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ARTICLE

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Channel activity of a phytotoxin of *Clavibacter michiganense* ssp. *nebraskense* in tethered membranes

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Abstract Solid-supported membranes immobilized on gold electrodes were used to detect and characterize the spontaneously inserting anion-selective protein channel (*Clavibacter* anion channel, CAC) present in the culture fluid of *Clavibacter michiganense* ssp. *nebraskense*. Three different membrane systems varying in the composition of the first chemisorbed monolayer were investigated by means of impedance spectroscopy. Conductance changes of the immobilized lipid membranes were sensitively detected after adding the culture fluid of the bacteria to the solid-supported membranes, indicating that the relative change in conductance is largest if the lipid layer is attached to the surface via a flexible lipid anchor. Variation in the d.c. potential revealed that CAC exhibits a voltage dependence in these tethered membranes which can be described by an exponential function in accordance with previous results obtained from patchclamp measurements and impedance analysis. The addition of an inhibitor that selectively blocks anion channels abolished the channel conductance almost completely, indicating that the increased conductivity can be attributed to the specific insertion of the CAC. A linear dependence of the channel conductance on the chloride concentration was found, which was modulated by the charges of the second lipid monolayer. The results demonstrate that tethered lipid membranes on gold surfaces in conjunction with impedance spectroscopy allows one to monitor and characterize water-soluble spontaneously inserting channels, providing an effective means to probe for bacterial toxins.

Keywords Bacterial toxin · Biosensor · Impedance spectroscopy · Protein channel · Solid-supported bilayers

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Introduction

Lipid bilayers have been used extensively to study the structure and function of biological membranes with regard to adhesion, fusion, molecular recognition events and ion transport (Lipowsky and Sackmann 1995; Rosoff 1996; Tien and Ottova 1998; Tien and Ottova-Leitmannova 2000). Though classical planarlipid bilayers are well suited for studying transport phenomena across lipid membranes, they suffer from limited long-term stability and are not applicable to the broad range of surface-sensitive methods developed in recent years for the characterization of ultrathin supported films. To overcome these major drawbacks, certain attempts have been made to immobilize lipid bilayers on solid supports. Nowadays, there are basically three different architectures of supported lipid bilayers available (Heyse et al. 1998): (1) floating bilayers on a hydrophilic surface, (2) hybrid membranes composed of a phospholipid monolayer on a hydrophobic support, and (3) covalently anchored bilayers on hydrophilic surfaces. On hydrophilic oxidized silicon or glass, a water layer with a thickness of approximately 1 nm is formed, which allows for free diffusion of phospholipid molecules within the bilayer (Bayerl and Bloom 1990; McConnell et al. 1986). However, applying electrochemical methods requires metal surfaces, on which lipid mobility is reported to be suppressed (Groves et al. 1997, 1998). To preserve the membrane's natural properties on metal surfaces, the lipid bilayer can be immobilized on a hydrophilic polyelectrolyte cushion that can act as a deformable and mobile substrate (Sackmann 1996), or tethered lipid membranes can be formed which are solid-supported lipid bilayers withhydrophilic spacer groups covalently linked to the support via Au-S, Si-O or Si-O-Si groups (Boden et al. 1998; Lang et al. 1994; Raguse et al. 1998). These systems have been shownto act as fluid lipid bilayers, possessing an aqueous compartment separating the membrane and the support. They enable one to incorporate ionophores such as valinomycin, alamethicin

and gramicidin (Cornell et al. 1997; Jenkins et al. 1998; Steinem et al. 1997a, 1998; Stora et al. 1999). Some examples are also given for a successful incorporation of complex proteins. For example, Salamon et al. (Salamon and Tollin 1996; Salamon et al. 1993) managed to insert rhodopsin and cytochrome *c* oxidase; Naumann et al. (1995, 1997, 1999) incorporated the F_0F-ATPase from chloroplasts and cytochrome *c* oxidase in peptide-tethered lipid bilayers; and Steinem et al. (1997b) inserted bacteriorhodopsin into lipid bilayers on gold surfaces. More difficult, however, is the insertion of a channel-active protein such as a bacterial toxin into solid-supported membranes after formation of the lipid bilayer by adding it from the aqueous phase.

Here, we describe the successful insertion and thorough characterization of an anion channel acting as a phytotoxin from the bacterium *Clavibacter michiganense* ssp. *nebraskense* (Schürholz et al. 1991, 1993) in solid-supported lipid bilayers by means of impedance spectroscopy. The *Corynebacterium Clavibacter michiganense* ssp. *nebraskense* causes Goss' wilt and blight in *Zea mais* (Metzler et al. 1997; Wysong et al. 1973). Members of the genus *Clavibacter* have been described to produce phytotoxins, which were classified as high molecular mass polysaccharides (*Clavibacter michiganense* ssp. *michiganense*) (Rai and Strobel 1968) or glycolipids (*Clavibacter rahtayi*) (Vogel et al. 1982). It is established that the isolated polysaccharides cause wilting in plant assays and degeneration of chloroplasts, which is discussed in terms of water stress (Krämer and Leistner 1986; Van Alfen and McMillian 1982). However, the presence of polysaccharides does not explain the discrete action on chloroplasts and their membranes. Schürholz et al. (1991, 1993) systematically searched for toxic activities excreted into the culture medium of *Clavibacter michiganense* ssp. *nebraskense* and identified a membrane-active component that forms anion-selective channels in planar lipid bilayers. By voltage clamp measurements they found that the channel exhibits anion selectivity ($Cl^- > F^- > SCN^- > I^- > C_2O_4^{2-} > SO_4^{2-}$) and that channel activity can be removed by protease treatment, anion-specific channel inhibitors such as indanyloxyacetic acid (IAA-94) and OH^- ions (pH > 10). The relative molecular mass of the *Clavibacter* anion channel (CAC) was determined to be 25 kDa by functional reconstitution of the channel protein from SDS gels.

