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Anion Channel Forming Activity from the Plant Pathogenic Bacterium *Clavibacter michiganense ssp. nebraskense*

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Summary. The plant pathogenic bacterium *Clavibacter michiganense ssp. nebraskense* secretes an anion channel forming activity (CFA) into the culture fluid. The CFA inserts spontaneously into planar lipid membranes when culture fluid of this species is added to the aqueous phase of the bilayer chamber. The channels formed are highly anion selective. The conductance decreases for larger anions ($Cl^- > SCN^- > SO_4^{2-}$) and is practically zero for gluconate. The channels show a unique voltage dependence: (*i*) The single-channel conductance increases linearly with voltage up to 200 mV saturating at 250 mV with 25 ± 1 pS (300 mM KCl). The channel is closed at negative voltage relative to the side of insertion (diode-type *I-V* curve). (*ii*) The average number of open channels also increases with voltage. The Poisson distribution of channel numbers indicates independent opening of the channels.

Channel activity can be abolished by protease treatment of the planar bilayer. The channels can be blocked by indanyloxyacetic acid (IAA-94) and by pH > 10. The CFA was purified yielding one major band on the SDS gel with a relative molecular mass of 65,000. The putative involvement of the CFA in the toxicity of this plant pathogen is discussed and compared to other toxins like colicins and to the diphtheria toxin group.

Key Words phytotoxin · planar membrane · channel reconstitution · voltage dependence · anion selective · rectifier

Introduction

Numerous members of the genus *Corynebacterium* are pathogens for man, animals, and plants while others are employed in biotechnology, for example in the production of aminoacids [2, 19, 32]. In an attempt to account for the taxonomic heterogeneity of this group of bacteria the plant pathogenic *Corynebacteria* have recently been assigned to the new genus *Clavibacter* [6]. The strain *Clavibacter michiganense ssp. nebraskense* (NCPPB 2581) used in this study causes Goss's wilt and blight in *Zea mais* [35]. Members of the genus *Clavibacter* have been described to produce phytotoxins which were classified as high molecular mass polysaccharides (*C. m. ssp. michiganense;* [25]) or glycolipids (*C. rathayi;* [33]). The isolated polysaccharides cause wilting in plant assays [31], and recently, for the polysaccharide produced by *C. m. ssp. michiganense* a degeneration of chloroplasts has been observed [16]. However, such an effect can be attributed to water stress rather than to a discrete action of the polysaccharide on the chloroplasts and its membrane. So far other types of phytotoxins produced by *C. m. ssp. nebraskense* have not been described. During a systematic search for toxic activities excreted into the culture medium by *C. m. ssp. nebraskense* we have identified a membrane-active component which forms anion channels in planar lipid bilayers. In this communication we describe the properties of this CFA.

Materials and Methods

Strain

The strain *C. m. ssp. nebraskense* (NCPPB 2581) was obtained from the National Collection of Plant Pathogenic Bacteria, Hatching Grenn Harpenden, England.

Chemicals

9-Anthracene-carbonic acid (9-AC) was purchased from Aldrich and diisothiocyanatostilbene-disulfonic acid (DIDS) from Sigma. Indanyloxyacetic acid (IAA-94) was a gift from Prof. Dr. D. Landry, College of Physicians and Surgeons, New York.

Culture Conditions and Purification of the CFA

Cultures (100 ml) of *C. m. ssp. nebraskense* were grown in a medium containing 0.5% yeast-extract (Difco), 0.5% glucose, 0.5% NaCl (Merck) for 7 days at 26°C with vigorous shaking.

Cells were separated from the culture medium by centrifugation (15,000 × *g*, 30 min) and sterile filtration of the supernatant through a filter with 0.45-μm pore size (Renner GmbH, Darmstadt, FRG).

The cell-free culture medium was then concentrated 10-fold under reduced pressure in a rotary evaporator. Voltage-clamp analysis can already be performed with this crude material. For purification the concentrated culture medium was dialyzed against distilled water at 4°C using a cellulose-acetate dialysis membrane (Serva, cut off mol wt 12,000). Exopolysaccharides (EPS) were precipitated by the addition of 2 volumes of acetone and incubation overnight at −20°C. After centrifugation (30 min, 50,000 × *g*) the pellet was discarded and the acetone in the supernatant was removed by rotary evaporation. The CFA-containing protein was precipitated by the addition of 2 volumes of ethanol and incubation at −20°C overnight. After centrifugation (30 min, 50,000 × *g*) the pellet was washed with the pure solvent and stored lyophilized until use. Determination of polysaccharides has been done by the anthrone method as described in [27]. Protein determination was done according to Miller [22].

