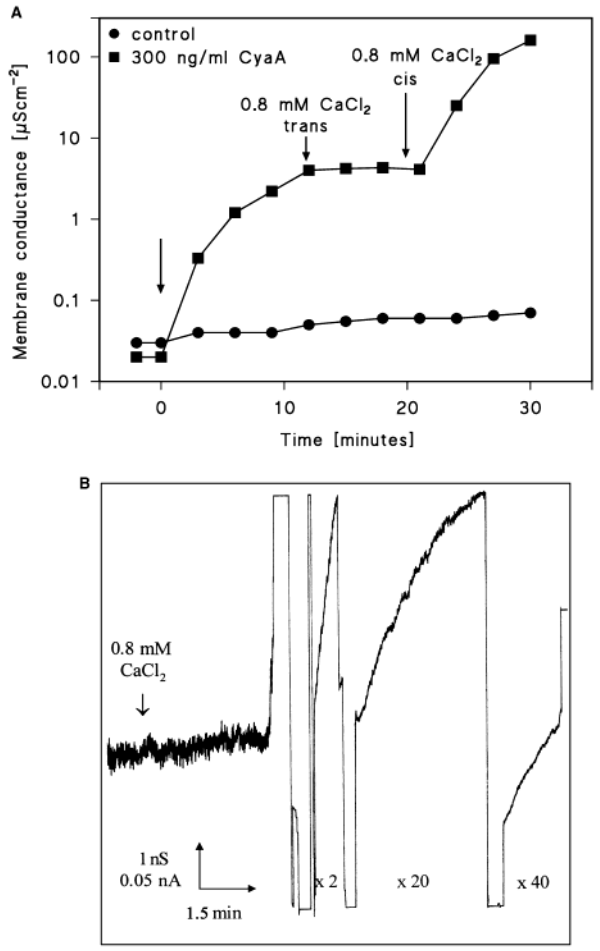


FIGURE 1: Effect of calcium on the channel-forming activity of CyaA. (A) The membrane was formed from asolectin/*n*-decane. The aqueous phase contained 1 M KCl, pH 7, and 300 ng/mL CyaA added to the *cis* side of the membrane (left-hand-side arrow). Twelve minutes after addition of CyaA 0.8 mM $CaCl_2$ was first added to the *trans* side (middle arrow), which did not influence the membrane conductance. Nine minutes later 0.8 mM $CaCl_2$ was added to the *cis* side (right-hand-side arrow), which led to a dramatic increase of membrane conductance (solid squares). The solid circles show a control experiment without CyaA. The temperature was 20 °C, and the applied voltage was 50 mV at the *cis* side. (B) This panel shows the original strip chart recording of membrane current after the addition of 0.8 mM $CaCl_2$ (arrow) to the *cis* side of the membrane. Note that the amplification of the signal was decreased several times during the subsequent strong conductance increase following the addition of $CaCl_2$ to the *cis* side (indicated by the vertical bars).

these conditions). As further shown in Figure 1A (arrow in the middle), addition of calcium ions ($CaCl_2$) to 0.8 mM at the *trans* side of the membrane, opposite to that at which CyaA was added to the membrane, had no effect on the steady-state conductance of the lipid bilayer. Hence, calcium as such did not affect the stability or permeability of the membrane. In contrast, addition of 0.8 mM calcium ions to CyaA present at the *cis* side of the membrane (right-hand-side arrow in Figure 1A) caused a steep increase of the bilayer conductance to about 50-fold within 10 min. The most plausible interpretation of this result is that interaction of calcium ions with CyaA strongly enhanced the specific membrane activity of CyaA either by increasing its membrane insertion and channel-forming capacity or by enhancing the unit conductance of the channels formed by calcium-activated CyaA or both. Interestingly, although the diffusion

