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Does VDAC insert into membranes in random orientation?

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Abstract

It is widely accepted that voltage-dependent anion-selective channel (VDAC) inserts into planar lipid bilayers in a random orientation. This is in contrast to the well-documented oriented insertion of various channel-forming proteins. Because of the potential importance of this issue, we have examined the orientation of VDAC inserted in membranes. The time constants of the VDAC-current relaxation in response to applied positive and negative voltage pulses were used to characterize the channel orientation.

We have found that VDAC channels can be separated into two groups according to differences in the time constant ratio. The difference in time constant ratio between the two main groups of VDAC channels was quantitative, and not qualitative as would be expected for opposite topologies. This finding allows us to hypothesize that both groups of VDAC channels possess a qualitatively similar asymmetry with respect to the localization of voltage-gated domains and, consequently, with respect to its entire molecular structure. The probability of having each type of VDAC channel conformation is predetermined by the protein structure in aqueous solution.

A striking resemblance between asymmetry in voltage sensitivity at the single-channel and multi-channel levels was also demonstrated. The first inserted channel seems to direct subsequent insertions of channels with a similar conformation.

Keywords: Ion channel; VDAC; Lipid bilayer; Oriented insertion

1. Introduction

Since the first report [1], porins isolated from eukaryotes have been frequently called voltage-dependent anion-selective channels (VDAC), on the basis of their electrophysiological characteristics. It is widely believed that VDAC is composed of one ~30-kDa polypeptide [2] and forms a large aqueous pore with a diameter that appears to vary along the pore axis from 2.0 to 4.0 nm [3,4]. VDAC channels are found in several cell membranes [5–8], including the mitochondrial outer membrane and the plasma membrane. The structure and electrophysiological properties are conserved in VDAC from different sources [5,9,10].

VDAC forms voltage-dependent channels, which can turn themselves into lower conductance states when positive or negative potentials (larger than 20 mV) are applied to the membrane. It is usually stressed (with rare exceptions [11,12]) that these two gating processes have virtually the same steepness of voltage dependence and the same midpoint voltage. As a consequence, it is widely accepted that VDAC inserts itself into planar bilayers in a random orientation (i.e., one channel opening does not preferentially face one aqueous compartment). This assertion stands in contrast to the well-documented oriented insertion of channel-forming proteins in planar bilayers [13–19] and to the oriented insertion (into planar lipid bilayer) of purified ion channels normally located in natural membranes [20–23]. Moreover, in many cases, when the authors did not investigate the orientation of the reconstructed porins from different sources, they noted that in response to positive and negative voltage pulses "the current decreased in a somewhat asymmetric fashion which suggested asymmetric insertion of the channels into the membrane" [7,24–26].

Recent studies involving site-directed VDAC mutants [27,28] have demonstrated that VDAC appears to insert in a

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single orientation in one individual bilayer, however, the orientation of VDAC is random from membrane to membrane. Because of the potential importance of this issue, we have further examined the orientation of VDAC in membranes. The time constants of the VDAC-current relaxation in response to applied positive and negative voltage pulses were used to characterize the channel orientation under different conditions.

2. Materials and methods

VDAC isolated from *Neurospora crassa* mitochondria was kindly donated by Prof. M. Colombini (University of Maryland, USA). Azolectin (Type II, Sigma) was purified by acetone extraction [29]. Agarose (Sigma) was dialyzed against 100 volumes of double-distilled water for at least two days at 4 °C. Water was changed every 12 h. Other chemicals were of analytical grade. Water, double distilled in glass, was used to prepare all buffer solutions. For bilayer experiments, basically two standard solutions were used. The first contained 1000 mM KCl, 10 mM acetic acid, 1 mM EDTA, pH 6.5 (buffer 1). The second contained 1000 mM KCl, 10 mM acetic acid, 6 mM $CaCl_2$, 1 mM EDTA, pH 6.5 (buffer 2).

