Evidence for a small anion-selective channel in the cell wall of *Mycobacterium bovis* BCG besides a wide cation-selective pore

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Abstract **Two channels were observed in extracts of whole *Mycobacterium bovis* BCG cells using organic solvents and detergents. The channels derived from organic solvent treatment had a single-channel conductance of about 4.0 nS in 1 M KCl in lipid bilayer membranes with properties similar to those of the channels discovered previously in *Mycobacterium smegmatis* and *Mycobacterium chelonae*. The channel was in its open configuration only at low transmembrane potentials. At higher voltages it switched to closed states that were almost impermeable for ions. Lipid bilayer experiments in the presence of detergent extracts of whole cells revealed another channel with a single-channel conductance of only 780 pS in 1 M KCl. Our results indicate that the mycolic acid layer of *M. bovis* BCG contains two channels, one is cation-selective and its permeability properties can be finely controlled by cell wall asymmetry or potentials. The other one is anion-selective, has a rather small single-channel conductance and is voltage-insensitive. The concentration of channel-forming proteins in the cell wall seems to be small, which is in agreement with the low cell wall permeability for hydrophilic solutes.**

Key words: Cell wall; Channel; Mycobacterial porin; Mycolic acid; Lipid bilayer membrane; *Mycobacterium bovis* BCG

1. Introduction

The last decade has seen a notable increase in the incidence of mycobacterial infections, particularly tuberculosis, as a result of the HIV/AIDS pandemic, and social factors such as poverty, alcoholism, large scale displacement of populations and the decline of disease control programmes [1–3]. Of particular concern is the increased prevalence of multidrug-resistant *Mycobacterium tuberculosis* strains and the lack of effective therapy to treat them [4–6]. Only a limited number of antimicrobial agents can be used for treatment of mycobacterial infections because mycobacteria are naturally resistant to a wide range of antibiotics [2,7–9]. The low permeability of the mycobacterial cell wall has been shown to play a major role in natural resistance to antibiotics [10–12]. It contains a large amount of long hydrocarbon chain lipids that form a hydrophobic layer and acts as a permeability barrier toward hydrophilic compounds [13]. The main lipids are mycolic acids that are covalently linked to arabinogalactan molecules that are esterified to mycolic acids (high molecular weight α-alkyl, β-hydroxy fatty acids). The chain length of these 2-branched, 3-hydroxylated fatty acids varies considerably within the mycolic acid-containing taxa and especially long mycolic acids have been found in mycobacteria (60–90 carbon atoms) [14,15]. Other complex lipids are also found in the cell wall but are not covalently linked. These lipids show a large variability of composition and polarity. It is believed that the mycolic acid layer and other free lipids are responsible for the permeability properties of the mycobacterial cell wall [16].

The permeability of the cell wall has been studied in *Mycobacterium chelonae* and *Mycobacterium smegmatis* [17,18]. The permeability coefficients for small hydrophilic solutes are considerably lower than those found for solutes diffusing across the outer membrane of Gram-negative bacteria, such as *Escherichia coli* [19] or *Pseudomonas aeruginosa* [20]. Small hydrophilic molecules permeate through water-filled channels. We have recently shown that the cell walls of *M. chelonae* and *M. smegmatis* contain pore-forming proteins, which have been named mycobacterial porin [12,18,21]. They are water-filled and allow the diffusion of small hydrophilic compounds. Very little is known about the molecular basis of the permeability of the cell wall of mycobacteria other than *M. chelonae* and *M. smegmatis* [11,12,17,18,21] and a major question is whether the cell walls of other mycobacterial species, in particular *M. tuberculosis*, behave as a permeability barrier and whether they also have pore-forming proteins. We investigated the cell wall of *Mycobacterium bovis* BCG, a member of the *M. tuberculosis* complex, for the presence of channel-forming proteins and found two channel-forming components. Our observation is of considerable importance because the mycobacterial cell wall is a main factor in the resistance and susceptibility of mycobacteria toward antimicrobial agents.

2. Materials and methods

2.1. Bacterial strain and growth conditions

M. bovis BCG Pasteur was grown for 7 days to mid-log phase in Dubos liquid medium supplemented with 10% Dubos albumin. The cells from 2 l of culture were harvested by centrifugation at 10000 rpm for 10 min at 4°C, yielding about 4 g (wet weight).

