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Voltage-dependent Closing of Porin Channels: Analysis of Relaxation Kinetics

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Abstract. The anion-selective porin Omp34 from *Acidovorax delafieldii* was unidirectionally reconstituted in planar lipid membranes. Pore closing was recorded particularly at low salt conditions for negative and positive membrane potentials in the range of ±10 to ±100 mV. Relaxation curves were fitted by exponential functions in order to describe and to analyze the voltage-dependent behavior. Omp34 exhibited the following characteristics: (i) The channels are asymmetric with respect to closing characteristics and corresponding functional parameters. (ii) Relaxation curves can be fitted by a single exponential function in the low voltage range only, at $\geqslant$40 mV combinations of two exponential functions are required. (iii) Beyond 60 to 70 mV a third exponential function is necessary to fit the fast closing components properly. The time constants differ by two to three orders of magnitude. (iv) Hysteresis in *I-V*-diagrams originate from slow relaxation components which are different for positive and negative voltages. The implications for models aiming at description of voltage-dependent closing are discussed.

Key words: Omp34 — *Acidovorax delafieldii* — Ion channel — Porin conductivity — Voltage gating — Omp32 — *Comamonas acidovorans*

Introduction

Porins are pore-forming proteins in outer membranes of bacteria, mitochondria and plastids (Benz, 1994; Jap & Walian, 1996; Popp et al., 1997). Bacterial porins are trimeric molecules with three water-filled channels which mediate the permeation of ions and small nutrients across the membrane. The channels of general diffusion porins are formed by 16-stranded β-barrels and the functional properties are mainly determined by the distribution and location of charged amino acids inside and outside the pores (Weiss et al., 1991; Cowan et al., 1992; Karshikoff et al., 1994). Since the first electrophysiological investigations of bacterial porins the phenomenon of voltage gating or voltage-dependent closing has been known (Schindler & Rosenbusch, 1978, 1981). Voltage-dependent effects have been observed with a number of different porins from various bacterial species, among them OmpF, PhoE and OmpC from *E. coli* (Schindler & Rosenbusch, 1978; Delcour, Adler & Kung, 1991; van Gelder et al., 1996), the porin of *Rhodobacter capsulatus* (Bishop & Lea, 1994), Omp34 from *Acidovorax delafieldii*, investigated here (Brunen & Engelhardt, 1993), Omp32 from the related bacterium *Comamonas acidovorans* (Mathes & Engelhardt, 1998), and others (Jap & Walian, 1996). Voltage-dependent closing in bacterial porins depends on experimental conditions, is modulated by the membrane type used for conductance measurements and by porin preparation (Lakey, 1986). The content of organic solvent in the membrane is critical (Lakey & Pattus; 1989; Brunen & Engelhardt, 1993) as well as lipopolysaccharide (LPS) which remains associated with the porin during purification (Buehler et al., 1991; Simón et al., 1996). The physiological importance of voltage-dependent closing could not yet be demonstrated, but it is likely that the open state is modulated by ligands *in vivo* (Delcour, 1997). A closer analysis of gating kinetics has become possible when patch-clamp investigations, digital data acquisition and processing were performed, although earlier investigations already provided a number of functional details (Schindler & Rosenbusch, 1978, 1981). Closing kinetics are complex, and cannot be described in a simple manner (Jones & Taylor, 1996), and very fast, as well as long-lasting open-closed transitions were observed (Schindler & Rosenbusch, 1978; Berrier et al., 1992; Berrier, Besnard & Ghazi 1997; Delcour, 1997).

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Detailed kinetic studies on voltage gating may help to understand what mechanism is responsible for the current-voltage behavior. This is particularly interesting now that the atomic structures of several porins are available. The benefit of patch-clamp measurements is that fast processes in the millisecond range and small currents of single channels can reliably be resolved. However, investigations of larger ensembles of molecules may be easier to perform with appropriate planar lipid membranes. The latter are usually stable enough to enable long-term measurements for the analysis of slow closing phenomena in porins.

In this study we report on voltage-dependent effects of current-time measurements with the anion-selective porin Omp34 from *A. delafieldii* (Brunen et al., 1991; Brunen & Engelhardt, 1995). The results obtained especially at low salt conditions illustrate that current-time and current-voltage characteristics of porin ensembles are even more complex than expected.

Materials and Methods

Porin Preparation

Growth of cells in complex medium, preparation of outer membranes, and isolation of porin Omp34 were previously described in detail (Brunen et al., 1991).