Experiments were conducted using planar lipid bilayers formed by the method of Montal and Mueller [30] as described earlier [31]. Briefly, a thin (20 μm) Teflon film with a small 0.2- to 0.3-mm diameter hole divided a Teflon chamber into two compartments, named *cis* and *trans*. The hole was pretreated with 2% solution of hexadecane in *n*-hexane and allowed to dry for 5–8 min. Appropriate aqueous solutions were then layered in the bottom of each compartment of the Teflon chamber, and 10–20 μl of a 2% (w/v) azolectin solution in hexane was layered on top. Ten to twelve minutes were allowed for hexane evaporation, leaving a phospholipid monolayer on the surface of the water solution in each compartment. The two monolayers were raised to form a phospholipid bilayer across the hole in the film. The final volume of a solution in each compartment was 2 ml. Electrical capacity of bilayers was about 0.8 $\mu F/cm^2$.

The azolectin membranes were chosen in our experiments in order to reproduce the conditions utilized by other groups in the investigation of VDAC insertion [27,32,33] and to better compare published data with results obtained in this study.

The voltage on the *trans* side was maintained at virtual ground by a current amplifier (K284UD1A, USSR), and the desired voltage was applied to the *cis* side. Ag/AgCl electrodes were used to interface with the solutions through salt bridges (3% agarose with 3 M KCl). Once the bilayer was formed, a 2–10-μl aliquot of *N. crassa* VDAC was added (unless stated otherwise) to the *trans* side of the chamber with stirring which continued for about 30 s. A stock 1% Triton X-100 VDAC preparation was utilized to get the multi-channel bilayers, while for single channel experiments the stock *N. crassa* VDAC preparation was previously diluted as much as 10-fold just before its addition. VDAC inserted spontaneously. Steady-state conductance of bilayers in the multi-channel studies was reached in 40–60 min. Channel insertion in the single channel experiments usually occurred between 20 and 30 min. Kinetic properties of VDAC were studied under voltage-clamp conditions (see Ref. [31] for details). Bilayers were not reformed when VDAC was already present in the bathing solution. The addition of a 20-μl aliquot of a pure 1% Triton X-100 solution to either compartment of the experimental chamber did not affect noticeably the bilayer properties.

The amplifier signal was filtered by a homemade passive filter with corner frequency equal to three-eighths of the sampling frequency, monitored with a Nicolet-2090-III storage oscilloscope (Nicolet Technologies, Madison, WI) and recorded on an IBM-compatible 486/487 100 MHz computer with DT01-EZ 12-bit A/D converter board (Data Translation), which could be operated at 12.5–27,500 Hz sampling frequency. Whole Cell Electrophysiology Program (WCP V1.7b, J. Dempster) software was employed.

Bilayers with steady-state conductance of 200 to 1300 nS were used to measure the kinetic features of the channels. On a step transition of the clamp voltage from 0 to ±40 mV, the current shows a large initial value, which then decreases in an essentially exponential manner to a lower steady-state value. The relaxation can be fitted adequately with one exponential component. The time constant of the process was used in this study to compare the relaxations observed.

3. Rationale

The central idea on which our experiments were founded is the determination of VDAC orientation in membranes based on a comparison of the kinetic constants of the channel transition from a high to a low conductance state. As a voltage-gated channel, VDAC must possess a voltage sensor that will respond to changes in transmembrane voltage. In theory, the voltage sensor could be a charged domain moving along the electric field, or a strong dipole that can reorient itself with respect to the electric field. VDAC channels have two separate gating processes: one at positive transmembrane potentials and the other at negative potentials. The fact that there is at least one mutation that affects both gating processes [34] indicates that the domains that move during the two gating processes may overlap. Instant and steady-state $I–V$ curves are symmetrical in most wild-type VDAC, so the orientation of the channel insertion cannot be inferred. On the other hand, the kinetics of VDAC channel transition from high to low conductance appears dependent on the sign of the voltage [7]. This makes it possible to utilize the ratio of time constants of the VDAC

current relaxation (τ) in response to applied negative (-40 mV) and positive ($+40$ mV) voltage pulses as a parameter indicating the channel orientation in membranes, denominated as asymmetry (A; $A=\tau_-/\tau_+$).