Different hybrid membranes composed of a phospholipid monolayer deposited on a hydrophobic thiol monolayer immobilized on electrically conducting gold electrodes have been employed to incorporate the CAC from the aqueous phase into these preformed bilayers. Changes in the membrane's conductance were followed by impedance spectroscopy (Gritsch et al. 1998; Hillebrandt et al. 1999; Jenkins et al. 1999; Raguse et al. 1998; Steinem et al. 1996, 1998) while applying a d.c. potential across the bilayer, indicating that the conductance change is largest if the lipid layer is attached to the gold surface via a flexible lipid anchor.

Materials and methods

Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dimyristoyl-*sn*-glycero-3-phosphothioethanol (DMPTE) were purchased from Avanti Polar Lipid (Alabaster, USA). Asolectin, diphenylamine-2-carboxylic acid (DPC) and octanethiol were from Fluka (Neu-Ulm, Germany). The strain *Clavibacter michiganense* ssp. *nebraskense* (NCPPB 2581) was obtained from the National Collection of Plant Pathogenic Bacteria, Hatching Green, Harpenden, UK. Purification of the CAC was performed as described by Schürholz et al. (1991). The gold used forthe working electrodes was a generous gift from Degussa (Hanau, Germany). The chromium was from Balzers (Balzers, Liechtenstein). Hellmanex used to clean the glass slides was purchased from Hellma (Mülheim, Germany). The reference electrode InLab 302 was cobtained from Mettler Toledo (Steinbach, Germany).

Methods

Impedance analysis

The electrochemical cell consisted of a gold working electrode with an area of 0.13 cm^2 deposited on a glass slide, a counter electrode (platinized platinum wire), and an Ag/AgCl reference electrode including a salt bridge containing potassium sulfate as electrolyte. Potassium sulfate was chosen in order to avoid conductance changes due to artifacts arising from an increase in electrolyte concentration during the experiments. It is known that the conductance of CAC for sulfate anions is negligibly small and the transfer numbers of potassium sulfate are approximately 0.5. Gold coating was performed in an evaporation unit (E 306, Edwards, UK). After applying a layer of chromium (10–20 nm) to improve the adhesion of gold, the gold layer was deposited subsequently with a final thickness of about 200 nm.

A.c. impedance analysis was conducted using a lock-in amplifier model 5210 and a potentiostat/galvanostat model 263A from EG&G (Princeton, USA). Impedance spectra were recorded in a frequency range of 10^{-1}–10^5 Hz with an a.c. amplitude of 10 mV (r.m.s.) and a d.c. offset potential between 0 and 200 mV. Data analysis was performed by means of a weighted non-linear least-square fit based on the Levenberg-Marquardt algorithm.

Preparation of solid-supported membranes (SSMs)

Immediately before use, the gold electrodes were exposed to an argon plasma with high energy (plasma cleaner, Harrick, USA) for 5 min. The precleaned gold electrodes were incubated in the electrochemical cell with (1) a 1 mM ethanolic solution of octanethiol for 30 min, (2) a 1 mM ethanolic solution of DMPTE for 10 min or (3) a 1 mM ethanolic spacerlipid A solution for 24 h (Fig. 1). Afterwards, the electrodes were thoroughly rinsed, first with ethanol and finally with buffer solution. Vesicles of asolectin and POPC with a nominal diameter of 100 nm were prepared by the extrusion method in the same buffer (MacDonald et al. 1991). The second monolayer was obtained by fusing these large unilamellar vesicles (1 mg/mL) on the preformed hydrophobic monolayer for 30 min. Remaining vesicles were removed by rinsing several times with buffer solution.

Insertion of the *Clavibacter* anion channel into preformed SSMs

Incorporation of the *Clavibacter* anion channel into preformed lipid bilayers immobilized on gold surfaces was obtained by adding an aliquot of the culture medium of *Corynebacterium Clavibacter michiganense* ssp. *nebraskense* containing a defined