2.2. SDS-PAGE

SDS-PAGE was performed according to [22] or, because of the poor resolution of proteins at low molecular mass on this gel system, according to Schägger and von Jagow [23] on tricine-containing gels. The gels were stained with Coomassie brilliant blue or with silver stain [24].

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2.3. Isolation of the channel-forming activity from the cell wall using organic solvent

The cell wall channel was directly extracted from whole cells. Four g wt weight cell suspension was washed twice in 10 mM Tris-HCl (pH 8). The final pellet (5 ml) was extracted with a 1:2 mixture of chloroform:methanol in a proportion of 1 part cells and 5 to 8 parts chloroform/methanol. The duration of the extraction was about 24 h at 40°C with stirring in a closed container to avoid loss of the chloroform. Cells and chloroform/methanol solution were centrifuged for about 15 min (10000 rpm in Beckman LH20) and the pellet was discarded. The supernatant contained the channel-forming activity. It was mixed in a ratio of 1 part supernatant to 9 parts ether and was kept overnight at −20°C. The precipitated protein was dissolved in a solution containing 0.4% LDAO and 10 mM Tris-HCl (pH 8) and inspected for channel-forming activity.

2.4. Isolation of the channel-forming activity from whole cells using detergents

The method consisted in washes of the cell pellet with a number of different detergent-free or detergent-containing buffers to remove most of the soluble components [25]. All buffers contained 10 mM Tris-HCl, pH 8.0 (buffer I). In a next step the buffer was supplemented with 0.2% SDS (buffer II). Buffer III contained besides the Tris-HCl 1% Genapol (Fluka, Buchs, Switzerland) and 10 mM EDTA. The final buffer contained besides the Tris-HCl only 1% Genapol (buffer IV). The treatment of the cells with the buffers was always followed by centrifugation at 14600×*g* in a Beckman J2-21M/E centrifuge (rotor JA20) for 10 min.

The first two steps (1 and 2) consisted in washes of the cells (1 g) for 30 min with 10 ml buffer I. The pellet was washed two times (steps 3 and 4) with 10 ml buffer II. The resulting pellet was then washed twice at 4°C (steps 5 and 6) with 10 ml of buffer III. The final pellet was incubated for 20 h at 50°C in 2 ml buffer IV (step 7). The channel-forming activity was preferentially present in the final supernatant. SDS-PAGE of step 7 did not show protein bands even when the gel was stained with silver [24] (data not shown).

2.5. Lipid bilayer experiments

The methods used for the reconstitution experiments using 'black' lipid bilayers have been described previously [26]. Membranes were formed from a 1% (w/v) solution of diphytanoyl phosphatidylcholine (PC) and phosphatidylserine (PS; Avanti Polar Lipids, Alabaster, AL, USA) in a molar ratio of 4:1 in *n*-decane across circular holes (surface area about 0.1 mm^2) in the thin wall of a Teflon cell separating the two aqueous compartments. The temperature was kept at 20°C throughout. The aqueous solutions were used either unbuffered and had a pH around 6, or were buffered with 10 mM MES at a pH of 6. Protein was added from the stock solutions to the aqueous phase of the cis compartment (the compartment to which the voltage was applied) after the membranes had turned optically black in reflected light. The current through the membranes was measured with two calomel electrodes switched in series with a voltage source and a model 427 current amplifier (Keithley, Cleveland, OH, USA). The amplified signal was monitored with a storage oscilloscope and recorded on a strip chart recorder. For macroscopic conductance measurements the current was measured with a Keithley electrometer (model 602).

3. Results

3.1. Extraction of channel-forming activity from whole M. bovis BCG cells using organic solvent

To identify the cell wall channel of *M. bovis* BCG we employed methods, which have been developed previously for the identification and purification of the cell wall porin of *Corynebacterium glutamicum* [27] and of *Nocardia farcinica* [25]. Both methods circumvent the difficulties encountered in lysing cells and the substantial losses of cell wall protein during sucrose density centrifugation of broken cells. In a first step the washed and centrifuged cells were extracted with chloroform/methanol as described. Interestingly channel-forming activity was found in the chloroform/methanol supernatant of the cell extraction. It contained only a few bands in SDS-PAGE (lane 2 of Fig. 1). Precipitation of the protein in the organic solvent could be achieved when ether was used in the cold for longer times. The protein pellet and supernatant after ether precipitation were again subjected to lipid bilayer studies. The channel-forming activity was localized mainly in the pellet and to a lesser extent in the supernatant of the ether precipitate. It is noteworthy that the protein content in the pellet was rather slight, which means that it was difficult to visualize the cell wall proteins.