At the experiments with a single VDAC channel, in the absence of a better criterion, we arbitrarily considered the asymmetry to be the ratio between the lifetime (t) that a channel spends in a fully open state (until first closing) at negative voltage, and that at positive voltage ($A^{\#}=t_-/t_+$).

The asymmetry could be a result of various factors, but it always indicates structural asymmetry of VDAC incorporated in a lipid bilayer. Any deviation of A from 1 (one) indicates asymmetry in conformational rearrangements associated with gating and asymmetry in the channel structure itself. If either of the two possible orientations of VDAC has A larger than 1, the VDAC with opposite orientation must have a qualitatively different A (smaller than 1). Quantitative, as opposed to qualitative, differences are interpreted as representing channels that have relatively small differences in structure and probably the same orientation in membranes.

To check whether the experimental apparatus itself is a possible source of the asymmetry, additional experiments were done in which the VDAC aliquot was added to the "cis" compartment of the experimental cell. To compare with the data obtained in the presence of VDAC in the "trans" compartment, the asymmetry of these "cis" experiments was defined as $A=\tau_+/\tau_-$. In both cases, the values for asymmetry obtained in the "cis"- and the "trans"-experiments for the respective pools were similar (data not shown).

Generally speaking, the detergent added to the VDAC aliquot may impact the asymmetry of VDAC via asymmetrical changes in bilayer properties since VDAC was always added from detergent solution at one side of bilayers only. At first glance, such a detergent effect would appear reasonable. However, as we will demonstrate below, the distributions of asymmetry in experiments at the single channel level and in experiments with multi-channel bilayers are analogous, although the final detergent concentrations differed about 100-fold. Hence, the detergent does not have a detectable effect on VDAC asymmetry in our experiments. Additional support for this conclusion comes from the fact that our experimental design reproduces as closely as possible the protocols of other researchers who saw a random orientation of VDAC.

4. Results and discussion

4.1. Role of pH

4.1.1. Qualitative analysis

After the bilayer membrane was formed, addition of wild-type *N. crassa* VDAC at a concentration of approximately 10 μg/ml led to a stepwise increase in transmembrane current, indicative of single channel formation (Fig. 1). At low voltage, most of these conductance steps were directed upward; the downward steps corresponding to closing events were observed only rarely. The cumulative histogram shown in Fig. 1 (inset) represents the conductance values for these unitary events. It is evident that VDAC channel conductance is homogeneous. The most frequent values were in the range of 3.0–4.5 nS with a mean value of ~ 4 nS.

After membrane conductance stopped increasing (which usually happened 40–60 min after the experiments were initiated and more than 50 channels had spontaneously inserted), voltage dependence was examined by applying an elevated potential. Current instantaneously increased as the increased voltage drove ions through the channels, and then diminished (Fig. 2) as the channels shifted to lower conductance states. All relaxations (recorded in response to ± 40 mV voltage pulses) have a single time constant and are fitted adequately with one purely exponential component.

Generally speaking, pH is important to protein secondary structure, and as a consequence, to channel insertion and orientation. We have found that at low pH (5.8), the VDAC-current relaxations observed in response to positive and negative voltage pulses are closer to each other than at pH