Planar Lipid Bilayer Techniques

Conductance measurements were performed with planar lipid membranes according to the technique of Montal & Mueller (1972) in Teflon chambers separated by a Teflon septum of 12-μm width with holes of 180-μm diameter as described in (Schindler, 1989). Ag/AgCl electrodes were used and 5-mM KCl plus 10-mM Tris, pH 8.3, or 30-mM KCl as an electrolyte. Treatment of Teflon septa and formation of lipid bilayers were essentially the same as described in detail elsewhere (Mathes & Engelhardt, 1998). Porin dissolved in 1% Genapol was added to one side of the membrane in amounts of 2 μl per 1-ml chamber volume. Voltage application and current measurement were performed via the same electrode (voltage side). The porin was added to the ground-side if not otherwise stated.

Data Acquisition and Processing

The data acquisition setup consists of a patch-clamp amplifier EPC7 (List-electronic, Darmstadt), a digital storage oscilloscope Tektronix 2211 (Tektronix, Heenveen, The Netherlands), a PC486 equipped with the data acquisition board AT-MIO-16F-5 from National Instruments (Munich), and a strip chart recorder for analog documentation. Programs for data recording and processing were written using the graphical programming language LabVIEW from National Instruments as described in (Mathes & Engelhardt, 1998). The EPC7 stimulus pathway was corrected by means of a software-controlled compensation, the accuracy was around 0.1% of the applied voltage.

Mathematical fits of the relaxation curves were performed using different fit algorithms implemented in the LabVIEW software. One to three exponential functions were approximated together with a constant offset (I_∞), representing the fraction of porins remaining in an open state. Equation 1 was used throughout, where i is 1 to 3, a_i denote the amplitudes and τ_i the time constants of the particular functions.

$$I(t) = I_\infty + \sum a_i \exp(-t/\tau_i) \quad (1)$$

Only those parameter combinations were recorded that were reliable with regard to their relative error and physical meaning (no change of sign), otherwise they were disregarded.

Results

Measurement of Current-Time Characteristics

Measurements of voltage-dependent closing of the *A. delafieldii* porin Omp34 were performed with planar lipid membranes of the Montal-Mueller type (Montal & Mueller, 1972). The lipid used was diphytanoyl phosphatidylcholine, which has a net charge of approx. zero over a wide pH range. We applied low salt buffers (5-mM KCl plus 10-mM Tris, pH 8.3, and 30-mM KCl, respectively) because previous studies have shown that high salt concentrations disturb the voltage sensitivity of Omp34 which also holds true for extreme pH values (Brunen & Engelhardt, 1993). About 250 porin molecules were inserted into the lipid membrane in an apparently unidirectional manner (Brunen & Engelhardt, 1993; Mathes, 1994) which enabled us to determine orientation-dependent closing characteristics.

Residual conductance in measurements with ensembles of porin molecules can derive from partial closing of single channels, from complete closing of a fraction of channels, or from intermediate situations. Single porin experiments with only a few (3 to 5) inserted molecules showed that ⩾95% of the conductance is lost due to closing of channels (*not shown;* Brunen & Engelhardt, 1993). Accordingly, histograms of conductance steps following insertion of porins into the membrane and subsequent closing steps exhibited almost identical average conductances and step size distributions. This also applies to the related porin Omp32 from *C. acidovorans* (Mathes & Engelhardt, 1998). The average conductance values for a single Omp34 trimer were about 25 pS in 5-mM KCl (plus 10-mM Tris) and 70 pS in 30-mM KCl.

Figure 1 illustrates the voltage profile applied to the membrane and the current response of porin molecules. Since the complete relaxation of current appeared to be a relatively slow process we attached importance to long-term measurements of each voltage step. For reasons of consistency and comparability, the complete set of curves was recorded with the same molecules in a single membrane. The experiments required more than 14 hr, which is extraordinarily long for these types of measurements. The voltage was increased from 0 to 100 mV in 10-mV steps, changing the polarity of the field after each

period. Application of zero Volts for about 1 min or changing the sign of the voltage caused closed porin channels to open immediately such that each new voltage period began with all open channels. The immediate response of closed channels to a short breakdown of membrane voltage is nicely illustrated in Fig. 3.