Voltage-Clamp Measurements

The planar lipid bilayer chambers contained 300 mM KCl and 10 mM HEPES (pH 7) as a standard solution. Changes of the aqueous solution were performed by continuous adding of the new buffer solution to the bilayer chamber and simultaneous removal of the stirred mixture using two syringes (5 volumes). Planar bilayers were obtained by apposition of two vesicle-derived monolayers [26]. Vesicles were formed from soybean phospholipids (Avanti Polar Lipids) and cholesterol (10%, if not stated differently). The solvent of the lipid, chloroform, was evaporated in a round bottom flask (rotavap) in order to form a thin film of the lipid on the glass. The lipid (30 mg/ml) was suspended in 80 mM HEPES (pH 7) by shaking with small glass beads. The suspension was homogenized by 1-min sonication in a bath and additional 3–4 freeze and thaw steps. The vesicles were stored at −80°C. The lipid samples were diluted 10–20 times in the appropriate salt solution before use.

For all current measurements a current-to-voltage converter was used with an OPA-104CM amplifier (Burr & Brown) and a 10^{10} Ω feedback resistor. The *trans* side of the bilayer chamber was connected to virtual ground, and voltage was applied to the *cis* side. In general the CFA was added on the *cis* side. However, to facilitate solution exchange the CFA was sometimes added to the *trans* side or the chamber was turned around. Then a negative voltage (but positive seen from the side where the CFA was added) had to be applied to open the channels. Therefore, currents are negative in Fig. 5, i.e., channels open downward. Data were stored on a video tape recorder and analyzed by the aid of a computer oscilloscope (E. Bablock, Augsburg, FRG) after filtering with a 4-pole Bessel filter at 100 Hz.

Protease K Treatment

Protease K (Sigma) digestions were performed by adding 80 μl protease K solution (10 mg/ml; final concentration 0.7 mg/ml) to the chamber buffer after channels were observed.

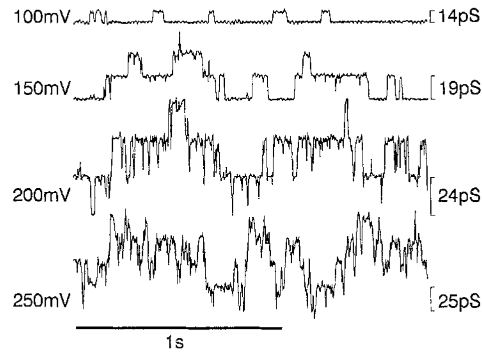

Fig. 1. Channel traces (current) induced by the channel forming agent (CFA) in a planar lipid bilayer. The voltage was clamped at the value indicated; channels open upward. The number of channels as well as the single-channel conductance increase with the voltage. For convenience, the conductance values are given instead of the current. In the last trace the current scale is reduced to half. The bilayer was formed by apposition of two vesicle-derived monolayers. Vesicles were formed from a natural mixture of soybean phospholipid (Avanti, for details *see* Materials and Methods). The aqueous solution contained 300 mM KCl and 10 mM HEPES, pH 7

Results

Spontaneous Channel Insertion

In our initial experiments we observed a channel forming activity (CFA) when 25–100 μl of the 10-fold concentrated culture fluid were added to the aqueous phase of a planar bilayer chamber. The first ion channels could be observed after 2–15 min (Fig. 1). The lag time depends on the amount of culture fluid added and differs between different preparations. In the control, fresh, sterile medium, as used for bacterial growth, was injected into the chamber and, as expected, no channels were seen. A negative result was also obtained with the culture fluid of *Clavibacter michiganense ssp. michiganense* (NCPPB 382), a closely related strain which is a pathogen for the tomato. Thus, the CFA is not a general metabolite of the genus *Clavibacter*.