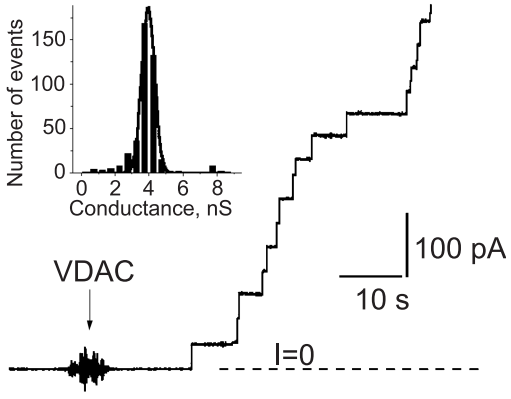

Fig. 1. A typical single-channel recording and the histogram of single-channel conductance of VDAC-channels (inset). A sample of an original single-channel recording at +10 mV fixed voltage is presented. The dashed line indicates the zero current level, and the arrow indicates the addition of the *N. crassa* VDAC (10 μM) to the *trans* compartment. The subsequent current noise is a result of solution stirring. Bathing solution contains 1 M KCl, 1 mM EDTA, 10 mM K-acetate buffer at pH 6.5. Bilayers were formed by the Montal method from purified azolectin. Experiments were done at room temperature, 25 ± 2 °C. Current and time scales are given in the figure. All other conditions are described in Materials and methods. Inset: The amplitude histogram of conductance steps induced by VDAC in voltage-clamped (+10 mV) bilayers is demonstrated. To build the histogram, the observed conductance steps like those shown in the current traces in the figure are utilized. Records were discarded if any of the open channels temporarily closed. More than 400 ion channels were registered (5–15 channels per membrane) under experimental conditions. Bin width was 0.5 nS. The line (drawn by Microcal Origin, version 5.0 software) indicates the theoretical normal distribution of the main pool of the channel events. The assay solution contained 1 M KCl, 1 mM EDTA, 10 mM K-acetate buffer at pH 6.5. All other conditions are described in Materials and methods.

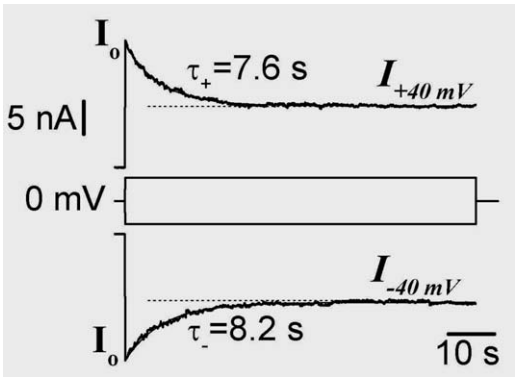

Fig. 2. Effect of voltage on the conductance induced by VDAC in lipid bilayers. Samples of the original time courses of the transmembrane current after application of voltage pulses of ± 40 mV on bilayer containing several dozens of VDAC-channels are presented. Final VDAC concentration was 50 μM. The voltage pulse protocol is shown in the central part of the figure. Dotted lines show steady-state value of transmembrane current at ± 40 mV. Current and time scales are given in the figure. The solid line represents the best fit of the current decay with mono exponential function. All other conditions are described in the legend to Fig. 1 and in Materials and methods.

6.5 or, especially, at pH 7.2. Significantly, asymmetry was found to be dependent on the experimental conditions. At pH 7.2 the asymmetry was larger than 1 in 100% of the observations (Table 1). Acidification results in the progressive appearance of bilayers with the opposite (low) type of asymmetry ($A<1$). Such asymmetry was observed in ~ 8% of cases at pH 6.5 and in ~ 25% at pH 5.8.

Experiments made with membranes containing only a single channel appear to be in accord with results obtained at multi-channel bilayers. So, at pH 6.5, the membranes with high and low asymmetry were seen in a proportion of ~ 9:1 and ~ 7:3, in the presence and absence of $CaCl_2$ in the bathing solution, respectively. This shows a striking resemblance to the asymmetry distribution at the multi-channel levels: ~ 9:1 and ~ 8:2, respectively (Table 1). This similarity appears to represent the oriented and self-catalyzed (at the same time) incorporation of wild-type VDAC, which had been shown earlier for mutated porin [27,32,33]. To further examine this phenomenon, another set of experiments was done in which the asymmetry was monitored during the process of VDAC channel incorporation. As a result, we found that asymmetry is actually independent of the number of channels presented. Two typical results for bilayers having different initial asymmetry ($A>1$ and $A<1$) are presented in Fig. 3. Thus, the results obtained are congruent with the hypothesis that incorporation of wild-type VDAC in lipid bilayers is an oriented and self-catalyzed process: the parameters of the first channel appear to determine the properties of subsequent channels.