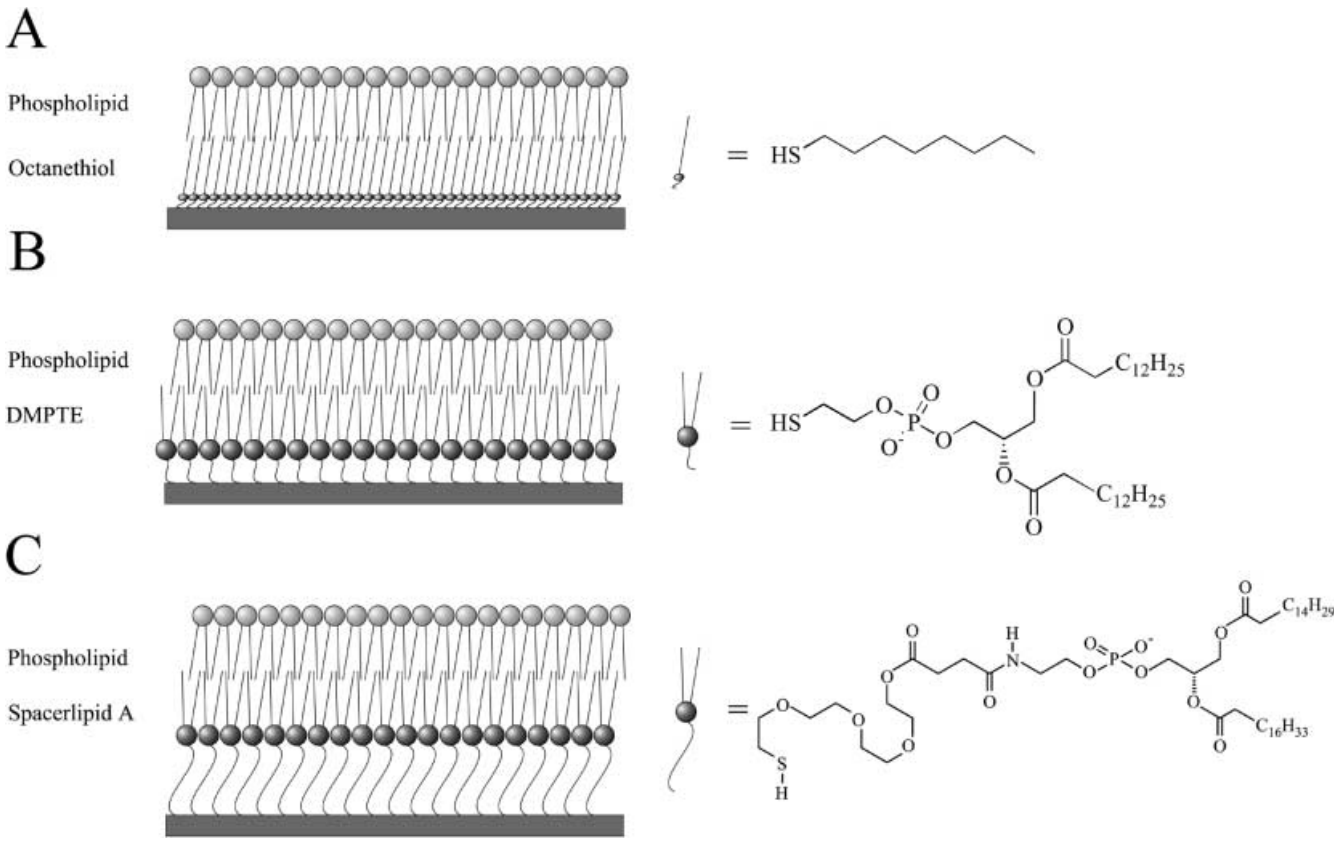

Fig. 1A–C Schematic representation of the three different lipid bilayer systems immobilized on gold surfaces used in this study. **A** A bilayer composed of an octanethiolmonolayer was formed by a self-assembly process, on which a second phospholipid monolayer was deposited by vesicle fusion. **B** Bilayer composed of 1,2-dimyristoyl-*sn*-glycero-3-phosphothioethanol (DMPTE) and a phospholipid. **C** A bilayer composed of spacerlipid A and a phospholipid

concentration of CAC to the aqueous phase under an applied d.c. potential of 50 mV. The incubation time was 15 min. If not indicated otherwise, 0.625 μg CAC were added to a total volume of 4.5 mL.

Results

Electrical properties of SSMs on gold surfaces

By means of impedance analysis, characteristic electrical parameters of the first chemisorbed monolayer and a lipid bilayer composed of the first monolayer and a second phospholipid monolayer subsequently fused onto the first one can be gathered. Three different thiol-bearing molecules were chemisorbed onto gold: octanethiol, DMPTE and spacerlipid A (Fig. 1). Octanethiol is known to form an almost defect-free crystalline monolayer on gold within minutes (Ulman 1996). The equivalent circuit representing an insulating lipid film is generally composed of a simple parallel RC circuit with a resistance R_m and a capacitance C_m. However, impedance spectra of alkanethiols can be described only by a capacitance C_m representing the thiol monolayer[1] in series with an Ohmic resistance R_e representing the bulk resistance and the wire connections (Fig. 2A). By fitting the equivalent circuit shown in Fig. 2A to the data, the capacitance of the octanethiol monolayer can be extracted from the impedance spectra (see Table 1). The chemisorption process of the thiol-bearing phospholipid DMPTE was limited to 10 min to generate defects within the first monolayer, in order to facilitate the subsequent incorporation of the CAC. In this case, the obtained impedance spectra were interpreted in terms of the equivalent circuit depicted in Fig. 2B, giving access to the membrane capacitance C_m and membrane resistance R_m. Due to the lower membrane resistance the capacitance of the electrochemical double layer C_{Au}, can be separated from the monolayer capacitance C_m. The capacitance of the DMPTE monolayer obtained after 10 min exposure of a gold surface to a 1 mM ethanolic DMPTE solution was determined to be 2.9 ± 0.5 $\mu F/cm^2$, which is rather large compared to that of a defectless DMPTE monolayer which exhibits a typical capacitance of 1.3 ± 0.1 $\mu F/cm^2$. The increased capacitance can be explained in terms of an increase in the mean dielectric constant due to a larger amount of water ($\epsilon_r \approx 80$) within the lipid monolayer ($\epsilon_r \approx 2$–3). Impedance