Omp34 is relatively sensitive to low membrane potentials and already begins closing at 10 to 20 mV (Figs. 1, 2) in agreement with previous observations (Brunen & Engelhardt, 1993). Figure 2 shows the relaxation curves for negative and positive voltages, here normalized with respect to the maximum current (amplitudes) in the very beginning of each curve in order to make them more easily comparable for further analysis. The maximum currents of open channels plotted in an *I-V*-diagram revealed a slightly asymmetric and nonlinear behavior. The latter is not an effect of channel closing but is due to nonlinearity of conductance (Mathes & Engelhardt, 1998). Figure 1*C* also shows that there is no indication of irreversible pore closing during the experiment. The following conclusions can be drawn from the results shown in Fig. 2. (i) The channels close at negative and at positive voltages. (ii) The higher the voltage the more porin molecules change from an open to a closed state, i.e., the amount of channels remaining open at equilibrium conditions decreases. (iii) Relaxation is apparently a slow process which requires measurements of $\geqslant$10 min for proper data analysis. (iv) Voltage-dependent closing exhibits asymmetry with respect to orientation, i.e., the relaxation is faster at negative voltages in our setup (the absolute orientation of porins is unknown). (v) Closing of porin channels is reversible and not static, fluctuations are the result of ongoing closing and opening events.

These results are consistent with all our experiments performed with different starting conditions (voltage), with single curves obtained in sessions of 1 to 6 hr or with complete data sets recorded over a shorter period of time ($\leqslant$10 min per individual condition). Again, the same principle results were obtained with porin measured in 30-mM KCl under the time conditions illustrated in Fig. 1; the normalized curves are very similar to those presented in Fig. 2.

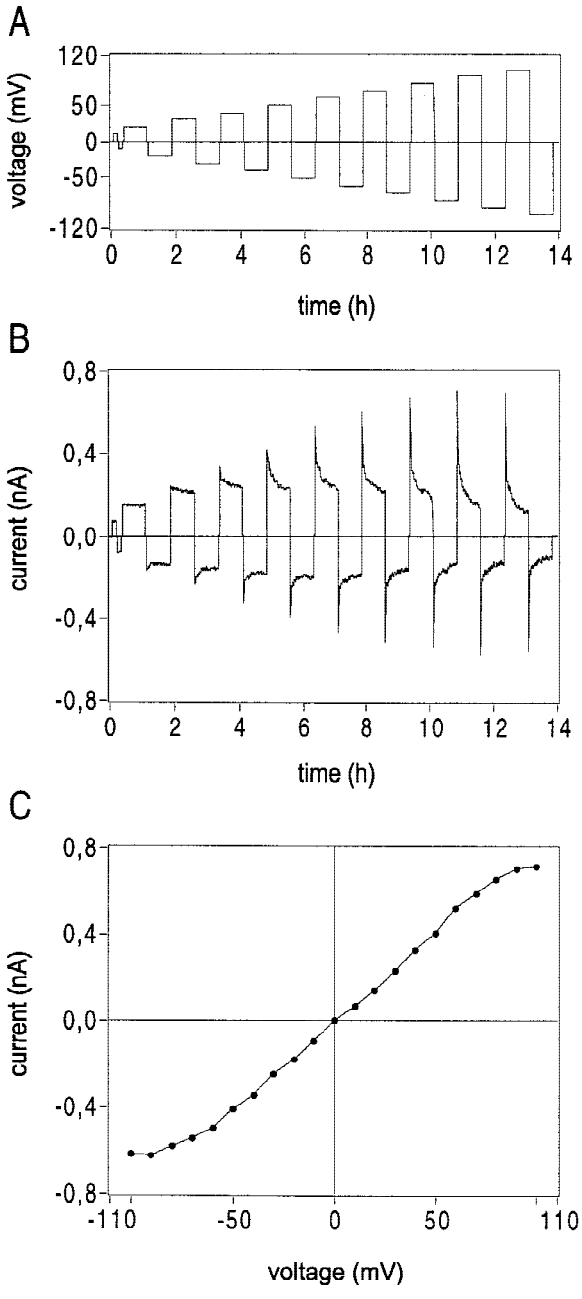

Fig. 1. Current response of porin Omp34 to constant voltages. (*A*) Voltage profile starting at 0 mV and increasing to ±100 mV in 10-mV steps with alternating sign. The steps are 43-min long and separated by zero potentials of a 1-min period. The entire experiment was performed with a single membrane. (*B*) Recorded current of about 250 porin molecules in 5-mM KCl, 10-mM Tris, pH 8.3. The relaxation is due to closing porin channels. (*C*) Maximum current amplitudes recorded at the very beginning of each voltage step (after capacitive currents have vanished). These open channel currents are used as normalization factors.