In summary, our results (based on qualitative analysis of the time constant of current relaxations) appear to demonstrate that at neutral pH there is unambiguously one type of structural asymmetry of VDAC in lipid bilayers. At acid pH, two clearly different structures of VDAC are seen. As established earlier [35], VDAC in

Table 1
Number of bilayers with different asymmetry in the time constant of VDAC current relaxations at different pH

pH	N_1 ($\tau_-/\tau_+>1$)	N_2 ($\tau_-/\tau_+<1$)	$P^{\#}$	N_1^s ($\tau_-/\tau_+>1$)	N_2^s ($\tau_-/\tau_+<1$)	$P^{\#}$
5.8	9	3	<0.083	–	–	
6.5	25	2	<0.00001	23	2	<0.00003
7.2	19	0	<0.00001	–	–	
6.5[a]	28	8	<0.00086	31	11	<0.00203

N_1 and N_2 are the number of experiments when the time constant at negative voltage (τ_-) was larger than the time constant at positive voltage (τ_+) and vice versa, respectively. N_1^s and N_2^s are the number of experiments when the lifetime of a single channel in a fully open state (until first closing) at negative voltage was larger or smaller than that at positive voltage, respectively. Solution contained 1 M KCl, 1 mM EDTA, 6 mM $CaCl_2$, 10 mM K-acetate buffer at the desired pH.

[a] Solution contained 1 M KCl, 1 mM EDTA, 10 mM K-acetate buffer at pH 6.5.

[#] The chi-square test was used for testing the distributions' similarity to the hypothetical 0.5: 0.5 ratio (number of events with $A>0$: number of events with $A<0$).

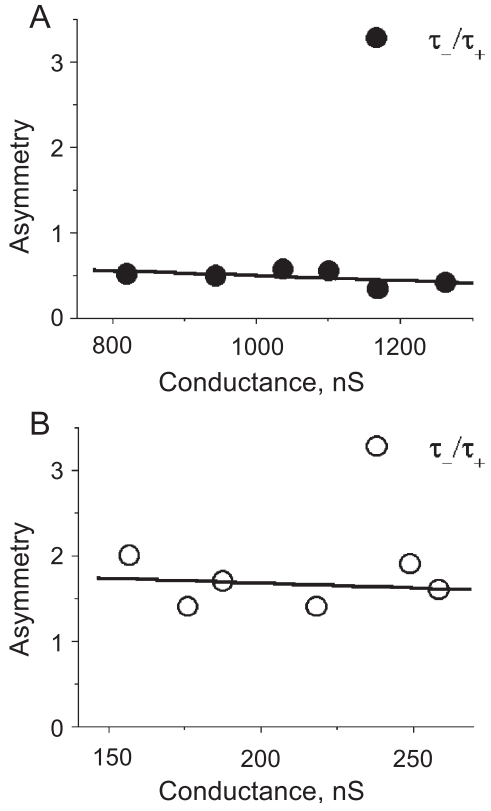

Fig. 3. The time constant ratio of the bilayer during the continued incorporation of VDAC. Presented are typical experiments done with bilayers having initial asymmetry smaller (A) and larger (B) than 1. The time interval between each asymmetry measurement was 10–15 min. Conductance was measured at 10-mV voltage pulses. Experiments were carried out in solution containing 1 M KCl, 1 mM EDTA, 6 mM $CaCl_2$, 10 mM K-acetate buffer at pH 6.5. All other conditions are described in the legend to Fig. 2 and in text.

detergent suspension undergoes a pH-dependent reversible conformational change. In that study, detectable changes were observed when the pH was decreased below pH 5. Detergents as well as lipid bilayers usually considerably increase the stability of membrane proteins to disturbing influences. However, in our experiments, the addition VDAC (that is initially at the optimal detergent concentration) in the bath solution is accompanied by considerable reduction of detergent concentration, which becomes far below its critical micellization concentration. Hence, one can suppose that in our case, weaker acidification already could promote certain conformational change of VDAC in solution, which allows for VDAC to be inserted in two different conformations. These two conformations can be seemingly identified with two hypothetical opposite channel structures [27]. A hypothetical localization of the VDAC channel in lipid bilayer is depicted in Fig. 4A.