3.2. Extraction of channel-forming activity from whole M. bovis BCG cells using different detergents

The cells were treated with different detergents, such as lauryl dimethyl amine oxide (LDAO) or Genapol X-80. Some channel-forming ability was observed in supernatants of these detergent extracts indicating that they may contain the cell wall channel. On the other hand, after SDS-PAGE of the supernatants many bands were visible, and it was impossible to relate one single band to the channel-forming activity (data not shown). To identify the protein responsible for the channel-forming activity we used a purification method that has been used in a previous study of the cell wall channel of *N. farcinica* [25]. The method consisted in washes of the cell wall pellet with a number of different detergent-free or detergent-containing buffers to remove most of the soluble cell wall components (see Section 2). Highest channel-forming activity was observed in the final supernatant (the supernatant of step 7). SDS-PAGE of concentrated step 7 did not show a defined protein band even if the gel was stained with silver [24]. This result indicated that the protein concentration in the supernatant of step 7 was rather small.

3.3. Investigation of the channel-forming activity of the organic solvent extract

The interaction of the *M. bovis* BCG cell wall channel with lipid bilayers was studied by the addition of small amounts of the detergent solubilized ethanol pellet to the aqueous phase

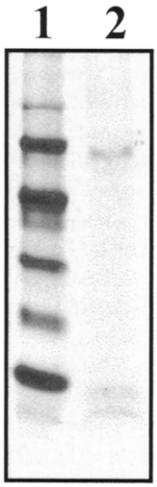

Fig. 1. 10% tricine-containing SDS-PAGE according to [23] of the cell wall extract of *M. bovis* BCG. Lane 1: Molecular mass markers 94 kDa, 67 kDa, 43 kDa, 30 kDa, 20.1 kDa and 14.4 kDa. Lane 2: 13 μl of the organic phase precipitated with ether in the cold, solubilized at 40°C for 30 min in 5 μl sample buffer. The gel was stained with Coomassie brilliant blue.

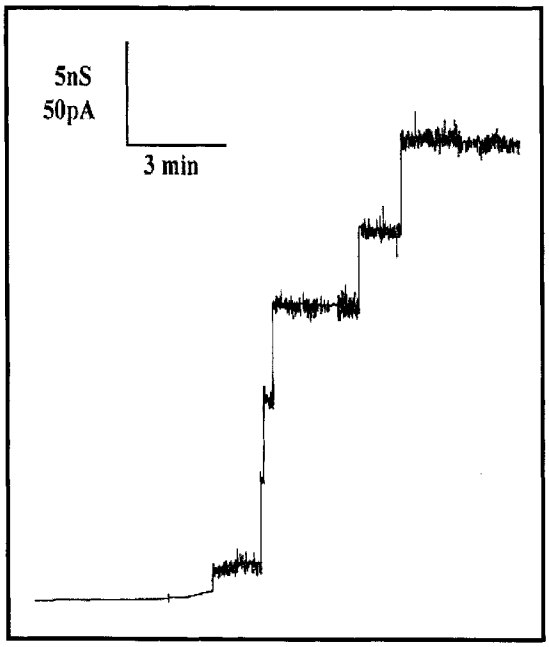

Fig. 2. Single-channel recording of pores generated by the organic solvent extract of whole *M. bovis* BCG cells in a black lipid bilayer membrane formed from PC/PS (molar ratio 4:1)/*n*-decane. The aqueous phase contained 1 M KCl and about 200 ng/ml (final concentration) of the protein precipitated from the organic solvent extracts with ether. The applied membrane potential was 10 mV; the temperature was 20°C.