Measurement of Current-Voltage Characteristics

Current-voltage relationships are usually determined with triangular voltages. Figure 3 shows a typical *I-V*-diagram for Omp34 with a particularly slow voltage triangle of a 100-min length period. Again, there is a clear asymmetry. At negative polarity the process of closing is more sensitive to voltage which corresponds to the observations in Fig. 2, i.e., closing is faster. The shape of the *I-V*-curve is a function of (slow) channel relaxation kinetics and the (relatively fast) voltage-profile applied to the membrane. The shorter the period of the triangular voltage the less time is available for the porin molecules to achieve their open-closed equilibrium level at a certain voltage. As a consequence the recorded cur-

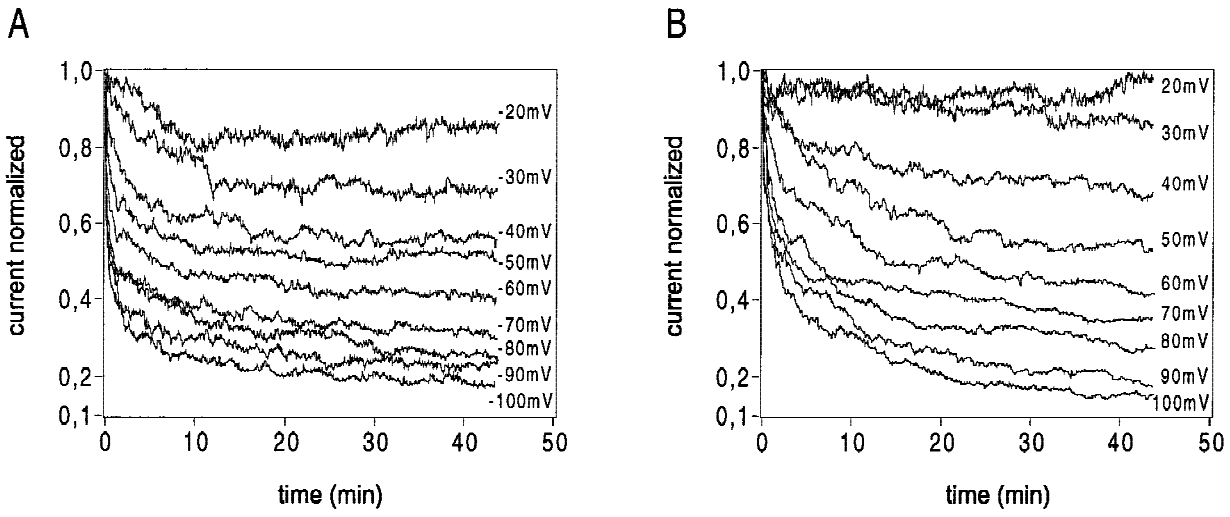

Fig. 2. Normalized current traces obtained from Fig. 1*B*. (*A*) Relaxation kinetics for negative and (*B*) for positive voltages. Curves for ±10 mV are not displayed. Normalization was done according to the maximum amplitudes shown in Fig. 1*C*.

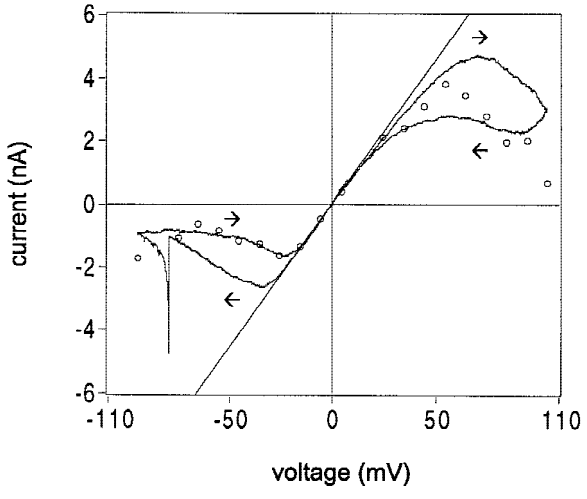

Fig. 3. *I-V* diagram of about 1,000 Omp34 molecules in 30 mM-KCl. The period of triangular voltage was 100 min with an amplitude of 100 mV. Arrows indicate the progress of the curve, circles denote the calculated equilibrium values for $t\text{->}\infty$ which were determined with the same membrane and voltage steps corresponding to the experiment in Fig. 1. The circles indicate the current-voltage trace of a theoretical experiment with an extremely long triangular voltage period ($t\text{->}\infty$). The spike at the lower left region of the curve originates from a black-out in the power circuit less than 0.1-sec duration. Note that closed porin channels opened immediately. The straight line illustrates the deviation of the curve from pure ohmic behavior. See text for an explanation for the asymmetric hysteresis effect.