The number of channels increased continuously up to at least 30 min. When the voltage was applied 10 or 20 min after the addition of the culture fluid, channels appeared immediately. The number of the channels was in the same range as when the voltage had been applied from the beginning. When the CFA-containing solution was exchanged against pure buffer the number of channels remained constant until the end of the measurements, i.e., half an hour. When membranes were formed after addition of culture fluid (e.g., after membrane rupture) it took again some time until the first channels appeared (at

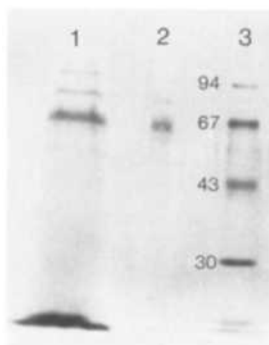

Fig. 2. SDS-polyacrylamide gel electrophoresis of the purified anion channel protein. Lane *1:* Culture fluid as prepared for ethanol precipitation after removal of EPS by acetone. Lane *2:* Channel forming protein purified by ethanol precipitation. Lane *3:* Protein standard with relative molecular masses ($\times$ 10^3). The running gel contained 15% polyacrylamide. The proteins were stained with Coomassie blue

least 1 min), and the same time-dependent increase in the number of channels was observed. However, a difference was seen with regard to rectification (*see below*).

Protein Purification

The SDS gel of Fig. 2 shows that the 10-fold concentrated and dialyzed culture fluid, from which the exopolysaccharides (EPS) have been removed, contains only a few proteins with a relative molecular mass higher than 10,000 (lane *1*). In the last purification step (ethanol precipitation) a rather pure preparation of the CFA was obtained (Fig. 2, lane *2*). After dialysis, 1 liter of culture fluid contains 460 mg $\pm$ 10% of protein and about 700 mg of EPS. The material of the final purification step contains 8 $\pm$ 2 mg of protein and is virtually free of EPS. The purified CFA had the same channel characteristics as observed in the crude extract.

To determine the increase in specific channel activity we have measured the average number of open channels per minute after an incubation time of 20 min (the number of channels increases with time). The specific activity was increased by a factor of 20 $\pm$ 5, i.e., the amount of protein needed to produce a certain number of channels was 20 times lower as compared to the culture fluid.

Characterization of the CFA

Interestingly, channel opening could be abolished by injection of protease K (0.7 mg/ml final concentration) into the aqueous compartment (*cis* side), supporting the notion that the CFA comprises a protein. The average number of channels was reduced to half its value about 5 min after addition of the protease. After 20 to 30 min of protease treatment the channel activity was found to be completely inactivated. In contrast no inactivation of the CFA could be detected, when the protease was added to the *trans* side of the bilayer chamber, regardless of the voltage applied (+125, 0, and −125 mV for 12 min, respectively).

Opening and Closing of the Ion Channel

Compared to most of the signal transducing channels the open time of the CFA channels was rather long. The exponential distributions can be satisfactorily fitted by two mean open times $t^{0,1} = 0.15 \pm 0.05$ sec and $t^{0,2} = 2 \pm 0.5$ sec. Channel openings often were interrupted by several closing gaps in the millisecond range. These short gaps are only partially resolved with the 100 Hz low-pass filter used (Fig. 1). The channels were grouped in bursts, i.e., channel opening is not randomly distributed. When the number of channels is small there are several openings in short periods interrupted by larger breaks. At higher channel numbers this behavior is manifested in large conductance fluctuations. Different sublevels of single-channel conductance have been recorded. However, they only account for less than 5% of the channels.

Voltage Dependence and Rectification

Channels could be observed only when voltage was positive on the side where the CFA was added, in general, the *cis* side. At negative voltage, even at −200 mV, the channels were closed. This strong rectification seems to be related to the voltage dependence of the single-channel conductance. Both, the probability of being open and the conductance/channel increase with voltage (Fig. 1). The latter is characterized by the semiparabolic *I-V* curve for single channels (Fig. 3). At zero voltage the channel is closed. The increase in conductance becomes saturated at about 250 mV. A higher voltage cannot be applied since bilayers then become unstable. In addition, the probability of channel opening increases with voltage. This is demonstrated in Fig. 4. The histograms are nearly ideal Poisson distributions, indicating independent channel opening. The average number of channels from the Poisson distribution μ increases from about 1 at 125 mV to more than 4 at 250 mV. This gives an additional rectifying moment to the whole system. When a new mem-