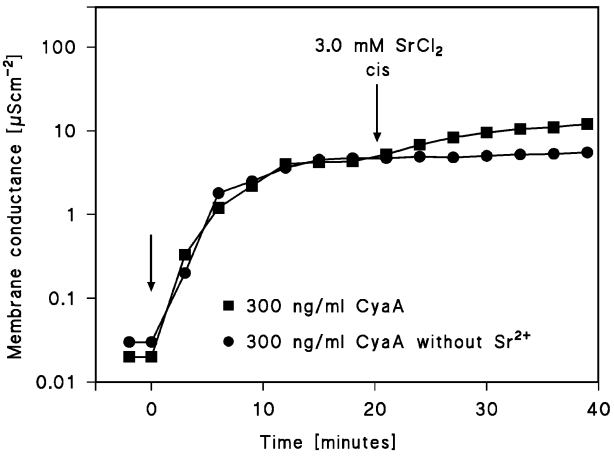


FIGURE 2: Effect of strontium on the channel-forming activity of CyaA. Membranes were formed from asolectin/*n*-decane. The aqueous phase contained 1 M KCl, pH 7, and 300 ng/mL CyaA added to the *cis* side of the membrane (left-hand-side arrow). Solid circles represent a control experiment where no strontium was added. Solid squares show an experiment where 20 min after the addition of CyaA, when the conductance was stationary, 3 mM $SrCl_2$ was added to the *cis* side (right-hand-side arrow). Subsequently, the membrane increased about a factor of 2. The temperature was 20 °C, and the applied voltage was 50 mV at the *cis* side.

of calcium ions even through unstirred layers adjacent to the membrane surface is expected to take less than 1 min and despite the use of stirring in the assay system, a typical delay of about 3 min was observed between the moment of calcium addition and the onset of the steep increase of the CyaA-mediated membrane current, as illustrated in Figure 1B. Several subsequent reductions of the current amplification were, hence, needed in order to record the conductance increase caused by calcium activation of membrane activity of CyaA, as also documented in Figure 1B.

*Other Divalent Cations Do Not Affect CyaA-Mediated Membrane Conductance.* It was important to test whether the calcium effect was on the level of the CyaA protein itself or whether it was rather resulting from an unspecific effect of the increased concentration of a divalent cation on the properties of the used lipid bilayers. Therefore, the effect of addition of other divalent cations on the membrane conductance mediated by CyaA channels was examined. In these measurements $Mg^{2+}$, $Sr^{2+}$, and $Ba^{2+}$ were added to the aqueous phase on the *cis* side of the membrane in concentrations up to about 20 mM following the addition of CyaA. However, at concentrations as high as 20 mM neither $Mg^{2+}$ or $Ba^{2+}$ exhibited any effect on membrane conductance mediated by CyaA nor did these cations inhibit the calcium-promoted conductance increase when added at such high concentration prior to the addition of calcium ions (data not shown). Only $Sr^{2+}$ ions caused a very small increase of CyaA-mediated membrane activity starting with about 3 mM (see Figure 2). The increase of the CyaA activity at 3 mM $Sr^{2+}$ was, however, about 500 times smaller as compared to the effect of $Ca^{2+}$ under otherwise identical conditions (see Figure 2, closed squares). It can be concluded that the effect of divalent cations on CyaA-mediated membrane conductance was strictly restricted to calcium. Indeed, if the calcium effect would be due to alteration of the general membrane properties, other divalent cations would be expected to substitute for it rather efficiently. The observed very high

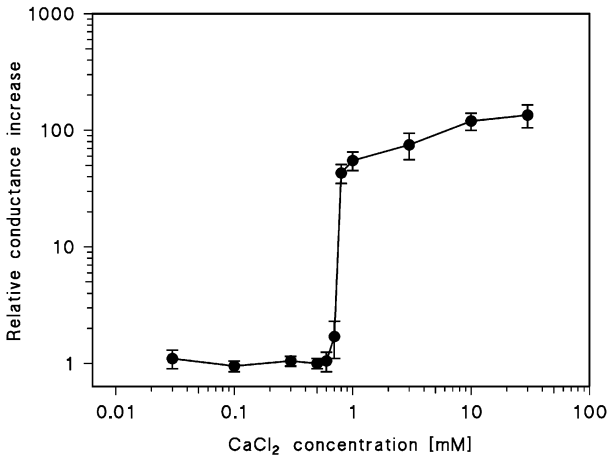


FIGURE 3: Dependence of the CyaA activity on calcium concentration. The calcium-mediated conductance increase of CyaA is given relative to that without calcium. The data were derived from the average of 10 titration experiments similar to that shown in Figure 1. Membranes were formed from asolectin/*n*-decane. The aqueous phase contained 1 M KCl, pH 7, and 300 ng/mL CyaA added to the *cis* side of the membrane. About 20 min after the addition of CyaA, $CaCl_2$ was added in the indicated concentration to the *cis* side of the membrane, and the conductance increase was taken when the current was stationary (usually 15−20 min after calcium addition). Means ± SD are shown from at least three experiments. The temperature was 20 °C, and the applied voltage was 50 mV at the *cis* side.

divalent cation specificity, typical for biological systems, strongly suggested that the conductance effect was due to selective binding of calcium to CyaA and to subsequent alteration of its membrane activity.