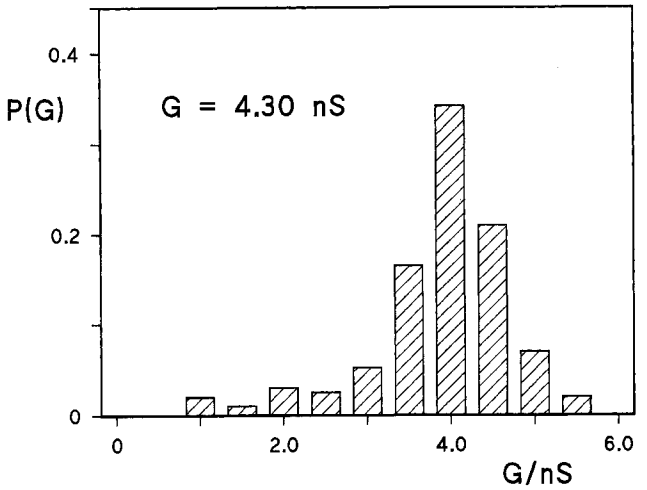

Fig. 3. Histogram of the probability $P(G)$ for the occurrence of a given conductivity unit measured after addition of protein from organic solvent extracts of whole *M. bovis* BCG cells to PC/PS (molar ratio 4:1)/*n*-decane membranes. The applied voltage was 10 mV; the aqueous phase contained 1 M KCl; $T = 20$°C. The average single-channel conductance of the channels was 4.3 nS for 185 steps.

bathing black lipid bilayer membranes. The membranes were formed from either a variety of pure lipids, such as phosphatidyl choline (PC) and phosphatidyl serine (PS), or of a mixture of both lipids in a molar ratio of 4:1. In all experiments including membranes formed of pure PC and PS and with the PC:PS mixture we observed a substantial increase of the specific membrane conductance in the presence of the precipitated cell wall protein. Highest membrane activity was obtained for membranes made of the PC:PS mixtures. About 2 min after addition of the protein, the membrane conductance started to rise and increased by several orders of magnitude in approximately 30 min (data not shown).

Single-channel measurements demonstrated that the membrane activity was caused by the formation of ion-permeable channels. Fig. 2 shows a single-channel recording in the presence of the cell wall porin. A few minutes after the addition of small concentrations of the protein (200 ng/ml) the current increased in step-like fashion. The channels had a very long lifetime at low voltages (mean lifetime more than 5 min). Most of the channels had a single-channel conductance of 4.0 nS in 1 M KCl, but we found also some smaller channels of the type shown in the first step in Fig. 2. Fig. 3 shows a histogram of the conductance fluctuations observed under the conditions of Fig. 2. Besides predominant conductance steps of 4.0 nS (more than 30% of all conductance fluctuations) we also observed channels with a smaller single-channel conductance which did not show a defined maximum. These current steps may represent substates of the 4.0 nS channel. It is also possible, however, that the 4.0 nS channel is formed by a bundle of small channels rather than a single large pore. In such a case the substate would represent different numbers of open channels. For larger membrane voltages the histograms of the conductance fluctuations became more complicated because the channels closed and additional smaller conductance levels occurred that were not observed at 10 mV (see below). In any case, the variations of the channel conductance suggested that the cell wall porin of *M. bovis* BCG did not form a

Table 1
Average single-channel conductance, G, of the channel derived from organic solvent extracts of whole *M. bovis* BCG cells in different salt solutions[a]

Salt	Salt concentration (M)	Single-channel conductance G (nS)	Hydrated cation radius a (nm)
LiCl	1.0	1.60	0.216
NaCl	1.0	2.2	0.163
KCl	0.30	2.5	0.110
	0.50	3.3	
	1.0	4.3	
	3.0	8.8	
KCH_3COO	1.0	4.0	
RbCl	1.0	4.5	0.105
Tris-Cl	1.0	0.4	0.321

[a]The membranes were formed of PC/PS (molar ratio 4:1) dissolved in *n*-decane. The aqueous solutions were buffered with 10 mM MES and had a pH of 6 unless otherwise indicated. The applied voltage was 10 mV, and the temperature was 20°C. The average single-channel conductance, G, was calculated from at least 80 single events. The radii of the hydrated cations were calculated using the Stokes equation and the limiting conductivity of the different ions [12].