rent is higher in case of increasing moduli of voltage than it would be if the triangular period were very long ($\geqslant$15 hr). Only at higher voltages, and especially at negative polarity in our setup where closing is considerably faster (Fig. 2), the curves could approximate to equilibrium conditions. This is illustrated in Fig. 3, showing that residual currents obtained from relaxation experiments almost fit the second leg of the *I-V*-characteristic where the voltage decreases with time. At positive polarity porin molecules respond more slowly (Fig. 2), so that neither with increasing nor decreasing voltages the open-closed equilibrium could be approached. Therefore, the extent of hysteresis differs with porin orientation in current-voltage diagrams (Fig. 3).

Analysis of Current-Time Characteristics

To describe the characteristics of the current-time curves by means of simple expressions we tried to fit exponential curves to the normalized data, assuming in a first approximation that the porin can be described by a two-state model (an open and closed state; Jones & Taylor, 1996). It was, however, not possible to fit the various current responses with a single exponential function. The shapes of the curves in Fig. 2, particularly those at higher membrane potentials, already suggested that a simple exponential function would not be sufficient. Therefore, we applied a least squares fit with a combination of two exponential functions to allow adjustment of the fast and slow relaxation processes. In Fig. 4 the corresponding parameters which possess the following features are displayed: (i) The equilibrium level of open channels (I_∞) is a function of voltage with a slight indication of asymmetry. More than 80% of the channels are closed at ±100 mV and even more tend to become closed beyond that value. At approx. ±50 mV half of the channels are in a closed state (Fig. 4*A*). (ii) There are two sets of time constants (τ) which differ by one to two orders of magnitude, τ is in the range of 6 to 60 sec for the fast component and *approx.* 300 to 3,000 sec for the slow transition process (Fig. 4*B*). The values vary

quite significantly within the two sets and exhibit apparently no clear tendency with respect to voltage, such that it was barely possible to extract or fit any functional dependence. (iii) The amplitudes reflect the observation that beyond −50 mV the fast process dominates the closing kinetics and is apparently a function of the membrane potential, while the contribution of the slow relaxation is approx. constant. On the positive side there are no obvious differences between the amplitudes, which appear to increase with the membrane potential (Fig. 4*C*). (iv) Below ±30 mV reasonable fits were not possible.

Particularly at higher voltages another fast component appeared in the closing kinetics which could not be covered by two exponential functions, while a third function approximated the curves between 60 and 100 mV quite well (Fig. 5). The time constant τ(fast) of the previous fit (Fig. 4) now splits into τ(fast) = 6 to 14 sec with amplitudes of 0.35 to 0.46 apparently being independent from the negative voltage, and τ(medium) = 40 to 280 sec with decreasing amplitudes. The parameters of the slow component remained almost unchanged. At positive potentials the values had approx. the same tendency although below ±40 mV fits were no longer reasonable. In Fig. 6 the least squares sums of all meaningfully calculated exponential fits are compared. The values were normalized with respect to the smallest error value of the correponding fits to achieve a better comparability of the data, the best fit is indicated by a relative error of 1. It is obvious, that at higher voltages there is a need for three exponential functions, while below 20 to 30 mV reasonable approximations could not be obtained with any of the expressions, although there was a slow decay of current to 80 to 90% of the starting level. The parameters from multiply reproduced measurements showed some variability but lay in the same orders of magnitude and exhibited the same tendencies and properties as illustrated in Figs. 4 to 6.

Discussion

The particular features of our data acquisition system and the characteristics of the porin Omp34 from *A. delafieldii* enabled us to measure closing kinetics of an ensemble of porin molecules over a long period at moderate voltages which are probably close to physiological conditions. The slow relaxation processes with time constants >600 sec suggest that data recording should at least be in this range, i.e., >10 min, so that slow decay components can be detected reliably. Since the time constants of slow relaxation processes do not decrease with increasing voltages, it appears not to be justified to perform shorter measurements even beyond ±100 mV.