[1] If R_m is too high to be determined in the observed frequency range, the equivalent circuit in Fig. 2B simplifies to that shown in Fig. 2A. In this case, C_m and C_{Au} cannot be distinguished. Since C_m is smaller than C_{Au} by a factor of approximately 10, the obtained capacitance can be approximated with C_m

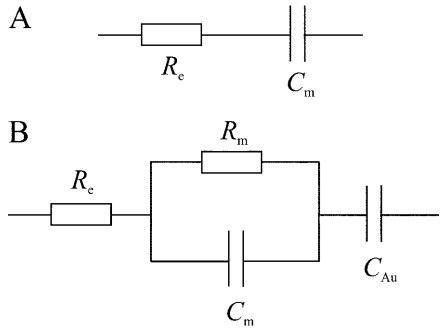

Fig. 2A, B Equivalent circuits representing the electrical behavior of the lipid films on gold surfaces. **A** Equivalent circuit representing an octanethiol monolayer and an octanethiol/phospholipid bilayer on gold. C_m represents the film capacitance and R_e the Ohmic resistance of the bulk solution and the wire connections. **B** Equivalent circuit representing a DMPTE or spacerlipid A monolayer and the corresponding lipid bilayers immobilized on gold surfaces. R_m represents the lipid layer resistance and C_{Au} accounts for the capacitance of the electrochemical double layer. For further details, see text

Table 1 Electrical parameters of the three different solid-supported lipid membrane systems. The data were obtained from fitting the parameters of the corresponding equivalent circuits shown in Figure 2 to the impedance data. The measurements ($n = 5$–7) were performed in 20 mM Tris, 100 mM NaCl, pH 7.0

Membrane system	Capacitance C_m (μF/cm^2)	Resistance R_m (Ω cm^2)
Octanethiol monolayer	2.4 ± 0.4	–
Octanethiol/asolectin bilayer	1.1 ± 0.3	–
Asolectin monolayer	2.0 ± 0.8	–
DMPTE monolayer	2.9 ± 0.5	2000–8000
DMPTE/asolectin bilayer	1.3 ± 0.5	100,000–600,000
Asolectin monolayer	2.3 ± 1.2	–
Spacerlipid A monolayer[a]	1.8 ± 0.3	3000–20,000
Spacerlipid A/asolectin bilayer	0.98 ± 0.06	80,000–300,000
Asolectin monolayer	2.1 ± 0.4	–
Spacerlipid A/POPC bilayer[a]	0.79 ± 0.05	20,000–30,000
POPC monolayer	1.4 ± 0.3	–

[a]Impedance analysis of spacerlipid A mono- and bilayers composed of spacerlipid A and a phospholipid were performed in 20 mM Tris, pH 7.0, without NaCl

spectra of spacerlipid A monolayers were taken in 20 mM Tris, pH 7.0, without addition of sodium chloride and evaluated using the equivalent circuit described above (Fig. 2B). A mean capacitance of 1.8 ± 0.3 μF/cm^2 was obtained. The results are summarized in Table 1. After formation of the hydrophobic monolayers, asolectin vesicles were fused to form the second leaflet and impedance spectra were again taken. Data evaluation was conducted using the same equivalent circuits as for the corresponding monolayers (Fig. 2B). In this case, C_m represents the capacitance and R_m the resistance of the lipid bilayer. Assuming a series connection of the capacitances of the two monolayers allows calculation of the specific capacitance of the second monolayer consisting of asolectin, which was determined to be 2.1 ± 0.4 μF/cm^2. The capacitance values for asolectin monolayers were independent of the first monolayer, though owing to defects in the DMPTE monolayer the formation of a second monolayer was less reproducible, leading to a larger standard deviation (see Table 1). Besides spacerlipid A/asolectin bilayers, we also investigated spacerlipid A/POPC bilayers by means of impedance spectroscopy. Capacitance values for POPC monolayers were determined to be 1.4 ± 0.3 μF/cm^2, smaller than the corresponding asolectin monolayers (Table 1).

