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Redistribution of the electric field within the pore contributes to the voltage-dependence of mitochondrial porin channel

Lev N. Ermishkin and Tajib A. Mirzabekov

Institute of Biological Physics, Academy of Science USSR Pushchino (U.S.S.R.)

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The effects of pH on the integral conductance and on the properties of single channels induced by porin from rat liver mitochondria in a lipid bilayer have been studied. When the membrane potential increases, the conductance of the multi-channel membrane decreases more sharply at acidic pH than at neutral or basic pH. The channel is shown to have several states with different conductance and selectivity. The number of levels and their conductance do not depend on pH, while the selectivity as well as the dependence of steady-state probabilities of different levels on the membrane potential are substantially affected by a pH change. This dependence curve steepens in the pH region where charges of carboxyl groups of aspartic and glutamic amino acids are neutralized. It is concluded that at neutral pH the channel gate is controlled by a great number of the positively and negatively charged groups. The high steepness of the conductance–voltage curve in the acidic region suggests that at least 60 positive charges participate in controlling the channel gate. This number, compared with that of the positively charged side chain amino acids per channel, according to the amino acid analysis of the porin, led us to conclude that almost all amino groups of the channel former must pass through the entire membrane potential difference upon random motion of the channel among the states. The assumption that channel closing leads to redistribution of the electric field within the pore, changing the energy of the charges on the voltage sensor, may be the only explanation of this phenomenon.

Introduction

The voltage dependence of the permeability of ionic channels in biological membranes results from the replacement of the charges or dipoles of channel-forming molecules in the membrane potential field upon a change in the conformational state of the channel [1]. A number of models of the channel gate and the voltage sensor have been suggested [2], however, the real mechanism of voltage-dependent gating is still unclear for any channel. This is due to (1) the complexity of these systems having, as a rule, a high molecular weight and (2) the necessity of diverse information on the channel structure as well as on the functioning of a single channel.

One of the channels with protein nature, studied by a variety of methods, is the VDAC (voltage-dependent anion-selective channel) formed by porin from the outer mitochondrial membrane [3–9]. Porins from mitochondria of different tissues and organisms have a similar molecular mass (30–37 kDa) and form large pores (about 20 Å in diameter) with similar permeability properties in the lipid bilayer. According to Linden and Gellerfors [10], the channel is formed by two identical molecules of protein. For porin from yeast mitochondria, the sequence has been determined, the analysis of which suggests β-barrel to be a plausible structure of the protein in the membrane [11]. In such a structure the charged side-chain amino acids face the water-filled pore.

The conductance of the porin-containing bilayer decreases with the membrane potential increase. This decrease results from the channel closing (its transition to the lower conductance states [7]). Zimmerberg and Parsegian [12,13] demonstrated that the channel closing is accompanied by a considerable change in volume of the pore, i.e., with major (not local) structural rearrangement. Increase of pH as well as conversion of positive charges of amino groups into negative ones by succinylation have been shown to decrease the steepness of the voltage-dependence curve [14,15]. In these studies, the authors came to the conclusion that the channel is controlled by 3–5 positive charges (presumably lysine

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Correspondence: L.N. Ermishkin, Institute of Biological Physics, Academy of Science U.S.S.R., Pushchino 142292, U.S.S.R.

ones). The same experiments suggest that these charges face the pore cavity and serve both as a voltage sensor and a selectivity filter.

We found that conversion of negative charges of carboxyl groups (of aspartic and glutamic amino acids) of porin into positive ones enhances, in its turn, the voltage dependence of the conductance [16]. The channel is, therefore, controlled by a great number of positive and negative charges.

In this paper we show that neutralization of the charges of carboxyl groups by lowering the pH also essentially enhances the channel voltage dependence. The study of the voltage dependence in a wide pH range allows estimation of the number of positive and negative charges in the voltage sensor and comparison of this number with that of charged side chain groups on the channel former according to the amino acid analysis of porin from rat liver mitochondria [6]. For this purpose, the effect of pH on the voltage dependence of transitions between the channel states was studied. In addition, due to a sharp voltage dependence at low pH, all the channel states (from the open to the completely closed one) can be studied in a limited range of the membrane potential values.

Methods and Materials

Materials. Azolectin (L-α-phosphatidylcholine Type II-S from soybean), Triton X-100 and DEAE-cellulose were purchased from Sigma, hydroxyapatite (Bio-Gel HTP) from Bio-Rad and Celite 535 from Roth. All other chemicals were reagent grade.