A second benefit of our system is that orientation-dependent effects could be measured reproducibly due to the unidirectional insertion of porin molecules into the

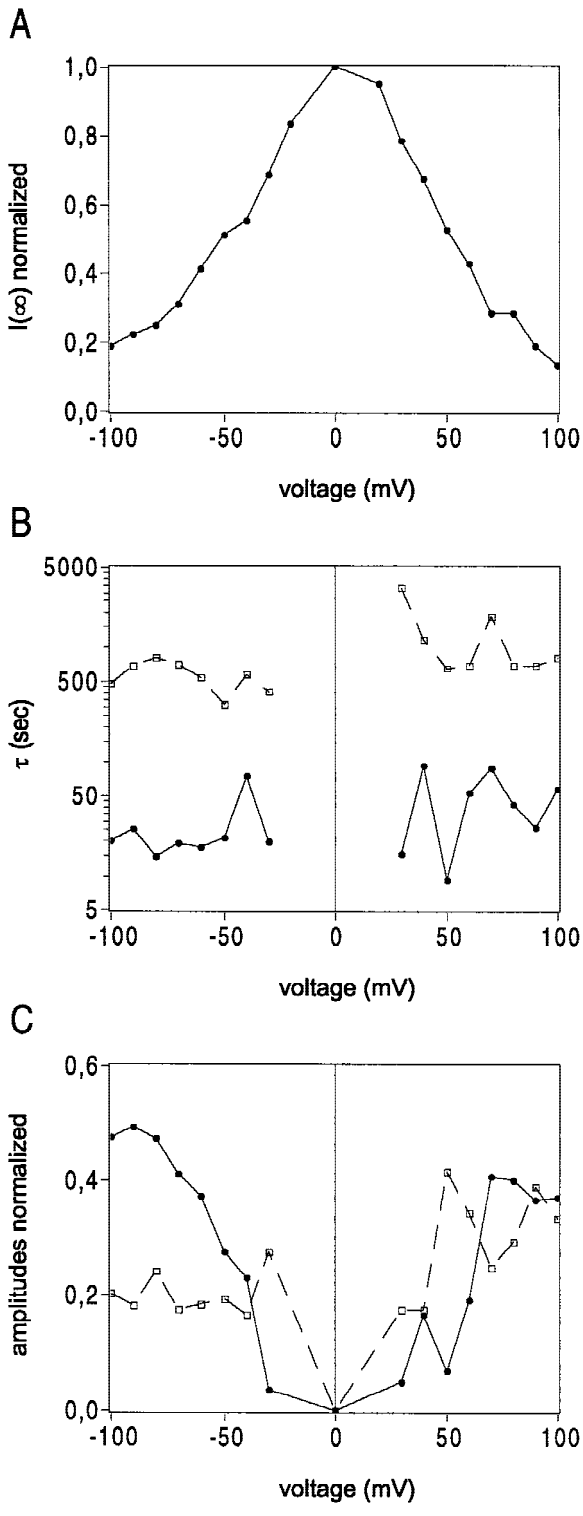

Fig. 4. Parameter values of curve fits of the normalized relaxation kinetics in Fig. 2, using a combination of two exponential functions according to Eq. 1. The diagrams contain only parameters of reliably fitted curves. (*A*) Equilibrium current I_∞ (normalized) for $t\text{->}\infty$ represents the fraction of permanently open porin channels. (*B*) Time constants τ according to Eq. 1 describe one slow (□) and one fast (●) relaxation process as indicated. (*C*) Amplitudes of the two exponential functions with fast and slow time constants as given in (*B*).