Impedance analysis of SSMs after incorporation of the CAC

According to Schürholz et al. (1993), the CAC spontaneously inserts into planar lipid membranes when culture fluid of this species is added to the aqueous phase. Our major objective was to detect the insertion of the CAC from the aqueous phase into solid-supported lipid bilayers immobilized on gold surfaces by its channel activity and thoroughly characterize the electrical parameters of this system. Preformed SSMs were incubated with the culture fluid containing CAC while applying a d.c. potential of 50 mV. After 15 min, impedance spectra were recorded at a d.c. potential of 50 mV and membrane-specific parameters were extracted using the corresponding equivalent circuits shown in Fig. 2. For octanethiol/asolectin bilayers measured in 20 mM Tris, 100 mM NaCl, pH 7.0, the addition of CAC to the aqueous solution did not alter the electrical parameters of the lipid layer. In the case of DMPTE/asolectin bilayers measured in 20 mM Tris, 100 mM NaCl, pH 7.0, a slight decrease in membrane resistance was observed from 425 kΩ cm^2 to 394 kΩ cm^2, which corresponds to a decrease of 16%. The largest increase in membrane conductance, however, was recorded for spacerlipid A/asolectin bilayers measured in 20 mM Tris, 20 mM NaCl, pH 7.0, exhibiting the lowest overall resistance. Upon addition of CAC to the aqueous phase, the membrane resistance decreased from 39.2 kΩ cm^2 to 26.5 kΩ cm^2, which is a decrease of 32%. A considerably larger decrease in R_m was obtained by applying a d.c. potential of 200 mV, yielding a decrease of the membrane resistance of 53% (Fig. 3). The membrane capacitance slightly decreases upon addition of the CAC, indicating that its insertion is not accompanied by formation of defects or rupture of the membrane, which would rather result in an increased capacitance due to an increased mean dielectric constant.

Voltage dependence

To investigate the change in membrane conductance in more detail, we applied different potentials prior and after incubation of the membrane system with CAC. The effect of an applied d.c. potential on the electrical parameters of immobilized spacerlipid A/asolectin

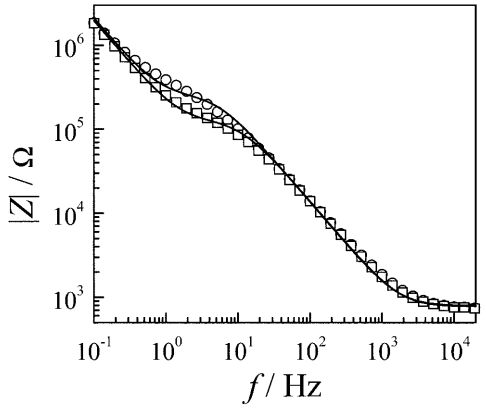

Fig. 3 Impedance spectra ($|Z|$ versus f) of a spacerlipid A/asolectin bilayer obtained at an applied d.c. potential of 200 mV before (○) and after addition of CAC (❑) in 20 mM Tris, 20 mM NaCl, pH 7.0. The most significant change in impedance is observed in the frequency range of 0.5–10 Hz. The *solid lines* are the results of the fitting procedure using the equivalent circuit depicted in Fig. 2B: (○) $C_m = 1.08$ μF cm^{-2}; $R_m = 29{,}700$ Ω cm^2, (❑) $C_m = 1.03$ μF cm^{-2}; $R_m = 14{,}020$ Ω cm^2. The capacitance C_{Au} was fixed at 6.2 μF/cm^2 during the fitting routines

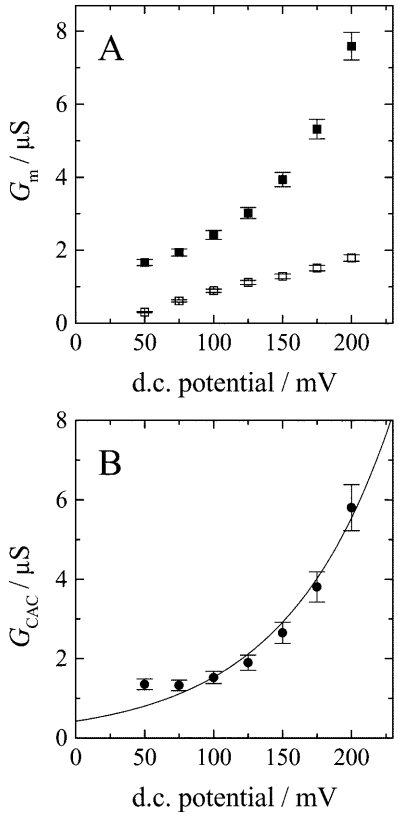

Fig. 4 **A** Conductivity G_m of a spacerlipid A/asolectin bilayer dependent on the applied d.c. potential in the absence (❑) and presence (■) of CAC. **B** (●) Conductivity G_{CAC} obtained according to Eq. (1) versus the applied d.c. potential. The *solid line* is the result of fitting the parameters of Eq. (2) to the data with the following results: $G_{CAC,0} = 0.5 \pm 0.1$ μS and $b = 13 \pm 1.3$ V^{-1}

bilayers was investigated by impedance spectroscopy up to values of 200 mV in 20 mM Tris, 20 mM NaCl, pH 7.0. Fitting the parameters of the equivalent circuit shown in Fig. 2B to the data revealed that C_m (data not shown) remains constant up to a potential of 200 mV, whereas $G_m = 1/R_m$ increases linearly (Fig. 4A). However, after adding the CAC to the lipid bilayer the membrane conductance G_m increases exponentially with increasing potential, indicative of the formation of voltage-dependent ion channels. Figure 4A depicts the conductivity G_m versus the applied d.c. potential. To solely account for changes in conductivity induced by CAC, we defined the conductance of CAC (G_{CAC}) as:

$$G_{CAC}(x) = \left(G_{m-1}(x) - G_{m-1,0}\right) - \left(G_{m-2}(x) - G_{m-2,0}\right) \quad (1)$$