Purification of rat liver porin. Porin was isolated by chromatography on columns with hydroxyapatite/Celite 535 (2 : 1, w/w) and DEAE-cellulose. The method was essentially the same as described elsewhere [17,18]. Rat liver mitochondria were solubilized for 30 min at 0 °C in 2.5% Triton X-100/20 mM Na_2SO_4/20 mM Mops-KOH (pH 6.7)/1 mM EDTA in a final protein concentration of about 10 mg/ml. The solution was centrifuged at $150000 \times g$ for 30 min and supernatant was applied to the column with hydroxyapatite + Celite. Eluate was applied to the column with DEAE-cellulose and the protein peak was collected and stored at −20 °C. Purity of the protein was tested by electrophoresis on a polyacrilamide-sodium dodecyl sulphate [19]. Porin concentration was determined by the Lowry method modified for the presence of Triton X-100 [20].

Membrane experiments. Black lipid membranes were formed from a solution of 1% azolectin in *n*-heptane. Crude soybean phospholipids were purified and stored as described in Ref. 21. Membranes had a diameter of either 0.5–1 mm for the macroscopic conductance measurements or about 0.2 mm for single-channel experiments. The construction of the cell used allowed to substitute the solution in one (cis) compartment within several seconds. After the membrane had turned black, the solution on the cis-side was substituted with the porin-containing one (1–10 ng/ml) of identical ionic content. The porin-containing solution was prepared immediately before use to avoid protein inactivation.

After the required conductance had been achieved, the solution was replaced again with the porin-free one in order to stop channel incorporation and to remove Triton X-100. After the substitution the number of channels in the membrane remained unchanged during 2–3 h. Aqueous solutions usually contained 1 M KCl, 10 mM buffer and were maintained at 25 °C. The cis-solution was taken as the virtual ground.

Results

Fig. 1 illustrates the pH effect on the dependence of the steady-state conductance of a porin-containing bilayer upon the membrane potential. The normalized conductance was obtained from recordings of the transmembrane current (like this shown in the inset) in response to a stepwise voltage change. As it is seen, increase of the pH to 9.0 diminishes the voltage dependence as previously observed by Bowen et al. [14]. On the contrary, the decrease of pH to 3 essentially enhances the voltage dependence of the conductance. It should be noted that in acidic media the conductance falls to much lower values and tends to zero at high

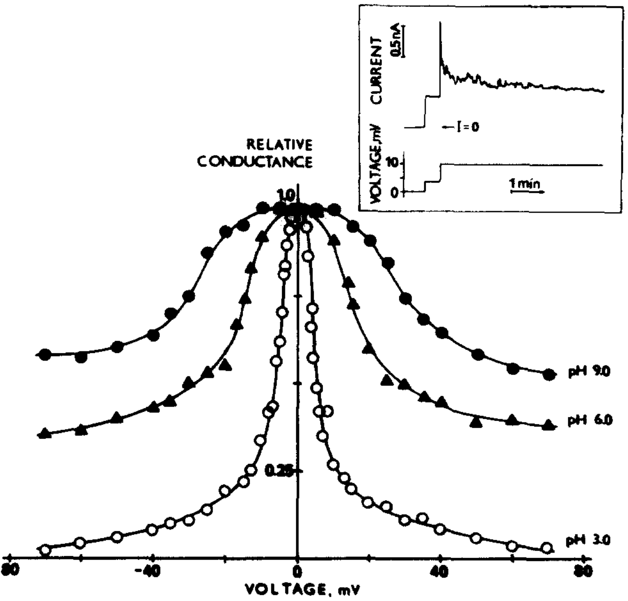

Fig. 1. Normalized steady-state conductance of a porin-containing bilayer vs. membrane voltage at different pH values. Inset: current through a multi-channel membrane at pH 4.0 in response to voltage step from zero to 3 mV and from 3 to 10 mV. At zero time the current through all the channels in the open state is measured. Steady-state current was measured after 5 min of maintained voltage. Normalized steady-state conductance was obtained as the ratio of the steady-state current to the initial current.