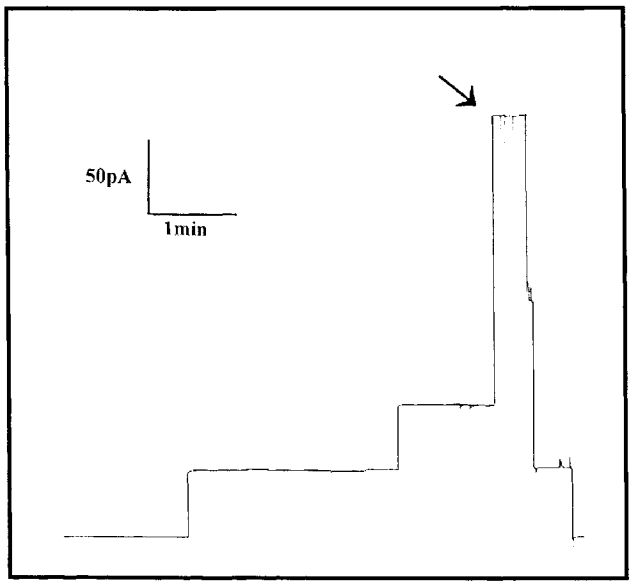

Fig. 4. Study of the voltage dependence of the channel derived from organic solvent extracts of whole *M. bovis* BCG cells at the single-channel level. After the addition of 50 ng/ml of the ether precipitated protein to the cis-side of a PC/PS (molar ratio 4:1)/*n*-decane membrane, two channels were reconstituted in the membrane (on-steps). After the reconstitution of the two channels the membrane potential was increased to 30 mV (arrow) and the membrane current was measured as a function of time. About 20 s after the increase of the voltage first one and 2 s later the other channel closed. The aqueous phase contained 1 M KCl and 10 mM Tris-HCl, pH 8; $T=20°C$. Note that the two channels closed almost completely.

rigid structure but showed conformation changes dependent on an applied membrane potential higher than 10 mV.

Single-channel experiments were performed in different salts to obtain some information on the size and other properties of the cell wall channels of *M. bovis* BCG. The results are listed in Table 1 and suggest that the influence of the cations on the single-channel conductance in different 1 M salt solutions was very high. The mobility sequence for the different cations within the cell wall channel was $Rb^+ > K^+ > Na^+ > Li^+ > Tris^+$, which means that the cation permeability follows the mobility sequence of these ions in the aqueous phase. Table 1 shows also the average single-channel conductance, G, for different KCl concentrations. Only at a few concentrations were sufficient current steps obtained to allow the calculation of a single-channel conductance. In particular, below 0.3 M KCl concentration, the reconstitution of channels was a very rare event. Nevertheless, it was possible to see from the concentration dependence of the single-channel conductance that the relationship between conductance and KCl concentration was not a linear function of the aqueous salt concentration. Instead, the slope of the conductance versus concentration curve on a double logarithmic scale was approximately 0.5, which indicated the influence of point net charges localized in or near the channels. It is noteworthy that negative point charges influence also the properties of cell wall channels of *M. chelonae*, *M. smegmatis*, *N. farcinica* and *C. glutamicum* [12,18,25,27]. The charge effects on the *M. bovis* BCG channels were caused by charges attached to the channel and not by the negatively charged lipids present in the membrane-forming lipid. This was the result of experiments with both neutral and negatively charged membranes, in which the cell wall channel had the same conductance.

3.4. *Voltages higher than 20 mV close the M. bovis BCG channel derived from organic solvent extract almost completely*

In single-channel recordings the cell wall porin exhibited some flickering when voltages higher than 10 mV were applied to the membrane. This means that it showed transitions between open and closed configuration, which could be caused by voltage-dependent partial pore closure or by the closing of individual channels in a bundle of channels. To study the voltage dependence in more detail we increased in single- and multi-channel recordings the transmembrane voltage. Fig. 4 shows an experiment of the first type. Two channels were reconstituted in a diphytanoyl phosphatidylcholine/*n*-decane membrane at a membrane potential of 10 mV (on-steps in Fig. 4), then the voltage was increased to 30 mV (arrow in Fig. 4). A short time after the application of the higher voltage, first one (after 20 s) and shortly after the second channel closed (after 22 s). The residual conductance of the two channels was only about 15% of the open channel (see Fig. 4). The results suggest that the cell wall channel of *M. bovis* BCG is not a bundle of channels, otherwise we would have observed several defined closing events as has been found in the case of Gram-negative bacterial porins (i.e. one third or two thirds of the opening of the channels [28]). Instead the cell wall channel closed almost completely at 30 mV.