*Increase of Calcium Concentration in a Very Narrow Range Causes a Major Elevation of CyaA Membrane Activity.* The concentration of free calcium ions determines both the specific hemolytic activity of CyaA and its capacity to bind and penetrate cellular membranes and to translocate the AC domain into the target cells (*17*, *25*, *26*). Previously, two affinity classes of calcium binding sites were found to be involved in CyaA activities in vivo (*26*). Indeed, as previously observed (*32*) and reproduced here, the presence of trace amounts of free calcium ions (free calcium concentration below 10 nM) contaminating the used salt solutions and asolectin preparations could cause calcium binding to the postulated three to five high-affinity binding sites on CyaA (*24*, *26*). In turn, the strong enhancement of CyaA-mediated membrane conductance observed at a calcium concentration higher than 0.7 mM goes well with the presence of a high number (≈40) of low-affinity binding sites with a half-saturation constant within the 0.5−0.8 mM range (*26*).

As shown in Figure 3, when the calcium dependency of the CyaA membrane activity was examined in detail, it was found to be an extremely cooperative function of the calcium concentration. Below 0.8 mM $CaCl_2$ an only insignificant conductance enhancement of CyaA activity was observed with rising calcium concentrations. A spectacularly steep, about 50-fold increase of membrane activity of CyaA was, however, reproducibly observed upon only a 15% increase in $Ca^{2+}$ concentration from 0.7 to 0.8 mM, which is an extremely narrow concentration range in which the massive impact on CyaA activity occurred. At above the 0.8 mM $Ca^{2+}$ concentrations, the CyaA-mediated membrane conductance continued to increase further, albeit in a less dramatic manner, by going up only 2−3-fold over a concentration range of 2 orders of magnitude wide (0.8−60 mM), respectively.

The minimum activating calcium concentration was slightly ionic strength dependent and decreased to 0.6 mM when the ionic strength of the aqueous phase was decreased to 150 mM KCl. The saturation concentration for the calcium effect was found to be about 10 mM. The effect of calcium on CyaA-mediated membrane activity was, however, somewhat difficult to quantify. Whereas a dramatic increase of membrane conductance at higher than 0.8 mM calcium concentrations was observed in all experiments, the absolute level of the final conductance differed somewhat from experiment to experiment. The reason for this is presumably the steep conductance versus concentration relationship, which suggests that several CyaA monomers could form a conductive oligomer (*18*, *31*). Nevertheless, it was possible to estimate on the basis of individual experiments the calcium concentration inducing the half-maximal activation effect on CyaA-mediated channel-forming activity, which ranked between 2 and 4 mM. This is compatible with the predicted number of low-affinity calcium sites in repeats and their estimated half-saturation constants starting at 0.5 mM. However, the somewhat higher half-saturation calcium concentration might be also indicative of an additional, third class of calcium binding sites with affinity constants higher than 1 mM. Collectively, these results suggested that calcium loading into the numerous low-affinity binding sites in the RTX repeats of CyaA accounted for the strong activation of the membrane activity of the toxin at submillimolar calcium ion concentrations.

*The High-Affinity Binding Sites Do Not Contribute to Calcium-Mediated Membrane Activity of CyaA.* The results presented above suggested that loading of the low-affinity binding sites of CyaA accounted for the steep increase of its capacity to induce membrane conductance. To examine whether the high-affinity binding sites could play also a role in this process, these were first loaded by incubation of CyaA with calcium prior to chelating free and loosely bound calcium ions with EDTA. This means that three to five calcium ions remain bound to CyaA even in the presence of 2 mM EDTA (*26*). When the CyaA-mediated membrane conductance induced by such calcium-preloaded CyaA was examined, it was, however, found to be approximately the same as that observed with the essentially calcium-depleted control CyaA used in the presence of 2 mM EDTA at the same toxin concentration. This result suggested that the trace amounts of calcium present within the buffers and in the asolectin preparation were, in fact, not accounting for the membrane activity of CyaA observed under calcium-depleted conditions. Moreover, upon addition of calcium a similar increase of membrane conductance and at similar $Ca^{2+}$ concentrations was observed with the initially calcium-preloaded and calcium-depleted CyaA not exposed to calcium prior to assay, respectively (data not shown). Hence, the preloading of the high-affinity binding sites by calcium ions was not a prerequisite for membrane insertion and channel formation activity of CyaA in the lipid bilayer system. It has to be mentioned that the length of the incubation time (i.e., the length of preloading) of CyaA in subcritical calcium concentration had no influence on CyaA-