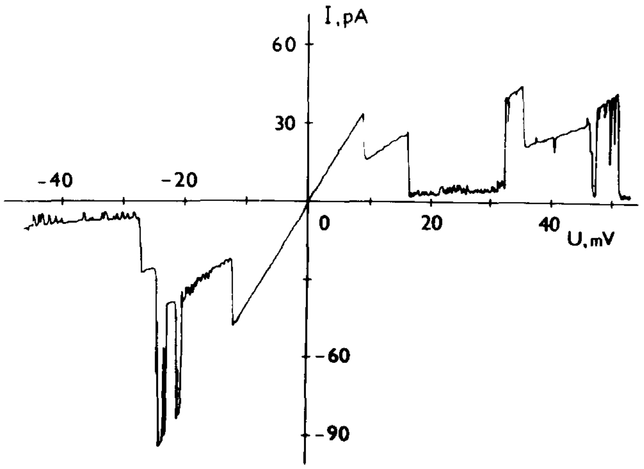

Fig. 2. Current–voltage relationship obtained on a membrane containing one porin channel. Triangular voltage with a rate of increase of 20 mV/min was applied starting from zero value at positive direction. Then voltage was set back to zero and swept at the negative direction. Both solutions contained 1 M KCl, 10 mM citrate-NaOH at pH 4.0.

voltages. The reason becomes clear from Fig. 2 which shows the current–voltage characteristics of the membrane with one channel at pH 4. The channel is seen to start closing already at a voltage of 10 mV. Repeated recordings of *I–V* curves of the same channel reveal more than ten conductance states, each possessing an ohmic conductance. The superposition of these recordings is symmetric, therefore, the gating system of the channel is symmetric as well. The latter, in its turn, accounts for the symmetry of *G–V* dependence of the multi-channel membrane in Fig. 1.

At higher voltages, both positive and negative, the channel tends to occupy the levels with a lower conductance. It is better seen from Fig. 3A which shows the currents passing through the channel in response to voltage steps from zero to the indicated values at pH 3.0. In all recordings the channel, just after the jump, is occupying the level with the highest conductance (shown on the right as level 0). At voltages close to zero the channel remains on this level (not shown). At low voltages (two upper recordings) the channel fluctuates between this open state and a substate with a lower conductance (about 40% of the open state conductance). Recording at 10 mV shows that the transitions between these two states are not simple one-step reactions. There is a certain period during which the channel fluctuates between the two states and then is stabilized in one of them for a long time. The higher the voltage is, the lower is the level on which the channel is stabilized. As can be seen from the lower recordings, the levels with the conductance of only several percents of the open-state conductance are filled at voltages as low as 30–40 mV. At 100 mV the states with a lower conductance and even a complete closing of the channel are observed.