4.1.2. Statistical analysis

To test the validity of this identification, statistical analysis of the asymmetry in time relaxation at different pH levels was performed. The asymmetry values for separate experiments were plotted in a cumulative histogram. Based on the hypothesis of the existence of two opposite VDAC structures in membranes, we expected to find the asymmetry to be uniformly distributed at neutral pH and to demonstrate multiple (at least two) peaks at acid pH. We also expected that each pool of VDAC would be fitted well with a Gaussian curve and that at the acid pH there would be two pools of VDAC, which could be fitted well with two Gaussians: one with the mean value of the asymmetry smaller than 1 (for the small pool of VDAC) and the second with the mean value of the asymmetry larger than 1 (for the larger pool) (Fig. 5H). Unexpectedly, it was found that the asymmetry in time constants is mainly distributed between two clearly visualized pools at all three pH levels (Fig. 5). Moreover, the mean values of the asymmetry for all peaks (pools) at all conditions were larger than 1. Additionally, we have discovered that a relatively small fraction of the channels with asymmetry smaller than 1 belong to the left "wing" of the first pool of VDAC with the mean value of the asymmetry larger than 1 in all conditions used. Therefore, one could not consider that these VDAC channels have qualitatively different orientations in the bilayer than the other channels of the same pool. This is a crucial point. To be a part of the pool means to have a qualitatively similar structure and consequently to be equally inserted with relatively small variation in localization of voltage-gated domains inside the channel. Therefore, it is reasonable to hypothesize that there are two principal thermodynamically favored structures of VDAC-channel in planar bilayers and they are topologically similar.

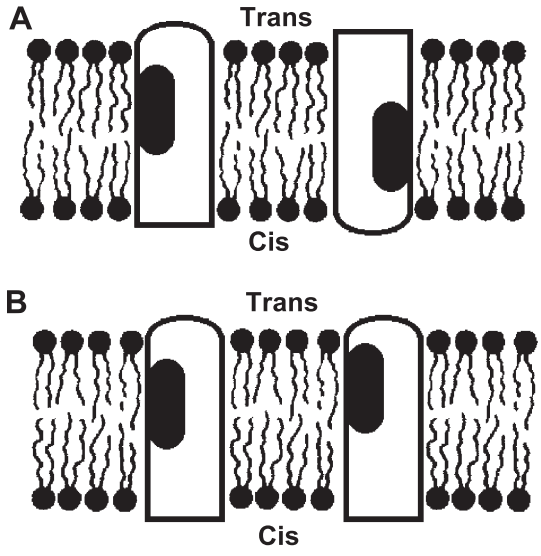

Fig. 4. A cartoon showing hypothetical VDAC channel localizations in lipid bilayer suggested by Zizi et al. [27] (A), and by the present study (B). White bullet represents VDAC-channel; black oval represents its voltage sensor. In all cases VDAC inserts from the *cis* side. (A) Two opposite VDAC topologies where one topology of the channel can be obtained by 180° rotation (relative the central membrane plane) of the second one and vice versa. The asymmetry of the channel in the first topology has to be inversely proportional to the asymmetry of the channel in the second topology. (B) Two conformations of topologically similarly inserted VDAC-channels with the localization of the voltage sensor at slightly different fixed position. The asymmetry of the channel in the first conformation is only quantitatively different from that in the second conformation.

It could be seen (Table 2) that although the localization of peaks is almost invariant, their sampling fractions are distinctly dependent on pH: with acidification, a portion of the first pool of membranes (with lower asymmetry) increases, while a portion of the second pool decreases. Of course, mean values of asymmetry naturally follow the redistribution of bilayers between those two pools with pH. Practically the same distribution of asymmetry was seen at the single channel experiments (Fig. 5D and F; Table 2), which additionally supports the hypothesis of self-catalyzed insertion of VDAC in membranes.