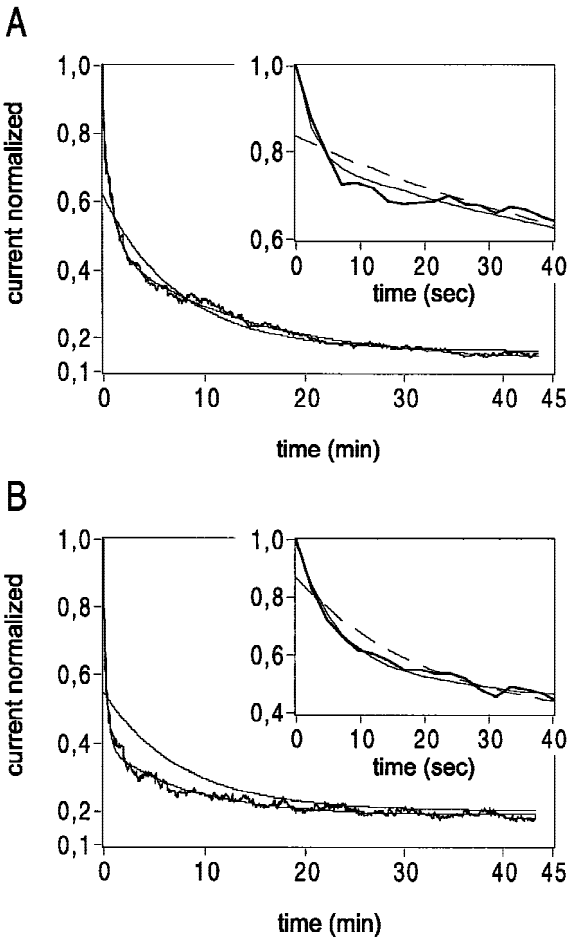

Fig. 5. Original data curves for −100 mV (*A*) and +100 mV (*B*) displayed together with fit curves consisting of 1 to 3 exponential functions. The fitted curves that obviously deviate from the original data contain only one single exponential function. The first 40 sec of the curves (insets in *A*, *B*) illustrate that the fit curves that consist of two exponential functions (dotted lines) deviate heavily from the measured data (bold lines), and indicate that a third exponential function is required to meet the fast closing components (smooth lines).

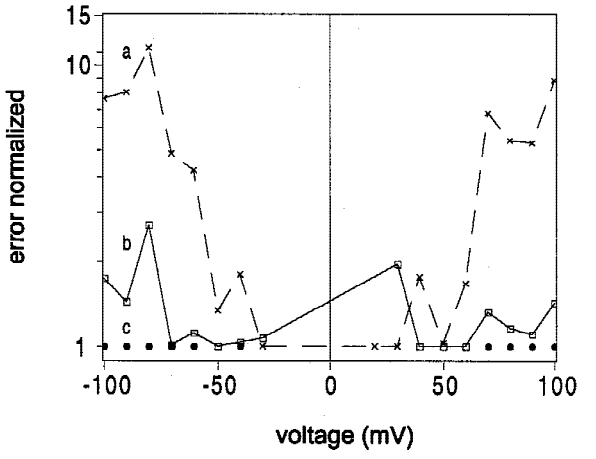

Fig. 6. Quality plot of fitted curves for one (*a*), two (*b*) and three (*c*) exponential functions. The plotted values are the sums of least squares normalized for the smallest value of each particular data curve, identifying the best fit with a relative error value of 1. At higher voltages three exponential functions are required to describe the relaxation kinetics with a combination of a fast, a medium and a slow closing curve, while two exponential functions or even a single one is sufficient at lower voltages.

membrane (Brunen & Engelhardt, 1993, 1995). This is of particular interest when functional data from electrophysiological measurements is eventually related to the asymmetric structure of porin channels (van Gelder et al., 1997). As a result the asymmetric hysteresis effects of current-voltage curves could be explained. Apparent hysteresis occurs if the increase (or decrease) of voltage per time unit is too high for the molecules to achieve their equilibrium level of closed channels at a certain membrane potential. The approx. identity of current values at $t\text{->}\infty$ with part of the *I-V*-diagram indicates that the curve could approach real equilibrium levels if it were measured with an extremely long voltage period (approx. 20 hr). This certainly denotes a challenge for the lifetime of membranes. However, it would be interesting to determine whether there is a real hysteresis because models describing voltage-dependent channel closing would have to include the history (voltage treatment) of porins or the membrane. Interestingly, Schindler & Rosenbusch (1981) observed residual hysteresis even after 6 hr in a comparable experiment with OmpF of *E. coli* which they assigned to clustering effects.