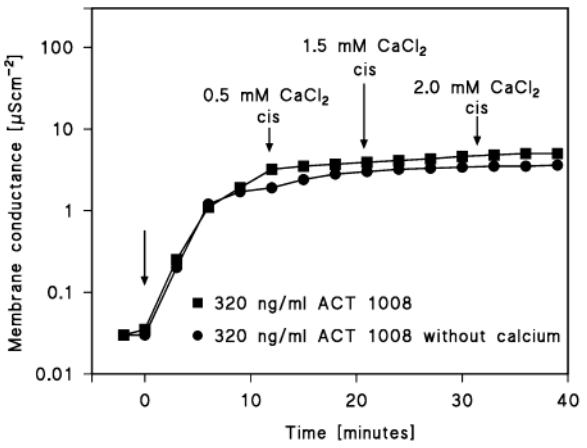


FIGURE 4: Effect of calcium on the channel-forming activity of the CyaA mutant ACT1008. Membranes were formed from asolectin/*n*-decane. The aqueous phase contained 1 M KCl, pH 7, and 320 ng/mL of the CyaA mutant ACT1008 added to the *cis* side of the membranes (left-hand-side arrow). Solid circles show the time course of the conductance in a control experiment without calcium. Solid squares show a similar experiment where calcium was also added to the *cis* side of the membrane. About 12 min after addition of the mutant protein, when the conductance was stationary, 0.5 mM $CaCl_2$ was added to the *cis* side (second arrow from the left-hand side). About 10 min later 1.5 mM $CaCl_2$ was added to the *cis* side (middle arrow), and again 10 min later the $CaCl_2$ concentration was increased to 2 mM (right-hand-side arrow). All additions had no influence on the membrane conductance. The temperature was 20 °C, and the applied voltage was 50 mV at the *cis* side.

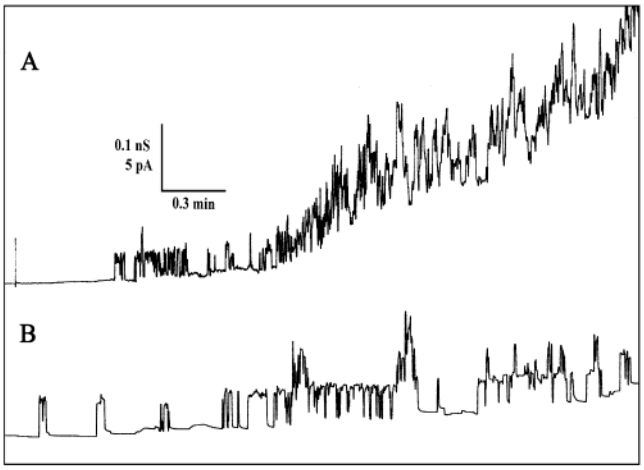


FIGURE 5: Calcium does not modulate the single-channel conductance of CyaA. Conductance experiments with high current resolution were performed with asolectin/*n*-decane membranes. (A) The aqueous phase contained 1 M KCl, 1 mM $CaCl_2$, pH 7, and 100 ng/mL CyaA added to the *cis* side of the membrane. The temperature was 20 °C, and the applied voltage was 50 mV at the *cis* side. (B) The experimental conditions were the same as in (A) with the exception that the aqueous phase contained only 1 M KCl, pH 7, as a control.

mediated channel formation. These findings are in some contradiction to earlier observations on erythrocytes (*26*, *28*), where previous exposure of CyaA to calcium was essential for making CyaA competent for subsequent membrane insertion and hemolytic activity even under conditions of very low free calcium concentrations (<10 nM) in the presence of 2 mM EDTA. This discrepancy could possibly be due to the different composition of the cellular and black lipid membranes, as well as to the different levels of the electrical potential on these membranes (about −10 mV on erythrocytes versus −50 mV on bilayers), and/or it could be due also to the presence of additional proteins and glycocalyx on the erythrocyte membranes, with respect to the naked black lipid bilayers.