In general, VDAC channels are mainly distributed between two groups by quantitative analysis of asymmetry in time constants. However, channels in these two groups only have quantitative differences in time constant asymmetry. Therefore, it appears that VDAC does not build opposite channel structures where one topology of the channel can be obtained by 180° rotation (relative to the central membrane plane) of the second one (Fig. 4A). Most probably it forms two topologically similar conformations of the channel (Fig. 4B).

4.2. Pre-incorporated VDAC and pH-shift

The analogy between distribution of the asymmetry seen for bilayers containing both single and multiple channels indirectly indicates the absence of the inter-

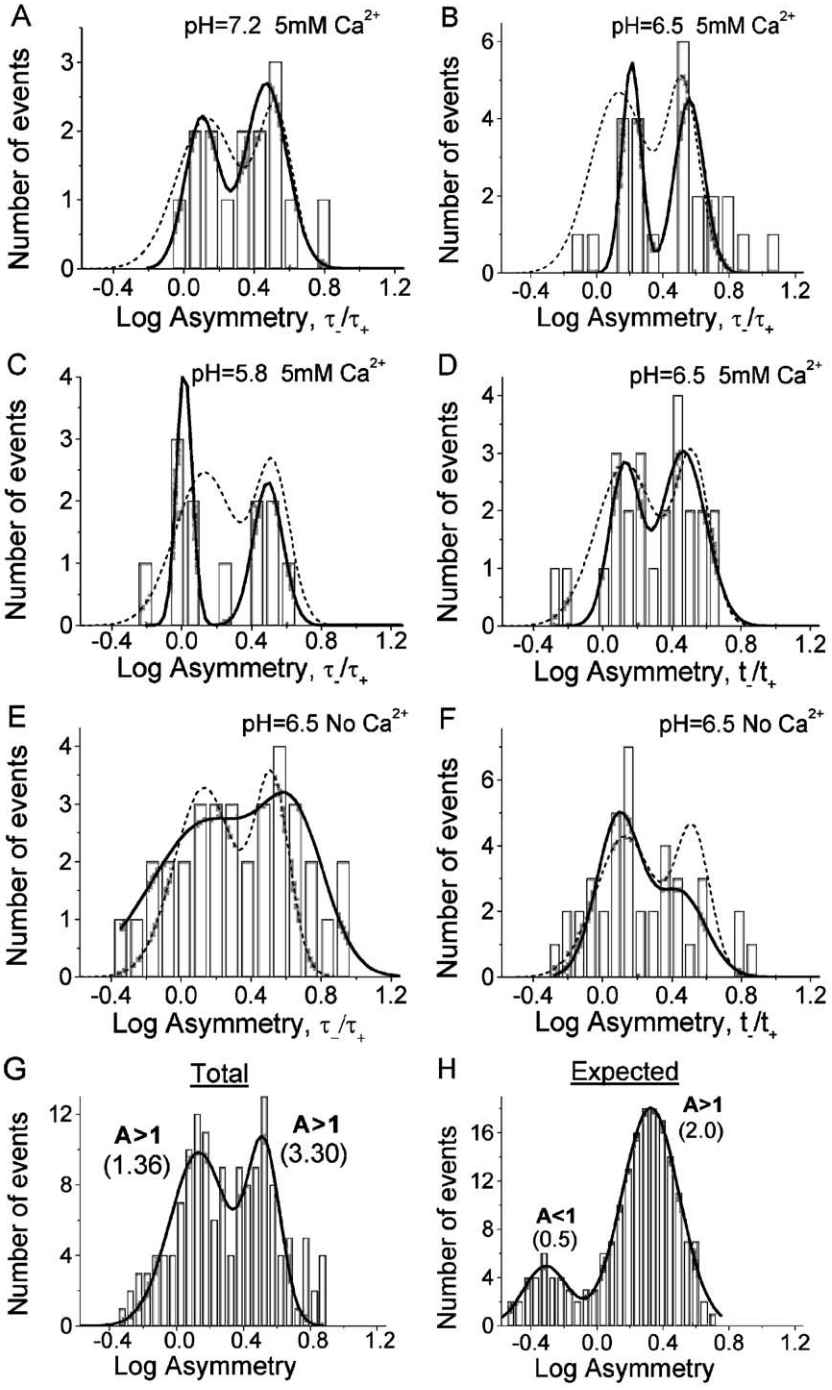

Fig. 5. Distribution of VDAC-modified bilayers according to their asymmetry at different pHs. Logarithm asymmetry of single channels ($A^*= t_-/t_+$; D and F) or multi-channel bilayers ($A = \tau_-/\tau_+$; A, B, C and E) was used to build the histogram under each set of experimental conditions. Bin width was 0.091 and 0.071 for membranes containing multiple channels and a single channel, respectively. Total histogram is presented at G, where the all data from A to E (148 experiments all in all) are summarized. H is the expected unreal histogram with two pools of VDAC: the small one with the mean value of the asymmetry smaller than 1 and the large pool with the mean value of the asymmetry larger than 1. The construction of this histogram was based on the hypothesis about VDAC insertion into planar bilayers in random orientation. The