A principle question is whether the current exclusively originated from ions passing through the porin channels in our experiments, or whether side activities contributed to the signal. There are three artificial current sources which have to be considered: leakage currents, activities created by other molecules or artificial effects of the porin itself, either following ohmic *I-V* characteristics or exhibiting voltage-dependent closing behavior. Leakage currents through the lipid membranes are very noisy and usually occur shortly before the membrane brakes down. They can easily be distinguished from channel signals and were not included in our analysis. Side activities not showing voltage-dependent closing would contribute to the residual conductance and could amount to a theoretical maximum of about 15% and presumably considerably less as judged from residual currents at ±100 mV and beyond (Fig. 4*A*). But as outlined above, the maximum level of side conductance should even be lower than 5% and would not pose a problem with the interpretation of data. The existence of conductance sources mimicking closing kinetics of Omp34 is very unlikely but would not be discernible. However, protein contaminants can be excluded here according to electrophoretic controls of porin purity.

The decay of current upon channel closing at a certain voltage cannot be described by a simple exponential

function with Omp34. This corresponds to the findings of Jones & Taylor (1996) with OmpF and OmpC from *E. coli*. They therefore suggested a model with two exponential functions representing two populations of porin molecules, one responding rapidly (τ = 1 to 50 sec) the other one slowly (τ = 40 to 900 sec). We found, however, that in the case of Omp34 even two exponential functions did not describe relaxation kinetics satisfactorily. Possible reasons for the observed differences are that very slow processes could be better resolved due to long term data recording, low salt conditions support effects that lead to different closing kinetics, or other reasons such as the presence of tightly bound ligands, differences in voltage sensibility, or purification effects were responsible. Particularly at higher voltages three functions could fit the data better due to the large differences between the time constants and the significant portion of rapidly responding channels (at negative potentials). This could mean that more than two porin populations, or at least four different states (closed and open) have to be postulated. Buehler & Rosenbusch (1993) showed that a single active channel in a trimer with two inactivated pores possessed properties other than that of a single channel of a fully active trimer. It is, however, unlikely that the three individual channels (or only one active, two active and three active channels per trimer) represent the three hypothetical porin populations investigated here. The channels of Omp34 behave in a strongly cooperative manner with the selected membrane potentials and close and open together. Less than 1% of closing events could be attributed to single channel (1/3 or 2/3 conductance) steps only (Mathes & Engelhardt, 1998).

Another observation which does not support the assumption that two exponential functions fully describe the relaxation kinetics of Omp34 is, that the parameter-voltage characteristics do not follow clear functions. One may argue that fluctuations of the curves prevent the fitting algorithm to find ideal values, but these fluctuations are an inherent property of the curves (Berrier et al., 1997) and may reflect the influence of an unknown but relevant parameter that affects the porin properties. Here statistics was not a problem because about 250 porin molecules were included. It is a challenge to develop a model that includes such fluctuations, and an intriguing question is what the various populations or porin states are, and how they are created. Several situations can be envisaged which are also relevant for porins in their natural environment, i.e., the outer membrane. (i) There are (two), three (or more) distinct porin populations that differ in their transition probabilities for closing. Since porins were shown to bind LPS and appear to depend on it for their particular functional properties (Hoenger et al., 1990; Buehler & Rosenbusch, 1991; Simón et al., 1996), LPS could contribute to the creation of different sets of porin molecules. The same holds true for other molecules or effectors which bind to porins (Kreusch et al., 1994; Delcour, 1997). It was shown that positively and negatively charged amino acid residues are involved in the gating process (van Gelder et al., 1997; Brunen & Engelhardt, 1993, 1995). Therefore, local physicochemical conditions and molecules altering the electrostatic properties of porins are likely candidates for creating different sets of porins. (ii) There are several open and/or closed states or substates inherent to each porin molecule with different conductance properties. The existence of various closed states has been reported for PhoE of *E. coli* (Berrier et al., 1997). (iii) Porins form static and/or dynamic clusters dependent upon the applied membrane potential (or other unknown parameters) that change the relaxation kinetics of a porin molecule upon interaction with its next neighbor. The existence of porin clusters in lipid membranes was shown for OmpF (Schindler & Rosenbusch, 1978, 1981) and is obvious for porins in the outer membrane. (iv) The lipid environment (in addition to the presence of LPS) alters the porin state. The capacitance of (planar) lipid membranes increases with applied voltage (Alvarez & Latorre, 1978; Toyama, Nakamura & Toda, 1991, *unpublished results*) which may result in differences in lateral tension and may in turn exert an influence upon porin molecules. To deduce or to exclude models, measurements with rigorously controlled experimental conditions are required which could reveal relevant and possibly hitherto unknown parameters for channel closing. The knowledge of conditions controlling channel gating will be of great value for understanding phenomena on a molecular basis when molecular models for gating are established (Watanabe et al., 1997).

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