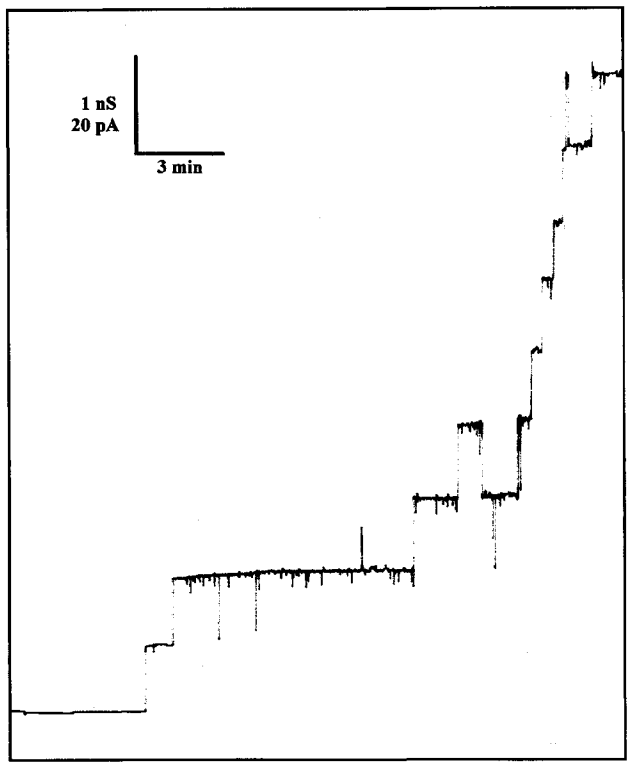

Fig. 5. Single-channel recording of channels generated by the detergent extract (step 7) of whole *M. bovis* BCG cells in a black lipid bilayer membrane formed from diphytanoyl phosphatidylcholine/*n*-decane. The aqueous phase contained 1 M KCl and about 100 μl/ml (final concentration) of the supernatant of step 7. The applied membrane potential was 20 mV; the temperature was 20°C.

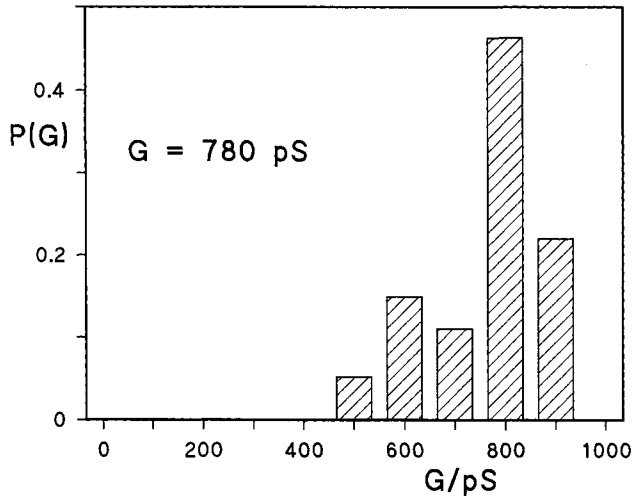

Fig. 6. Histogram of the probability $P(G)$ for the occurrence of a given conductivity unit measured after addition of the supernatant of detergent extract step 7 to diphytanoyl phosphatidylcholine/*n*-decane membranes. The applied voltage was 20 mV; the aqueous phase contained 1 M KCl; $T = 20°C$. The average single-channel conductance of the channels was 780 pS for 230 steps.

3.5. Investigation of the channel-forming activity of the detergent extract of whole cells

The detergent extracts of whole cells exhibited also channel-forming activity. Fig. 5 shows a single-channel recording in the presence of the supernatant of step 7 of whole *M. bovis* BCG cells using a diphytanoyl phosphatidylcholine/*n*-decane membrane. After a delay of several minutes probably caused by slow aqueous diffusion of the protein we observed channels with a single-channel conductance of about 800 pS in 1 M KCl. The channels had also a long lifetime but closed also occasionally (see Fig. 5). A histogram of the conductance fluctuations observed with the supernatant of step 7 is shown in Fig. 6. Most conductance fluctuations had a value of 800 pS (more than 40%) but we observed also smaller channels, which may represent substates of the channel because channel closures were sometimes smaller than channel openings. Similar results were also obtained for other salts. Table 2 shows the single-channel conductances derived from the experiments with the detergent extract of the *M. bovis* BCG cells. Its dependence on the different salts suggests that the channel is anion-selective because its single-channel conductance in 1 M potassium acetate (about 400 pS) was smaller than that in 1 M LiCl (650 pS). The selectivity for anions is presumably caused by an excess of positively charged amino acids in or near the channel. The single-channel conductance was a linear function of the bulk aqueous concentration of potassium chloride (see Table 2), which means presumably that we have here a similar situation as has been observed previously for the anion-selective PhoE of *E. coli*, where both types of charges are present inside the channel, but its anion selectivity is caused by an excess of positively charged amino acids [29,30].