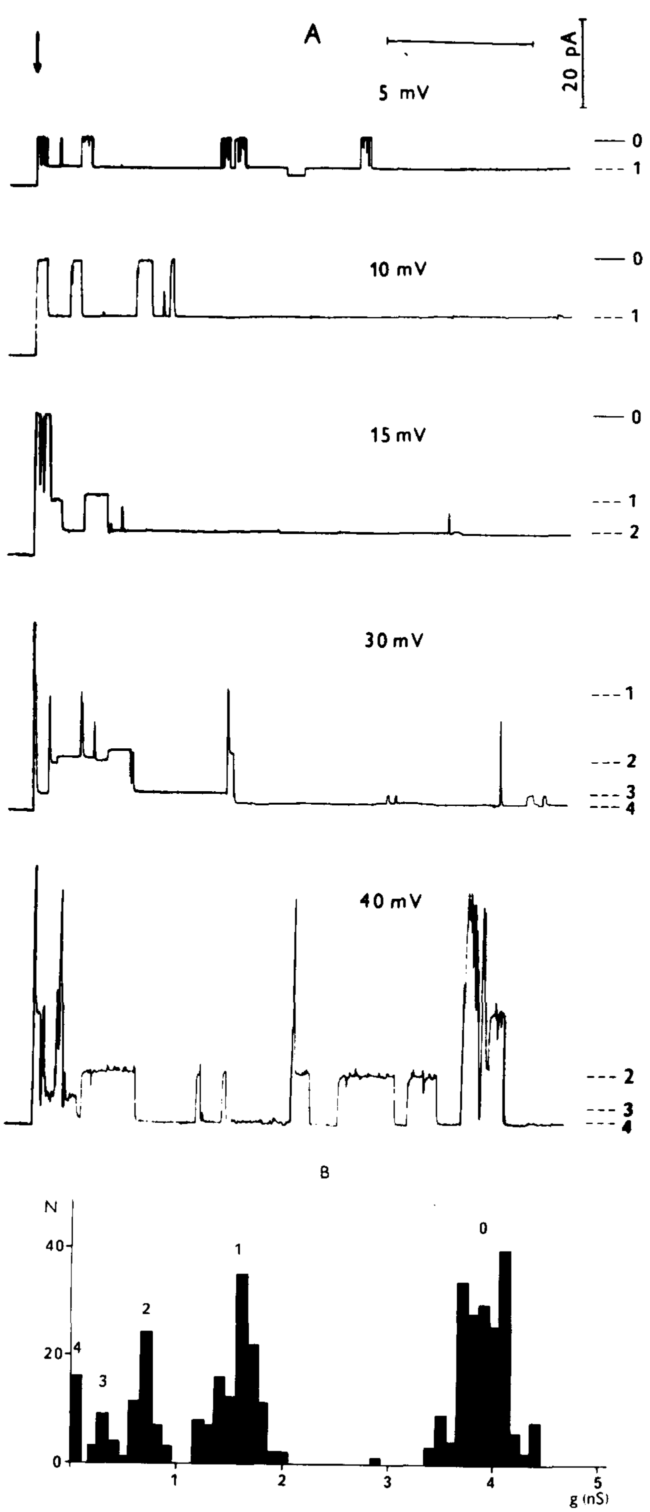

Fig. 3. (A) Recordings of single porin channels at different membrane potentials at pH 3. Voltage was applied at the time points shown by arrows. The horizontal bar is 1 min for the upper record and 15 s for the others. Lines and values on the right show conductances of the main substates related to the open-state conductance. It is seen that (1) the higher the voltage the lower the levels that are filled; (2) fluctuation pattern changes with time: after several fast switchings the channel is stabilized in a long-living state. (B) Histogram of conductance levels in steady state (5 min after voltage was applied). The data were obtained on 188 membranes each containing one channel at voltages from 5 to 100 mV. It is seen that the channel has not less than five levels of different conductance in a steady-state.

Fig. 3B demonstrates a histogram of the steady-state levels obtained from the recordings like those in Fig. 3A at pH 3. The steady state has been reached by 5 min as follows from the multi-channel conductance kinetics. After this time the conductance remained constant during at least 30 min. The histogram shows five conductance peaks with the following mean values: 4.0, 1.6, 0.7, 0.3 and 0–0.1 nS. Within the first 50–100 s after the voltage jump the levels with a conductance of 3.1, 2.5, 2.0, 1.2 nS and others may be also observed, however, these levels never appear in a steady state. Fig. 3B summarizes the data obtained at voltages from 5 to 100 mV. As the conductance of each level does not depend on voltage (see Fig. 2), all possible steady-state levels can be seen in one histogram. However, such a histogram does not give any information on distribution among the states depending on the membrane potential. The data for such a dependence at different pH values are summarized in Table I. It is seen that the channel has the same set of conductance levels at acidic, neutral and basic pH. The pH effects only the voltage value at which a given state is filled. Table I shows the number of observations of a given level in a steady-state at different voltages. For example, at pH 3 the transition from 0 to 1 level occurs at 5–10 mV while a pH 6 and 9 it takes place at 30–40 and 50 mV, respectively. Such a behaviour of a single channel is in agreement with the integral dependence in Fig. 1.

Discussion

The results obtained indicate that a pH shift both to acidic and basic region does not practically affect the conductance of individual states, but it markedly changes the distribution of the channels among the states at non-zero values of the membrane potential *. The following diagram of the channel states is consistent with the data in Figs. 2 and 3.

⇋(−2)⇋(−1)⇋⓪⇋①⇋②⇋

Here central state 0 has the highest conductance, substates −1 and 1 have an equal conductance of approx. 0.4 times the 0-state conductance. The conductance of substates −2 and 2 is about 0.18 times the 0-state conductance and so on. The presence of separate states with equal conductance (for example, −1 and 1) which are occupied at negative and positive voltages, respectively, as well as the absence of direct connections between them are suggested by the kinetics of a multichannel membrane upon a change of voltage polarity. In such an experiment the conductance rapidly rises from the level, corresponding to the voltage before jump, to the conductance of all channels in the open state and then returns to the initial level. The existence of separate states 1 and −1 is also confirmed by Doring and Colombini [25] who showed that the modification of the channels with succynic anhydride from one side of the membrane causes an assimetry of the G–V curve.