*The Repeats Are Required for the Calcium Effect on CyaA Membrane Activity.* There exist several possibilities for the effect of calcium on the channel-forming activity of CyaA. The channel is cation selective, which is probably caused by negatively charged groups in or near the channel as has been shown previously (*18*, *32*). The CyaA molecule also contains about 38 glycine and aspartate-rich nonapeptide repeats characteristic of all RTX toxins, which have been shown to be essential for biological activity (*26*, *28*). To check whether the negatively charged groups in or near the channel or the repeats are responsible for the calcium effect, experiments were performed with the mutant ACT1008, in which the last 698 amino acids comprising the C-terminal repeats were removed, while the fatty acid acylation site is still present (*35*). Figure 4 shows measurements of this type in which the conductance increase was followed in a control experiment (closed circles) and for another membrane (closed squares). In the control (closed circles) ACT1008 showed approximately the same conductance increase as observed with wild-type CyaA (see Figure 1A). In the other experiment (closed squares) concentrated $CaCl_2$ was added to the *cis* side of the membrane in increasing concentration from 0.5 to 2 mM (arrows). Calcium ions had virtually no influence on the conductance of the ACT1008 mutant. This result suggested that the repeats are required for the calcium-mediated conductance increase of wild-type CyaA channels because the ACT1008 mutant lacks the repeats.

*Calcium Ions Do Not Change the Conductance of the CyaA Channels.* It was important to determine whether the effect of calcium on membrane conductance induced by CyaA was due to increased amounts of channel formed in the presence of calcium ions or whether it was due to the alteration of channel conductance and/or their size or both. To examine these two possibilities, the conductance of single CyaA channels was assessed at different concentrations of calcium ions and in 1 and 0.1 M KCl of different ionic strength, respectively. Figure 5 shows recordings with (A) and without 1 mM $CaCl_2$ (B) measured in a 1 M KCl solution. The channels had virtually the same conductance, irrespective of the presence and absence of calcium. This is also documented in Figure 6, which shows histograms of the current fluctuations with and without 1 mM calcium ions. The single-channel conductance was somewhat higher here as compared to a previous study at pH 6 (*32*) because the pH of the aqueous was set to pH 7, which led in a conductance increase from 27 to ∼50 pS. The results of these measurements at different calcium concentrations are summarized in Table 1, further suggesting that calcium ions had at the tested concentrations no influence on the unit conductance of the CyaA channels.

*Calmodulin, the Other CyaA Ligand, Does Not Influence Channel Formation by CyaA.* Since calmodulin has a high binding affinity for the AC domain of CyaA (*36*−*39*), we examined whether binding of such a large ligand (∼17 kDa) would affect channel formation by CyaA. Experiments with high and low conductance resolution were performed to test whether calmodulin interfered with channel formation medi-