where G_{m-1} and G_{m-2} are the conductances of the bilayers in the presence and absence of CAC, dependent on the varied parameter x. $G_{m-1,0}$ and $G_{m-2,0}$ are the conductances of the tethered lipid bilayers before addition of CAC to account for variations in R_m of different bilayer preparations. Each conductance was determined by fitting R_m to the corresponding impedance spectrum. Calculating G_{CAC} according to Eq. (1) leads to the plot shown in Fig. 4B. The obtained voltage-dependent conductance can be formally described by an exponential function:

$$G_{CAC}(V) = G_{CAC,0}\exp(bV) \quad (2)$$

where $G_{CAC,0}$ is the conductance at $V = 0$ V and b is a constant. Fitting Eq. (2) to the data results in a conductance $G_{CAC,0} = 0.5 \pm 0.1$ μS at zero d.c. potential with $b = 13.0 \pm 1.3$ V^{-1}.

Selective blocking of the channel activity by DPC

To ensure that the increased conductivity is due to the specific transport of chloride ions across the lipid bilayer facilitated by CAC, the channel activity of the protein was blocked by an anion-selective channel inhibitor. Recently, we found that the *Clavibacter* anion channel can be inactivated by DPC, which was shown to inhibit single-channel activity when added to the *cis* side (Michalke et al. 2001). DPC is soluble in buffer up to a concentration of 0.24 mM, as determined by UV-Vis spectroscopy. In the experiments, DPC was adjusted to a final concentration of 0.14 mM, which is above its IC$_{50}$[2] of 0.1 mM. The conductance G_{CAC} was monitored with and without DPC dependent on the applied d.c. potential (Fig. 5). The addition of DPC results in an almost complete loss of channel activity. This result rules out that the increase in membrane conductance is caused by non-specific defects, which are formed due to the incubation of the solid-supported membrane with the culture fluid of *Clavibacter michiganense* ssp. *nebraskense*.

[2]The IC$_{50}$ (inhibitory concentration 50%) is the concentration required for 50% inhibition

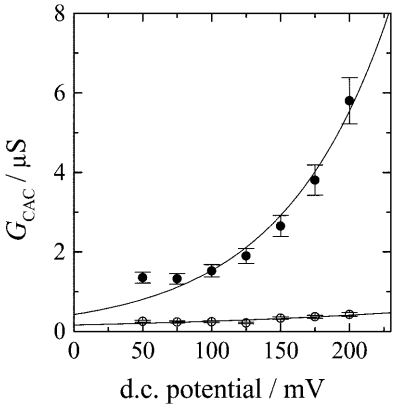

Fig. 5 Conductivity G_{CAC} dependent on the applied d.c. potential; (●) represents the data obtained in the presence of CAC and (○) those in the presence of CAC together with 0.14 mM of the anion channel inhibitor diphenylamine-2-carboxylic acid (DPC). The experiments were performed in 20 mM Tris, 20 mM NaCl, pH 7.0. The *solid lines* are the results of fitting the parameters of Eq. (2) to the data with the following results: (●) $G_{CAC,0} = 0.5 \pm 0.1$ µS and $b = 13.0 \pm 1.3$ V^{-1}; (○) $G_{CAC,0} = 0.16 \pm 0.03$ µS and $b = 4.6 \pm 0.9$ V^{-1}

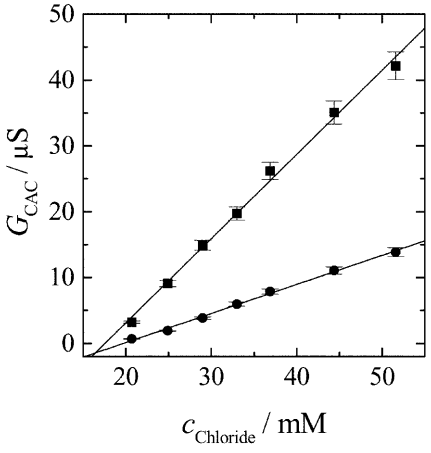

Fig. 6 Conductivity G_{CAC} obtained according to Eq. (1) versus the chloride concentration in solution. A d.c. potential of 50 mV was applied. The *solid lines* are the results of linear regressions with $m = 0.44 \pm 0.01$ µS mM^{-1} for the spacerlipid A/asolectin (●) and $m = 1.28 \pm 0.04$ µS mM^{-1} for the spacerlipid A/POPC bilayer (■)

Variation of the chloride concentration

To further support the experiments indicating that CAC is incorporated into solid-supported spacerlipid A/asolectin bilayers, we investigated the influence of increasing chloride concentrations on the conductance of the lipid bilayer. If CAC forms anion-selective channels within the lipid bilayer, an increase in conductance is expected with increasing chloride concentration in solution. Since the membrane resistance itself is sensitive to changes in ion concentration, R_m was monitored in the absence and presence of CAC at a constant d.c. potential of 50 mV. A linear increase in conductance was observed for both the lipid bilayer in the absence and presence of CAC. However, the slope is considerably steeper if CAC is present. Calculating G_{CAC} according to Eq. (1) results in a linear increase in conductance with increasing chloride concentration, with a slope of $m = 0.44 \pm 0.01$ µS mM^{-1} (Fig. 6).