3.6. Attempts to purify the cell wall channel protein

Several different strategies were employed to identify the channel-forming protein(s) from the cell wall of *M. bovis* BCG. One of the attempts was the excision of different molecular mass bands from tricine-containing preparative SDS-PAGE of the organic solvent extracts without heating of the samples. Addition of some of the extracts to planar lipid bilayers resulted in reconstitution of channels of the detergent type. However, it was not possible to relate the channel-forming activity to a defined molecular mass. The reason for this is presumably the small concentration of channel-forming protein in the cell wall and the possibility that it is an oligomer similar to the situation in *C. glutamicum* [27] and *N. farcinica* [25]. We tried also to use cation and anion exchange chromatography for the identification and purification of the cell wall channel(s). Again we found single-channel activity in some of the fractions but it was impossible to identify a specific protein responsible for the channel formation. This result represents another indication that the concentration of the channel-forming protein is probably very small.

4. Discussion

4.1. The cell wall of M. bovis BCG contains ion-permeable channels

In this study we have investigated *M. bovis* BCG cells for the presence of channel-forming activity using methods, which have successfully been applied for the purification of the cell wall channel protein of *C. glutamicum* [27] and of *N. farcinica* [25]. These methods do not affect the properties of the cell wall channel, when channel formation by detergent extracts of the cell wall or from the protein purified from the organic phase is considered [25,27]. The reason for this is presumably that the cell wall channel of the mycolic acid-containing members of the suborder *Corynebacterineae* of the actinomycetes [31] is deeply buried within the lipid layer of the cell wall, and therefore possesses properties which are related to very hydrophobic proteins. The organic extracts of whole *M. bovis* BCG cells contain only a few proteins. Nevertheless it was not possible to identify the channel-forming component for the wide 4 nS channel. Similarly, the protein responsible for the anion-selective 780 pS channel could not be identified. It is also possible that the channels are composed of subunits sim-

Table 2
Average single-channel conductance, G, of the channel derived from detergent extracts of whole *M. bovis* BCG cells in different salt solutions[a]

Salt	Salt concentration (M)	Single-channel conductance G (pS)
LiCl	1.0	650
KCl	0.10	85
	0.30	250
	1.0	780
	3.0	1800
KCH_3COO (pH 7)	1.0	500

[a]The membranes were formed of diphytanoyl phosphatidylcholine/*n*-decane dissolved in *n*-decane. The aqueous solutions were used buffered and had a pH of 6 unless otherwise indicated. The applied voltage was 20 mV, and the temperature was 20°C. The average single-channel conductance, G, was calculated from at least 120 single events.

ilar to the situation of the cell wall channels in *C. glutamicum* [27] and *N. farcinica* [25].

This means that the data presented in this paper are consistent with cell wall of *M. bovis* BCG acting as a permeability barrier towards hydrophilic compounds in a similar way to the outer membrane of Gram-negative bacteria [32,33]. A similar result was obtained when the permeability of the cell wall channels of *M. chelonae* [12], *M. smegmatis* [18], *C. glutamicum* [27] and *N. farcinica* [25] has been studied. All these members of the suborder *Corynebacterineae* of the order *Actinomycetales* within the class *Actinobacteria* as recently defined [31] contain a thick lipid layer on the surface. The mycobacterial cell wall harbors large amounts of unusual lipids, including mycolic acids that are linked through ester bonds to the arabinogalactan that for its part is attached to the murein of the cell wall [13,14,34]. The chain length of these 2-branched, 3-hydroxylated fatty acids varies considerably within the mycolic acid-containing taxa and are especially long in mycobacteria (60–90 carbon atoms) [13–15,35–39]. Such a thick hydrocarbon layer considerably impedes the diffusion of hydrophilic compounds, including most antibiotics. An aqueous channel is required to overcome this permeability barrier as has been discussed in different studies [11,36,40]. Here we have identified two of these proposed pathways, one has a high permeability at small membrane potentials and is cation-selective and the other has a small permeability, is anion-selective and has no voltage dependence.