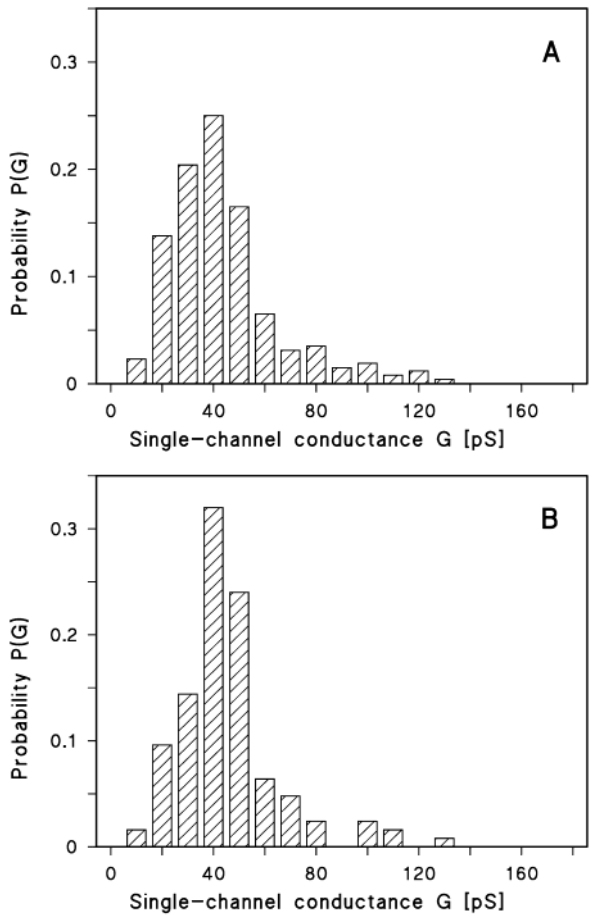


FIGURE 6: Histogram of the probability of the occurrence of certain conductivity units observed with membranes formed of asolectin/*n*-decane in the presence of 100 ng/mL CyaA. The aqueous phase contained 1 M KCl, pH 7, and 100 ng/mL CyaA. The applied membrane potential was 50 mV at the *cis* side; $T = 20$ °C. The average single-channel conductance was 53 pS for 260 single-channel events [(A) 1 M KCl and 1 mM $CaCl_2$] and 49 pS for 125 events [(B) control without $CaCl_2$]. The data were taken from at least five different membranes in both cases. The conductance records of each membrane had a length of at least 5 min.

Table 1: Average Single-Channel Conductance, *G*, of the Channel Formed by CyaA in Different Salt Solutions with and without Calcium[a]

| KCl *c* (M) | $CaCl_2$ *c* (mM) | single-channel conductance *G* (pS) |
|---|---|---|
| 0.1 | 0 | 6 |
| 0.1 | 1 | 10 |
| 1.0 | 0 | 49 |
| 1.0 | 1 | 53 |

[a] The membranes were formed of asolectin dissolved in *n*-decane. The aqueous solutions were buffered with 10 mM HEPES−KOH and had a pH of 7. The applied voltage was 50 mV, and the temperature was 20 °C. The average single-channel conductance was calculated from at least 100 single events; *c* indicates the concentration of KCl and $CaCl_2$ in the aqueous salt solutions. The pH of the unbuffered aqueous salt solutions was about 7.

ated by CyaA. Multichannel experiments were performed in a similar way as described above with calcium. In these measurements calmodulin (in a concentration of 2 $\mu$M) was added to the *cis* and to the *trans* sides of preformed CyaA channels. However, no effect of calmodulin on formation of CyaA channels was observed (data not shown). Similarly, the presence of 1 mM calcium ions did not lead to any calmodulin effect on the formation of channels when both were added to the *cis* side. In addition, preincubation of CyaA with calmodulin before addition of the toxin to the aqueous phase bathing lipid bilayer membranes did not affect the steep calcium-dependent conductance increase as compared to control experiments where only CyaA was added to the aqueous phase. Since binding of calmodulin to the AC domain did not affect the single-channel conductance of CyaA, these results suggested that the AC domain is not involved in formation of CyaA channels.

*The AC Domain Is Not Essential for Calcium-Mediated CyaA Activity.* To test whether the AC domain could play any role in the activation of CyaA by calcium, experiments with high and low current resolution were performed with the ACTΔ1−373 mutant, which lacks most of the AC domain. However, no difference in channel properties and calcium dependence of their respective membrane activities could be observed between the intact CyaA and its truncated ACTΔ1−373 variant (data not shown). Both proteins could be activated by addition of calcium ions to the *cis* side starting at about 0.6−0.8 mM calcium ion concentration depending on the ionic strength of the buffer used (data not shown). This result suggested that the ACT domain is not at all required for CyaA channel formation.