The steepness n_0 of the voltage-dependence of transition 0–1 can be calculated from the G–V plot (Fig. 1) by a method similar to that used previously [3,26]. At low positive voltages only states 0 and 1 are occupied. Therefore we can write, assuming the Boltzmann's distribution between the states 0 and 1

$$\frac{N_1}{N_0} = \exp[n_0 \cdot (\varphi - \varphi_0)] \qquad (1)$$

were $\varphi = VF/RT$ is dimensionless membrane potential, n_0 is the number of gating charges moving through the entire transmembrane potential for channel switching between states 0 and 1, and φ_0 is dimensionless potential at which the channels are equally distributed between 0 and 1. N_0 and N_1 are the number of channels in states 0 and 1, respectively.

The total number of channels in the bilayer

$$N = N_0 + N_1 \qquad (2)$$

It follows from Eqns. 1 and 2 that

$$N_0 = N/[1 + \exp(n_0 \cdot (\varphi - \varphi_0))] \qquad (3)$$

The measured integral conductance is

$$G_{\mathrm{meas}} = g_0 \cdot N_0 + g_1 \cdot N_1 \qquad (4)$$

where g_0 and g_1 are channel conductance in level 0 and 1, respectively.

We have, taking Eqns. 3 and 4 into account,

$$G_{meas} = g_0 N[1 + \exp(n_0 \cdot (\varphi - \varphi_0)) \cdot g_1/g_0]/[1 + \exp(n_0 \cdot (\varphi - \varphi_0))] \qquad (5)$$

Factor $g_0 N$ is conductance of all N channels in state 0, which in our case may be measured as a membrane conductance at very low voltage values so that we may calculate the normalized conductance

$$G = G_{\mathrm{meas}}/N \cdot g_0 \qquad (6)$$

When deriving relationship 5 it was assumed that the channels are distributed between only the two states (either 0 and 1 or −1 and 0). So care must be taken to satisfy this condition when determining the n_0 value.

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* Enhancement of the channel voltage dependence at acidic pH has been shown for channels formed by bacterial porin OmpF from *E. coli* [22], hemocyanin [23], colicin Ia [24] and others. However, calculation of the number of gating charges in a way proposed in this paper is not possible until it is stated that the set and conductance of channel levels are pH-independent.

TABLE I

Characteristics of steady-state levels of channels formed by porin from rat liver mitochondria in azolectin bilayers

Data are taken from records like those shown in Fig. 3A for 188 membranes at pH 3, 77 membranes at pH 6 and 30 at pH 9. L is the number of the level. Level conductances were found by averaging of data in a given peak of hystogram at different voltages. At voltages less than 15 mV at pH 6 and 9 practically all channels remained open during tens of minutes and their number was not counted. The ratio of permeability coefficients for chloride and potassium in the last column was calculated using Hodgkin-Huxley-Katz equation from values of the zero-current potential arising on a membrane in the presence of 2–3-fold difference of KCl concentrations in the two aqueous solutions. Values of the potential for different substates were obtained on single-channel membranes from I–V curves taken at slow voltage sweep (20 mV/min).

pH	L	Conductance (nS) (mean ± S.E.)	Number of channels at a given level							P_{Cl^-}/P_{K^+}
			5	10	15	20	40	50	100 mV	
3	0	3.9 ± 0.3	13	0	0	0	0	0	0	4.9 ± 1.2
	1	1.6 ± 0.2	21	50	17	10	1	0	0	1.8 ± 0.4
	2	0.7 ± 0.1	2	4	20	11	7	0	0	3.1 ± 0.9
	3	0.3 ± 0.1	1	2	4	1	6	1	2	6.3 ± 2.2
	4	⩽ 0.1	0	0	0	0	2	6	9	
6	0	4.0 ± 0.3	–	–	–	9	1	0	0	1.9 ± 0.2
	1	1.6 ± 0.2	–	–	–	3	17	21	4	
	2	0.8 ± 0.1	–	–	–	0	0	1	13	
	3	0.3 ± 0.1	–	–	–	0	0	0	5	
	4	⩽ 0.1	–	–	–	0	0	0	2	
9	0	4.0 ± 0.2	–	–	–	9	4	2	0	1.2 ± 0.1
	1	1.8 ± 0.2	–	–	–	1	6	12	1	
	2	0.7 ± 0.1	–	–	–	0	1	2	5	
	3	0.4 ± 0.1	–	–	–	0	0	0	2	