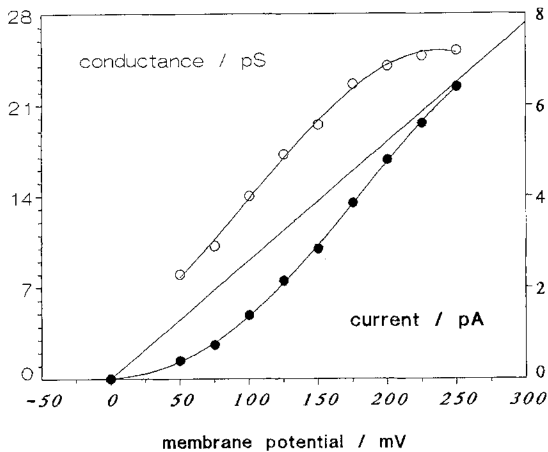

Fig. 3. Current-voltage curve of single channels. The filled circles show the *I-V* curve. At lower voltage it has a semiparabolic form. At 250 mV, when the conductance (open circles) becomes constant it meets the line which characterizes ohmic behavior. The conductance was calculated from the current data according to $G(V) = I(V)/V$; ($V = U_{cis} - U_{trans}$ = membrane voltage). Buffer: 300 mM KCl, 10 mM HEPES, pH 7.1

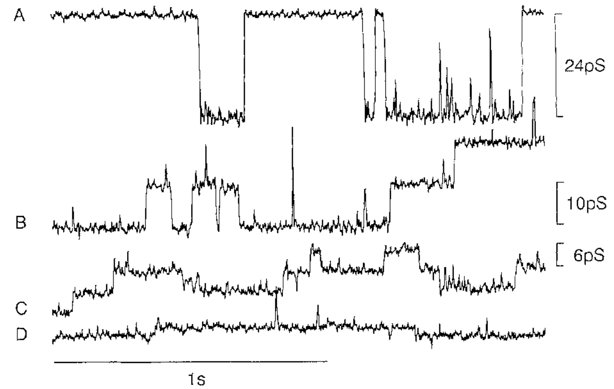

Fig. 5. Channel selectivity. The channel traces correspond to different salt solutions on the *cis* side of the planar lipid membrane: (*A*) 300 mM KCl, (*B*) 300 mM KSCN, (*C*) 300 mM Na_2SO_4, and (*D*) 300 mM K-gluconate. The *trans* side always contained 300 mM KCl. The data, which show that the channel is anion selective, were recorded from the same membrane. In *this* experiment, the CFA was added on the *trans* side. In this configuration, which was chosen to facilitate buffer exchange, the channels open downward because a negative voltage had to be applied to open the channels (V = −200 mV). However, the voltage is positive with respect to the CFA. No current could be measured at 0 mV. The aqueous solution in the *cis* chamber was continuously exchanged (two syringes) with 5 volumes of the indicated salt solution containing 10 mM HEPES, pH 7

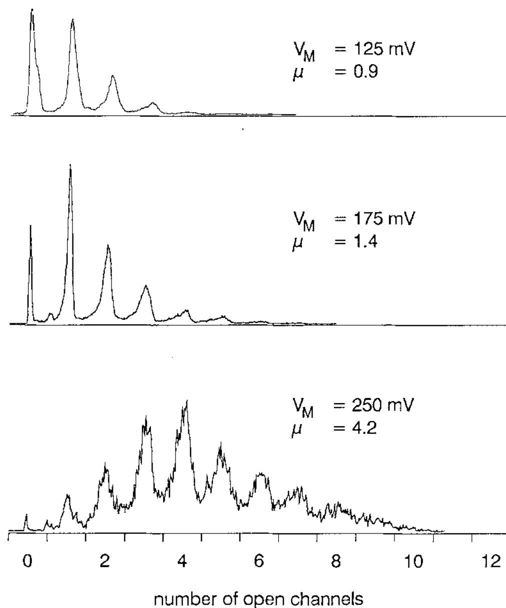

Fig. 4. Probability function of open channel number (channel histogram). With increasing voltage the average number of open channels increased too. The peaks correspond to the relative probability that 0, 1, 2, . . . , *n* channels were simultaneously open. The measured values match a Poisson distribution with the average number of open channels μ. To get equally distant peaks in the three histograms the current values on the original *x*-axis were divided by the current carried by a single channel at the applied voltage. This gives the (dimensionless) number *i* of open channels; μ therefore is $\Sigma\, i \cdot n_i/N$. (n_i = number of sample points of value *i*; *N* = number of all sample points)

brane is formed, after the CFA had been added, few channels also appear at negative voltage. This can be explained by the diffusion of the CFA to the *trans* side after membrane rupture, or an exchange between monolayers may have occurred.