Recently, it has been suggested that an OmpA-analogue (OmpATb) of the cell wall of *M. tuberculosis* is responsible for the cell wall permeability [41]. The gene of the protein was amplified by PCR and cloned into *E. coli*. The overexpression of the gene allowed the purification of the protein, which had a molecular mass of about 38 kDa in SDS-PAGE. It was active in planar lipid bilayers and had a single-channel conductance of 700 pS in 1 M KCl with unknown ion selectivity. This could mean that it is identical to the anion-selective channel characterized here in lipid bilayer experiments. It is noteworthy, however, that in a recent X-ray diffraction study of the crystallized membrane domain of OmpA, it has been demonstrated that this protein is not a channel-forming component in *E. coli* outer membrane [42], an observation that is in agreement with results of our own reconstitution experiments with OmpA [33]. On the other hand, it cannot be excluded that OmpATb has a different function than OmpA of *E. coli*.

4.2. Voltage control of the large channel in the cell wall

The channel from the organic solvent extract of *M. bovis* BCG cells has a high single-channel conductance, similar to that of cell wall channels from *M. chelonae* [12], *M. smegmatis* [18], *C. glutamicum* [27], and *N. farcinica* [25]. These channels are wide and water-filled and have diameters between 1.4 to 2.5 nm. The analogy of these channels to that of *M. bovis* BCG means that it probably has a similar, large diameter. This result tends to contradict the small permeability of the cell wall for hydrophilic solutes. However, the high conductance channel of *M. bovis* BCG was found to be voltage-dependent starting with voltages of about 20 mV. This result could explain the broad distribution of single-channel conductances in the histograms, particularly in the range between 1 to 3 nS (see Fig. 3). We do not know if an asymmetry potential of more than 20 mV exists across the cell wall of *M. bovis* BCG in the form of an intrinsic membrane potential (asymmetry of lipid distribution) or a Donnan potential (asymmetry of charge distribution). However, such a low voltage across a lipid layer is more likely to exist as needed for voltage-dependent control of outer membrane permeability in Gram-negative bacteria [28,43]. In any case, the voltage-dependent closure of the cell wall permeability in *M. bovis* BCG could indeed account for the extremely limited permeation of hydrophilic solutes through the mycobacterial cell wall because it is almost completely closed at low voltages as the reconstitution experiments clearly indicated. It is also noteworthy that cell wall channels of other mycolic acid-containing bacteria showed voltage-dependent control, e.g. *M. chelonae* [12], *M. smegmatis* [18] and *N. farcinica* [25]. The interesting point of the voltage dependence of these channels is that it is asymmetrical in such a way that the channel preferentially closed when the side of the addition of the protein has negative polarity. If we assume that these channels have the same orientation in vivo and in vitro, voltage-dependent control would require a negative potential at the inner side of the cell wall.

4.3. Implications of the cell wall channel properties for the antibiotic resistance of M. bovis BCG

Infections caused by members of the *M. tuberculosis* complex, represented here by *M. bovis* BCG, and other slow growing mycobacteria are characterized by pronounced treatment problems. These are due in part to a broad, often high level, natural resistance to most antibacterial chemotherapeutics currently available [9]. As far as penicillins and cephalosporins are concerned the resistance has been attributed to the production of potent β-lactamases. Little is known about natural resistance mechanisms to other antibacterial drugs although permeability problems could account for this [44]. These could be related to the selectivity of the cell wall channels of *M. bovis* BCG as an adjunct or an alternative possibility to the production of antibiotic degrading or modifying enzymes, because the cell wall should have a low permeability for anionic antibiotics whereas its permeability should be much higher for neutral, zwitterionic and positively charged antibiotics. This is consistent with the use of several potent antibiotics, such as isoniazid, ethambutol, rifampin, and pyrazinamide to cure tuberculosis [45]. These antibiotics are either positively charged or they are neutral. Among them the positively charged isoniazid is particularly effective against the biosynthesis of mycolic acids [46].

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