As it follows from Eqns. 5 and 6, n_0 can be determined from a linear dependence:

$$Y = \ln((1 - G)/(G - g_1/g_0)) = n_0 \cdot (\varphi - \varphi_0) \tag{7}$$

Ratio $g_1/g_0 = 0.41$ and the voltage region, in which only states 0 and 1 are filled (6, 25 and 40 mV for pH 3, 6 and 9, respectively), were found in the single-channel experiments (see Table I). $Y(\varphi)$ dependences at three pH values are shown in Fig. 4. The data for each curve at pH 6 and 9 were obtained on a single membrane. At pH 3 the data from several membranes were collected. The values of n_0 and $A = -n_0 \cdot \varphi_0$ were found from the model of simple linear regression $Y_i = n_0 \cdot \varphi_i + A$, $i = 1\ldots, k$, where k is the number of measurements. The standard errors S.E.(n_0) and S.E.(A) were found using estimation dispersions (see Ref. 27)

$$s = (k - 2)^{-1} \cdot \Sigma(Y_i - A - n_0\varphi_i)^2, \qquad x^2 = \Sigma(\varphi_i - \bar{\varphi})^2,$$

$$\psi^2 = \Sigma\varphi_i^2,$$

where

$$\bar{\varphi} = k^{-1} \cdot \Sigma\varphi_i.$$

$$\text{S.E.}(n_0) = S/x$$

and

$$\text{S.E.}(A) = S \cdot \psi / x \cdot \text{SQR}(k)$$

The determined values of n_0 are equal to 3.1 ± 0.2 at pH 9 and 6.1 ± 0.4 at pH 6, compared to the values 2.0 and 4.5, respectively, obtained by Bowen et al. [14] on bilayers of the same lipid composition. De Pinto et al. [17] reported a lower value of 2.3 obtained at neutral pH using bilayers of diphytanoylphosphatidylcholine/phosphatidylserine 4 : 1. Value $n_0 = 32 \pm 2.5$ at pH 3 is unexpectedly large *.

The number of charges in voltage sensor

Fig. 5 represents the pH dependence of n_0. A sharp change of n_0 is seen to occur at low pH, in the region of titration of the carboxyl groups of amino acid residues. This means that the channel voltage sensor includes a great number of carboxyl groups. However, it was shown that in the neutral region the channel is controlled by a positive charge. In other words, at neutral pH the channel voltage sensor includes both negative and positive charges. On the assumption that at pH 3 the charges of all carboxyl groups are neutralized, the num-

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* Mangan and Colombini [26] observed very steep voltage-dependence of mitochondrial porin channels at neutral pH in the presence of polyanion-dextran sulfate. Their explanation of the phenomenon based on potential-dependent concentration of the polyanion near the channel entrance and stabilization of the channel closed state seems to be quite reasonable. However, the possibility that polyanion near the channel may influence the voltage dependence via local pH increase also can not be excluded and requires experimental check.