Ion Selectivity

Exchange of K^+ against Na^+, $Tris^+$, or $choline^+$ did not change the conductance.[1] Hence, the currents through the channels formed appear to be carried by anions. To prove this, Cl^- was exchanged against other anions on the side opposite to the one containing CFA.[2] The result is shown in Fig. 5. With increasing size of the anion the conductance becomes smaller, $Cl^- > SCN^- > SO_4^=$ and is zero for gluconate. Substitution of Cl^- on the side where the CFA was added had no significant effect. To compare the absolute conductance of the channels for different anions, membranes were formed with

[1] Even in a large water-filled pore the large cations should show a significant lower conductance than K^+ when permeant.

[2] This time the CFA was added on the *trans* side to facilitate the exchange of buffer. Therefore, channels open at negative voltage and anions move from *cis* to *trans*. Bi-ionic potentials could not be determined as the conductance is too tiny when the voltage approaches zero.

Table. Single-channel conductance of different salts[a]

Salt	Conductance/pS membrane voltage		
	100 mV	150 mV	200 mV
NaF	12	16	17
KCl (NaCl)	14	19	24
KBr	10	16	22
NaJ	3	4.5	8.5
$NaNO_3$	18	24	31
NaN_3	9	14	20
KSCN	4	7	11
Na-formiate	7	10	14
Na-acetate[b]	—	—	<1
Na-pyruvate[b]	—	—	<1
K_2-oxalate	—	4	6.5
K_2-malonate	—	2.5	5
K_2SO_4	—	3.5	5.5
K-gluconate	No measurable conductance		

[a] Both sides of the membranes contained 300 mM of the salt indicated and 5 mM Na-PIPES, pH 6.8. To reduce the formation of $(I_3)^-$ 10 mM Tris-EGTA was added (final pH = 8.0). The conductance values are average numbers of 10 channels. The SD is always in the range of 5–10%. However, for the small values the error arising from the confined resolution (≈ ±0.05 pA) has to be taken into account.

[b] 2 ± 0.5 pS at 250 mV.

symmetric solutions of the salts listed in the Table. Among the halides tested chloride showed the maximal conductance. Only with NO_3^- was a higher value observed. Some anions of organic acids (formiate > oxalate > malonate > pyruvate) can pass the channel at least at high membrane voltage. Gluconate showed no measurable conductance.

Application of a Cl^- gradient (15 mM *cis*/150 mM *trans*) was not sufficient to produce a detectable channel conductance. This behavior can be understood when assuming that channels are closed at ≤0 mV.

Investigation of pH Dependence

A drastic effect of pH on insertion, respectively adsorption of the channel forming peptide to the membrane, has been reported for a number of toxin channels especially for the colicins, the diphtheria toxin group and the alamethicin group (*see* ref. [10]). The pH also influenced ion selectivity.

Between pH 4 and 9 no change in channel formation or ion conductance was detected with the CFA from *C. m. ssp. nebraskense*. At pH > 9 the lipid bilayers become unstable. However, when the aqueous solution was exchanged for Na-bicarbonate buffer of pH 10 or 11 only on one side of the membrane (alternative *cis* or *trans*) channel measurements were possible. At pH 10 the conductance was reduced to 7 pS at 100 mV (half-maximal value) and to ≤2 pS at pH 11.

Chloride Channel Blockers

We have tested three different channel blockers known to be most effective on chloride channels of *Torpedo* electric organ (DIDS), of muscle (9-AC) and of kidney (IAA-94), all with inhibition constants of μM [4, 17, 23]. None affected channel activity when added on the *cis* side, even at millimolar concentrations. However, when added on the *trans* side a block of CFA activity by IAA-94 was observed, starting at 10^{-5} M. At this concentration the single-channel conductance was reduced to about 70% and channel flickering between the open and closed state indicated competitive binding at the chloride binding site. With increasing concentration of IAA-94 the single-channel conductance decreased correspondingly. At 10^{-4} M IAA-94 the channels were totally blocked. No effect was seen with DIDS and 9-AC when added to either side.