Since the chloride concentration at the membrane interface is influenced by the charge density of the lipid layer (Bard and Faulkner 1980), we hypothesized that changing the second leaflet of the bilayer exposed to the aqueous phase from asolectin, which is a mixture of different lipids in which part of them are negatively charged, to a neat zwitterionic phospholipid such as POPC shouldresult in an increased slope of G_{CAC} versus the bulk chloride concentration. Indeed, the slope for a spacerlipid A/POPC bilayer was determined to be $m = 1.28 \pm 0.04$ µS mM^{-1}, which is by a factor of three larger than that of a spacerlipid A/asolectin bilayer.

Variation of the CAC concentration

In the next set of experiments, we investigated the influence of different CAC concentrations in solution on the electrical parameters of the two lipid membrane systems, spacerlipid A/asolectin and spacerlipid A/POPC. From the fact that CAC spontaneously inserts into lipid bilayers after being added to the aqueous phase, we conclude that the channel partitions between the water and the membrane phase. Hence, an increased number of incorporated channels canbe obtained if the CAC concentration in solution is raised. Bilayers were incubated with different amounts of CAC in the aqueous phase for 15 min at 50 mV d.c. potential. Impedance measurements were performed at different sodium chloride concentrations and the conductance G_{CAC} was calculated from the obtained impedance spectra (Fig. 7). The conductivity G_{CAC} depends on the CAC concentration in a linear fashion in the observed concentration range for both lipid systems. Besides changes in membrane conductance, a slight decrease in membrane capacitance was observed upon increasing the CAC concentration,independent of the lipid composition. Incubating the tethered membranes with a CAC-amount of 1.25 µg results in a decrease of C_m by 7%.

Comparing the changes in conductivity versus the amount of channel protein in the aqueous solution of spacerlipid A/asolectin and spacerlipid A/POPC again demonstrates that for POPC, as the second monolayer, the slope is slightly steeper than for asolectin. Since both lipids, asolectin and POPC, are in the fluid state at room temperature and partition of the protein in the membrane is assumed to be mainly driven by hydrophobic interactions, it can be assumed that the partition coefficient of CAC is similar for both membrane systems. However, one cannot rule out that, due to the different surface charge densities of the lipid bilayers, the partition coefficient is modulated by the surface potential. Another explanation for the different slopes might be again the different local chloride concentrations at the membrane's interface.

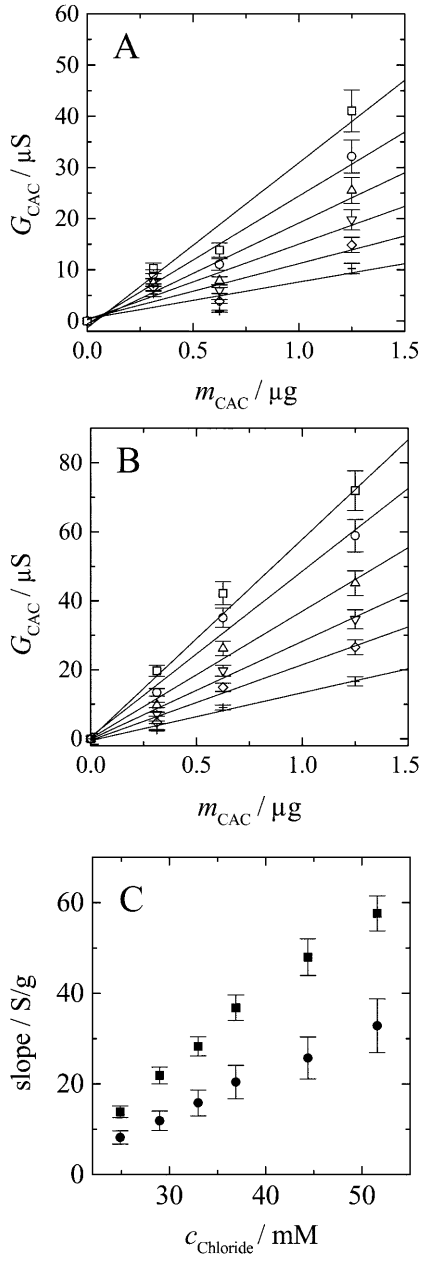

Fig. 7 **A** Conductivity G_{CAC} of a spacerlipid A/asolectin and **B** spacerlipid A/POPC bilayer dependent on the amount of CAC in solution. Different chloride concentrations were used for each set of experiments. (+) 24.9 mM, (◇) 27.9 mM, (▽) 33.0 mM, (△) 36.9 mM, (○) 44.4 mM, and (□) 51.6 mM. The applied d.c. potential was set to 50 mV. The *solid lines* are linear regressions, showing that the conductance depends linearly on the CAC concentration in solution in the investigated concentration range. **C** Comparison of the slopes obtained from **A** and **B** for different chloride concentrations: (●) spacerlipid A/asolectin and (■) spacerlipid A/POPC bilayer