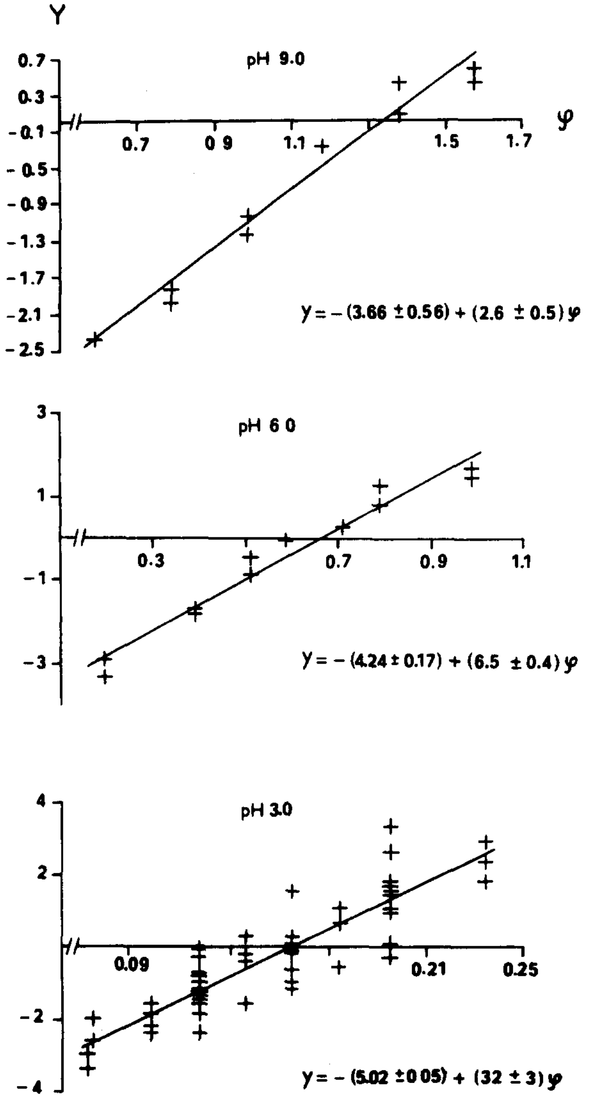

Fig. 4. Determination of the steepness of voltage dependence for transition 0–1 from the linear regression ($Y = n_0\varphi + A$ where $\varphi = VF/RT$, $Y = \ln((1-G)/(G - g_1/g_0))$. Magnitudes of the normalized conductance are obtained as in Fig. 1. Values of g_0 and g_1 of the channel conductance in state 0 and 1 are taken from Table I. Experimental points at pH 6 and 9 obtained on single membranes, data at pH 3 are collected from 12 membranes. Mean and S.E. of the parameters A and n_0 are given next to the regression lines. Correlation coefficients are 0.985, 0.982 and 0.887 at pH 9, 6 and 3, respectively. At each pH value only those voltage values were taken into consideration at which occupation of low-conducting levels 2, 3 and 4 was negligible (see Table I).

ber of positive charges controlling transition 0–1 can be evaluated. This number must be equal to n_0, if during the transition each of these charges passes through the entire potential difference. So, at pH 3 the transition from -1 to 1 must be accompanied with replacement of not less than $2n_0 = 64 \pm 5$ positive charges. This is the minimal estimation of the number of positive charges in voltage sensor. The number could be underevaluated by the following reasons: (1) only a fraction of the externally applied voltage may be felt by gating charges, which is supported by a conclusion made by Mangan and Colombini [26], that only 70% of voltage drop occurs within the channel; (2) the charges may pass only a fraction of the potential difference even if the entire external voltage drops on the channel; (3) dependence n_0(pH) in Fig. 4 has not reached the saturation at pH 3, so not all the carboxyl groups have been neutralized; (4) transitions -1–0 and 0–1 are not the only voltage-dependent ones. For example, as seen from Table I the voltage increase shifts the equilibrium from 1 to 2 at pH 3. The steepness of voltage dependence of this transition was estimated from the Boltzmann distribution between states 1 and 2 as $n_1 = 6 \pm 2$.

To compare the number of charges in the voltage sensor with the total number of the charged groups per channel, the channel stoichiometry should be known. Symmetry of the gating system suggests that the channel is formed by an even number of molecules with a symmetric arrangement relative to the channel center. On the basis of sedimentation analysis Linden and Cellerfors [10] came to a conclusion that porin solubilized with Triton X-100 exists as a dimer. As the conductance of the lipid bilayer increases linearly to the concentration of the porin-Triton extract in aqueous solution [7], the channel may be concluded to be formed by the two porin molecules. According to the amino acid analysis [6], one porin molecule contains about 6 arginine, 27 lysine and 4 histidine residues, which are positively charged at low pH. Therefore, the dimer contains about 74 side chain groups which may be positively charged. Thus, almost all residues which may be positively charged are involved in the voltage sensor and their charges must be transferred through the entire membrane field upon the transition of the channel from -2 to 2.

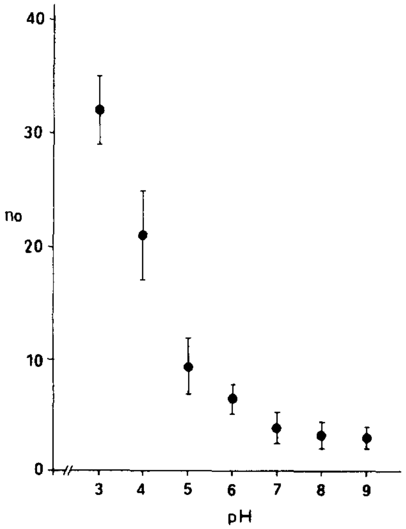

Fig. 5. pH dependence of n_0. Data from Fig. 4 are included as well as data obtained on single membranes at different pH values.