Investigation of Cholesterol Effects

In the standard lipid mixture cholesterol was added at a concentrations of 10%. Neither increasing the cholesterol content to 33% nor its omission had any measurable effect on the channel formation and the single-channel conductance.

Discussion

Protein Purification

The purified protein shows only one major band (M_r = 65,000) and two to three fainter bands on the SDS-polyacrylamide gel. As the difference from lane *1* (*see* Fig. 2) is relatively small, we conclude that the main protein fraction which has been removed has a relative molecular mass below 10,000 and appears on the bottom of the gel in lane *1*. Obviously the bacteria do not excrete many different proteins into the medium. Respectively, the increase in specific channel activity is not very large. However, one should take into account that a large amount of bacterial exopolysaccharides was removed.

Comparison with Other Toxins

There are many different toxins known and many different mechanisms of action. The source can range from procaryotes (colicins, alamethicin, α-

toxin, etc.) to mammals as part of a complicated defense mechanism (complement system, C9, and perforin/cytolysin [5, 21, 30]). So far, not so many channel forming toxins directed against plants have been reported [12, 15]. Here we report on a CFA secreted from a phytopathogenic corynebacterium. In general, interaction with membranes is an important step of toxin action. Characteristic for channel forming toxins is that they insert into membranes spontaneously as we have shown for the CFA.

Small peptides like alamethicin or melittin have been proposed to form channels by aggregation of at least six molecules which adopt an α-helical conformation in the bilayer [3, 8, 9]. For channel opening a voltage induced reorientation of the peptides is necessary. At high concentration and high voltage very large unspecific pores can be formed finally leading to membrane rupture. Similar properties have been reported for cytotoxic agents from Hydra or sea anemones [20, 34]. The alamethicin type of channel formation is not likely for the CFA from *C. m. ssp. nebraskense*. Different from alamethicin, the single-channel conductance of the CFA is a continuous function of voltage at least up to 0.2 V. Channel induction by voltage is much less steep and, importantly, not symmetric as shown for alamethicin. From this point of view, the CFA of *C. m. ssp. nebraskense* has more similarity with the colicins where the channels are also induced at positive voltage. However, the gating kinetics of colicin channels are rather slow as compared to the CFA. It was shown by protease digestion experiments that the gating process involved the translocation of a small hydrophilic domain across the hydrophobic bilayer, associated with a high activation energy [29]. A slow process, regarding the CFA, is the increase in conductance (number of channels) after addition of the culture fluid. This irreversible accumulation of the CFA in the bilayer is independent of voltage. A similar kinetic behavior of channel formation was found for the HmT-toxin of *Helminthosporium maydis* [14, 15]. In contrast, the partition of the antibiotic monazomycin between the lipid bilayer and the aqeuous phase is in equilibrium. The equilibrium strongly depends on voltage.

Compared to the CFA, channel formation is fast for gramicidin, e.g., where channels appear after about 1 min. This time is needed for the diffusion of the peptides across the unstirred layer [28]. Assuming the CFA is a larger protein diffusion could account also for the longer lag time seen with this system. After reformation of a membrane in the presence of gramicidin, channels can be seen immediately (Th. Schürholz, *unpublished results*). The formation of channels by the CFA apparently needs a time-consuming reorganization step into a conducting state which cannot be adopted in the monolayer. An irreversible structural change of the CFA in the monolayer might be another explanation.

Again similar to colicin it was possible to close channels by protease digestion. Therefore, a more hydrophilic domain which is susceptible to protease digestion seems to protrude from the membrane into the aqueous phase. The protease has no effect on the *trans* side of the membrane, irrespective of membrane voltage. This is another evidence that transport of a cleavable hydrophilic domain is not part of the gating process. Second, this result corroborates the asymmetric nature of the channel.

Voltage Dependence, Rectification and Ion Selectivity

Voltage dependence of single channels and rectification of the CFA channels reported here are rather unique for toxin channels reported so far. It seems that the rectification and the semiparabolic voltage dependence of the single channel are based on the same molecular mechanism, but the rectification could be related to the voltage-dependent gating (number of channels) as well. The channel is closed at $\leq$0 mV and successively opens with increasing voltage. This behavior is different from the all-or-none process of colicins which can be compared to a flip-flop switch. The channel we describe here closes without application of a negative voltage. One possible explanation would require that the open channel is virtually the fast flickering of a channel of larger conductance which cannot be resolved at 100 or 300 Hz. The voltage-dependent increase in conductance then would rely on changes in open or closed times of the flickering process. The partly resolved short closing gaps and the increased noise in the open state support this possibility. However, we think that a continuous conformational change with voltage cannot be excluded (*see below*).