Discussion

The first report from Schürholz et al. (1991, 1993) on the electrical properties of an anion channel from *Clavibacter michiganense* ssp. *nebraskense* provided results of voltage clamp experiments. This technique delivers conductances of single channels, its lifetime, and the average number of open channels. In contrast to voltage clamp experiments, impedance spectroscopy provides an integral value for channel activity as a change in membrane conductance G_m that is affected by all of the above-mentioned characteristic parameters. The significant increase in membrane conductance upon addition of CAC indicates a successful insertion of the channel into solid-supported membranes. In order to solely account for the conductance induced by CAC, the conductance G_{CAC} was extracted from G_m. By monitoring the conductance of the lipid layers by impedance spectroscopy, it was shown that the fluidity and flexibility of the immobilized lipid bilayer is of paramount importance for a successful incorporation of the CAC exhibiting channel activity. While the specific conductance of octanethiol/asolectin bilayers was not affected by the addition of CAC, a slight increase was observed in the case of DMPTE/asolectin bilayers. The most pronounced increase in membrane conductance was monitored for lipid bilayers composed of spacerlipid A/asolectin bilayers, which were thought to exhibit the largest flexibility due to the hydrophilic ethylene glycol spacer of the first monolayer. It is known that the insertion of proteins requires a deformable lipid membrane (Bretscher and Munro 1993), which can only be achieved on a solid support if the distance between the solid substrate and the lipid layer is at least some Ångstroms and the membrane is in the fluid state.

To further corroborate the channel activity of CAC in these tethered lipid membranes, the voltage dependence of CAC was investigated. From single-channel analysis it is known that the single-channel conductance increases exponentially with voltages up to 200 mV, saturating at 250 mV, and the channels are closed at negative potential relative to the side of insertion. The average number of open channels also increases with the applied potential. We assumed that the channel conductance G_{CAC} versus the applied potential – though an integral value – also follows an exponential function, which was supported by the results of impedance analysis.

In order to rule out that the increased membrane conductance is due to the formation of defects induced by incubating the lipid layers with the culture fluid of *Clavibacter michiganense* ssp. *nebranskense*, we showed that channel activity can be blocked by DPC, an anion channel inhibitor acting on the *cis*, water-exposed, side. Since the channel conductance is almost zero and does not show the characteristic exponential dependence on the applied potential, we conclude that the change in membrane conductance can be specifically attributed to the insertion of the protein channel.

In contrast to the results obtained by Schürholz et al. (1993), who found a Michaelis-Menten type relationship (Eq. 3) for the dependence of the single-channel conductance on the chloride concentration in solution:

$$G(a) = G_{\max} \frac{a}{K_{0.5} + a} \quad (3)$$

our results revealed a linear dependence on the anion concentration in solution. $G_{\max}$ is defined as the maximum conductance at infinitely high salt concentration, a the activity of the anion, and $K_{0.5}$ is the activity at which the conductance reaches half the maximum value. However, taking into account that the highest investigated chloride concentration was by a factor of 50 smaller than that used in Schürholz's experiments (Schürholz et al. 1993), this apparent discrepancy can be easily explained. Due to the low salt concentration the activity a can be neglected in the denominator of Eq. (3), leading to a linear relation of G_{CAC} on the chloride concentration. Nevertheless, one should keep in mind that single-channel conductance is compared with an integral conductance comprising single-channel conductance, lifetime and open state probabilities.

The increase of the CAC concentration in the aqueous phase leads to an increased channel conductance G_{CAC}, revealing that the channel protein partitions spontaneously between the aqueous and the membrane phase. Notably, the membrane capacitance was slightly decreased upon addition of higher CAC concentrations, indicating that the insertion of the channel-forming compound does not generate membrane defects, which would rather result in an increased membrane capacitance than a smaller value for C_{m}. The origin of a slightly decreased membrane capacitance is speculative. It is conceivable that the channel preferentially inserts into membrane defects; thus the overall number of defects is decreased, leading to a decreased overall dielectric constant as water exhibiting a dielectric constant of $\epsilon_{\mathrm{r}} \approx 80$ is removed from the bilayer membrane. It is also possible that the membrane thickness is increased upon insertion of the channel owing to lipid-protein mismatches (Killian 1998) or partial adsorption of the proteinat the lipid interface.

Dependent on the chloride concentration, two different lipid systems, spacerlipid A/asolectin and spacerlipid A/POPC, were compared, showing that G_{CAC} is larger in spacerlipid A/POPC membranes than in spacerlipid A/asolectin membranes. Since both systems are supposed to be in the fluid state, the differences might not be attributed to differences in the incorporation rate of the channel protein. It is also conceivable that the negative charges of the asolectin (asolectin is composed of phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol in a ratio of 1:1:1) monolayer influences the interfacial chloride concentration, leading to a decreased apparent channel activity. That the protein activity is influenced by the lipid composition due to an accumulation or reejection of specifically transported ions was also reported for bacteriorhodopsin (Alexiev et al. 1994; Steinem et al. 1997b).

Conclusion

This work describes the successful combination of tethered lipid bilayers on gold surfaces with electrochemical analysis tools for the detection and thorough characterization of a protein channel excreted into the culture fluid of *Clavibacter michiganense* ssp. *nebraskense*. As shown by impedance spectroscopy, which allows for the determination of capacitance and conductance values of the membrane system in one single experiment, the flexibility of the lipid membrane is of paramount importance for a successful insertion of a water-soluble protein from the aqueous phase. Due to its long-term stability, inserted channel proteins can be thoroughly characterized and be used for a long period of time. The detection of toxic channel activity in bacterial fluids by means of solid-supported membranes combined with impedance spectroscopy might also be a promising step for the development of new biosensor devices scanning a solution with unknown composition for toxic ingredients.

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