When pH of aqueous solutions increases from 3 to 5, the number of positively charged residues does not seem to change (pK values of free amino acids are 6.0, 10.8 and 12.5 for hystidine, lysine and arginine, respectively). At the neutral region (pH 6–8) a further diminishing of the voltage dependence occurs due to neutralization of positive charges of hystidine residues. At pH 9 the channel is controlled by the net positive charge approximating $n_0 = 6$ (see Fig. 5). Hence, in this pH region the voltage sensor contain not less than 60 positive and 50 negative charges. Such a great number of amino acids involved in the voltage sensor suggests that the sensor is not localized but distributed along the entire channel structure.

Field redistribution within the pore

A strong voltage-dependence observed can be not explained assuming that distribution of the electrical field in the region of voltage sensor does not depend on a channel state (for example, if the channel has cylindrical pore with a linear distribution of the membrane potential in all the states, while the states differ only in a pore diameter). In this case almost all positively charged groups with a symmetric arrangement relative to the central plane of the membrane in state 0 should be transferred to one surface of the membrane in state 1 and to the opposite surface in -1. Only so all these charges could pass through the entire membrane field upon the transition -1–0–1. Therefore, the whole channel former should appear on the membrane surfaces in states 1 and -1. Nevertheless, the channel conductance should remain high.

These results can be easily explained in another model previously proposed by Colombini [25, 28]. It is assumed that the charges of the voltage sensor face water-filled pore and, therefore, their energy depends on the membrane potential distribution along the pore. The pore is symmetric only in state 0. In states 1 and -1 it has a cone shape (Fig. 6) with non-uniform electrical resistance along the pore. This results in non-uniform distribution of the potential difference most of which drops in a narrow part of the pore. Thus, closing of the channel leads to redistribution of the field within the pore and a change of energy of the charges of voltage sensor even if these charges do not replace along the pore axis.

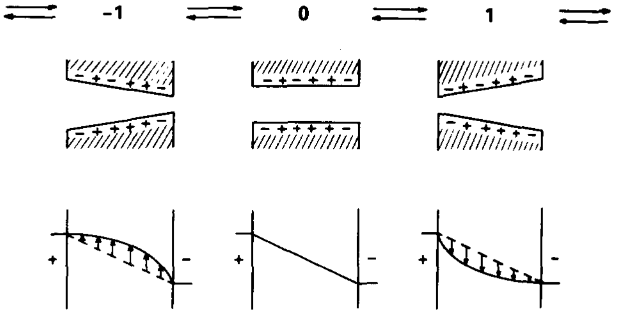

Fig. 6. Schematic representation of the three main states of the channel (above) and distribution of the potential along the pore (below) according to the model suggested by Colombini [28]. The figure shows that redistribution of the field within the pore upon channel closing results in stabilization of one of the closed states.

Distribution of charges along the pore

In the model considered charges on the pore walls serve both as voltage sensor and selectivity filter (see Adelsberger-Mangan and Colombini [15]). Therefore, neutralization of negative charges influences not only the gate but the selectivity as well (see Table I). As almost all positive charges of the channel former are involved in voltage sensor the same charges are responsible for anionic selectivity. However, contribution of a charge in voltage dependence and selectivity may be different depending on its position along the pore. As is seen from Fig. 6, most of the potential change occurs at the central part of the pore. So, charges in this part contributes much in voltage dependence while the charges near the pore entrances do not. On the contrary, channel selectivity in states 1 and -1 are influenced mainly by charges in more narrow entrances. As all positive charges contribute to the voltage dependence, they must be situated in the central part of the pore as well as the charges of carboxyl groups, neutralization of which influences the voltage sensitivity of the channel. Some carboxyl groups appear to be situated near the entrances. Negative charges of this groups at neutral pH should result in an increase of cationic over anionic selectivity. This is in agreement with the data by Ludwig et al. [29] who showed that transition 0–1 at neutral pH leads to a reverse of selectivity. At pH 3 charges at the entrances are neutralized and channel closing is accompanied only with a decrease of anionic selectivity, as is seen from Table I.

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