The increase in the number of channels with increased voltage has also been reported for monazomycin. The membrane conductance induced by this antibiotic channel former shows an *e*-fold dependence on the membrane voltage. The channels are closed at negative potentials [24]. In contrast to the CFA channel, monazomycin is *cation* selective [1, 24]. The voltage dependence of monazomycin was shown to reside on a shift in the partition of the peptide between the membrane and the aqueous phase, which equilibrates in the second range [24]. The CFA channels cannot be removed by buffer exchange. Therefore, rearrangements of the proteins inside the lipid phase are more probable.

A further remarkable feature of the CFA is the

practically ideal ion selectivity for anions over cations. A cation conductance was not measurable (K-gluconate, the Table). Between different anions the selectivity is similarly weak as in the chloride channels found in epithelial cells [17]. The conductance sequence of the different anions (the Table) shows remarkable parallels to the sodium channel [13]: (*i*) The shape of the minimum pore size that will pass the permeant ions should also be noncircular (e.g., $G(NO_3^-) > G(Cl^-)$)[3]. (*ii*) The conductance of the halides falls with increasing crystal radius, indicating a strong electrostatic interaction. The relative low conductance of F^- may be due to rate-limiting strong electrostatic interactions[4]; $P(Li^+) \leq P(Na^+)$ for the sodium channel, respectively [13]. (*iii*) The conductance of anions containing a methyl group (acetate, pyruvate) is also very low. This has been attributed to their inability to participate in hydrogen bonding [13]. The small F^- ion shows saturation of voltage-dependent conductance already at about 150 mV. The larger molecules with three C-atoms show measurable conductance at high voltage. Both data suggest that the pore size of the molecular sieve increases with increasing voltage. Colicin shows only a fairly good selectivity for chloride over potassium at low pH and allows for the passage of molecules larger than gluconate.

The channel histograms of Fig. 4 can be well matched by a Poisson distribution, indicating independent openings of channels. This could be a contradiction to the nonstatistical opening behavior of the channels which most often open in groups. However, this behavior probably is caused by opening bursts of *single* molecules from a favored closed state. Therefore, the channels can adopt at least two closed states, one with short closed times and one with low open probability as an inactive state.

Interaction of the CFA with Other Molecules

Cholesterol has been shown to be necessary for channel formation as for membrane proteins like the acetylcholine receptor [7] and for simple toxin molecules like amphotericin B [11]. The chloride channel we have described here obviously does not interact with cholesterol. The significance of other lipids, especially charged lipids remains to be tested.

The competitive blocking of the channel by IAA-94 is observed only when the blocker is added at the *trans* side. This is not surprising, since also chloride can enter the channel only from this side. IAA-94 reduces chloride conductance of mouse muscle cells only from the cytoplasmic side (E. Wischmeyer & H. Jockusch, *personal communication*). In contrast, we have shown that protease K is only effective from the *cis* side.

As channels are open at positive voltage on the side of insertion it is entirely possible that the CFA could be harmful to plant cells by energy depletion as has been proposed for colicin channels [18], which also open at positive membrane voltage. Regarding the unique gating and ion selectivity of this CFA the purified molecule might become an interesting model system for voltage-dependent anion channels.

Furthermore, it may be interesting to elucidate which role the CFA plays in the pathogenic interaction of *C. m. ssp. nebraskense* with its host plant.

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[3] As NO_3^- has a planar structure, the smallest opening that can pass such a molecule must have the form of a slot. Whereas a minimal pore size is a condition *sine qua non,* molecular interactions of the permeant ion inside the pore determine the transport rate. In the present case, e.g., one could imagine that NO_3^- can form hydrogen bonds at the small ends of the slot which are too far away for chloride. In the case of the sodium channel it has been supposed that a water molecule fits into the residual area not occupied by the alkali ions.

[4] It should be noted that the relative conductance as measured by bi-ionic potentials could be higher for F^- than for Cl^- or NO_3^-. *See also* footnote 2.

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