## DISCUSSION

We have analyzed here the major impact of submillimolar concentrations of calcium ions on the channel-forming capacity of CyaA. The observed activity enhancement of CyaA due to calcium binding is rather consistent with the in vivo situation, where free calcium is present at millimolar concentrations in plasma and body fluids bathing the surface of CyaA target cells, while typically a very low calcium concentration is present in the cell cytosol (around 100 nM). Particularly noteworthy is the extreme (∼50-fold) increase of the channel activity of CyaA upon increase of the calcium concentration within a very narrow range from 0.7 to 0.8 mM, hence by only as little as 15%. This strongly indicates that the toxin molecule undergoes a true conformational switching from the essentially "off" state to the "on" conformation that accounts for its high membrane activity and which occurs at higher than 0.6−0.8 mM free calcium concentrations. The most plausible interpretation of this toggle-like behavior of CyaA is that in the range of 0.6−0.8 mM concentrations of $Ca^{2+}$ the binding of calcium ions to CyaA proceeds in a highly cooperative manner and at numerous binding sites concomitantly. Such cooperative calcium binding can be expected to cause a major conformational change and/or even partial refolding of the protein. This might possibly consist of formation of parallel $\beta$-roll structures upon calcium binding to the numerous low-affinity calcium binding sites within the RTX repeats of CyaA, as predicted by analogy to the parallel $\beta$-roll motifs of the RTX repeats of *Pseudomonas* and *Serratia* proteases, where calcium is bound within the turns connecting the $\beta$-strands (*21*). In parallel, this conformational change might involve also mutual positioning of the calcium-bound $\beta$-sheet blocks within the CyaA molecule, possibly due to formation of helical structures within the loops linking the repeat blocks, as suggested by our earlier results on calcium-induced

conformational changes in the CyaA molecule as observed by circular dichroism (CD) spectroscopy (*26*).

As further shown here, a delay of several minutes was reproducibly observed upon addition of higher than 0.6−0.8 mM calcium concentrations to CyaA before the onset of the steep increase of the channel activity of CyaA. This delay could potentially reflect the time needed for the conformational change within the CyaA molecule upon calcium binding. However, conformational changes induced by ligand binding are typically very rapid processes. It appears, therefore, more likely that the observed delay in CyaA activation corresponds to the time needed for membrane insertion and/or oligomerization of additional calcium-bound CyaA monomers. In this respect, it is important to note that the significant increase of CyaA-mediated membrane conductance in the presence of calcium was not due to a change of the unit CyaA channel conductance (size). This remained virtually the same in the presence or absence of 10 mM $Ca^{2+}$ ions. Indeed, we have previously observed that the conductive CyaA oligomer could be a tetramer or an octamer even at trace concentrations of free calcium ions (*18*). The present results suggest that binding of calcium to CyaA does not lead to formation of larger CyaA oligomers. Instead, it appears to importantly enhance the propensity of CyaA to insert into the membrane and to form the oligomeric channel.

The effect of calcium on membrane activity of CyaA appeared to be highly specific. Other divalent cations, such as $Mg^{2+}$, $Sr^{2+}$, and $Ba^{2+}$, had no or very low effect on formation of CyaA channels, and there was no competition between calcium ions and the other divalent cations. Even at very high (20 mM) concentrations the $Mg^{2+}$, $Sr^{2+}$, and $Ba^{2+}$ cations did not interfere with the enhancement of CyaA-mediated channel-forming activity by calcium present at submillimolar concentrations. It is noteworthy in this respect that a similar cation selectivity has been found also for the activity of *E. coli* HlyA (α-hemolysin) on model membranes, although strontium and barium induced some HlyA activity (*40*). In sharp contrast, however, channel formation by HlyA in lipid bilayer membranes did not require the presence of calcium ions, and the channel-forming activity of HlyA remained unaltered upon deletion of the RTX repeats or removal of free calcium ions (*19*, *41*). This represents a remarkable difference between the two RTX toxins, which could be accounted for by the specific structure of the RTX domain of CyaA that contains many more calcium binding repeats (∼40), as compared to the 13 repeats found in HlyA (*19*).

As also shown here, the intact CyaA and the mutant ACT1008, in which 698 amino acids of the repeats were removed, exhibited, indeed, the same small channel-forming ability when no calcium ions were added to the assay system (*32*). At calcium concentrations higher than 0.6 mM, however, the membrane activity of intact CyaA was strongly increased compared to ACT1008, which did not respond to increased calcium concentrations at all. This strongly indicates that binding of calcium ions to the repeats of CyaA accounted for the calcium effect on its membrane activity.

Another conclusion deduced from the experiments reported here is that the AC domain of CyaA is not involved in formation of the membrane channels. No interference of calmodulin binding to the AC domain with the formation of channels by CyaA could be observed, and the removal of the AC domain did not affect channel formation, either (*32*). This provides further support for the recently proposed model suggesting that channel formation and translocation of the AC domain through cellular membranes may represent two parallel and unrelated, if not mutually exclusive, membrane activities of CyaA (*18*).

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