lines were drawn by Microcal Origin, version 5.0 software. The solid lines show the fitting with the sum of two Gaussians. The dashed line in Fig. 5A–E is superimposition of the curve in Fig. 5G drawn to demonstrate consistency of the data with two populations of channel conformers. By the criterion of the sum (S) of square deviations of observations from appropriate theoretical Gaussian distributions ($\Sigma(X_T - X_E)^2$), the fitting of all experimental histograms with the sum of two Gaussians (S_{Gauss2}) was always better with only one Gaussian (S_{Gauss1}). For A, B, C, and D, the solution contains 1 M KCl, 1 mM EDTA, 6 mM $CaCl_2$, 10 mM K-acetate buffer with desired value of pH. For E and F, the solution contains 1 M KCl, 1 mM EDTA, 10 mM K-acetate buffer at pH 6.5. All other conditions are described in Materials and methods.

Table 2
Main asymmetry pools of VDAC-modified bilayers and relative sampling fractions

pH	*A* for peak 1	τ, sec peak 1	*A* for peak 2	τ, sec peak 2	*R*	A_1	A_2
5.8	1.03 (0.93 ÷ 1.15)	(+)10.9 ± 3.2 (−)11.4 ± 3.1	3.12 (2.56 ÷ 3.79)	(+)3.6 ± 1.4 (−)12.9 ± 4.7	0.94	2.0 ± 1.2	2.0 ± 1.2
6.5	1.62 (1.42 ÷ 1.85)	(+)9.0 ± 3.9 (−)12.7 ± 7.5	3.62 (2.95 ÷ 4.43)	(+)4.4 ± 2.4 (−)13.0 ± 5.6	0.79	2.7 ± 1.5	
7.2	1.26 (1.02 ÷ 1.57)	(+)6.5 ± 2.6 (−)8.5 ± 2.9	2.94 (2.24 ÷ 3.86)	(+)5.1 ± 2.0 (−)13.3 ± 3.4	0.65	2.5 ± 1.4	2.8 ± 2.1
6.5[a]	1.34 (1.09 ÷ 1.63)		2.92 (2.17 ÷ 3.94)		0.61	2.2 ± 1.1	
6.5[b]	1.44 (0.68 ÷ 3.07)	(+)12.5 ± 5.9 (−)11.9 ± 3.5	4.48 (3.00 ÷ 6.71)	(+)4.7 ± 1.9 (−)14.5 ± 6.4	2.32	2.3 ± 1.6	
6.5[a,b]	1.24 (0.93 ÷ 1.66)		2.8 (2.00 ÷ 3.93)		1.64	2.0 ± 1.6	

'*A* for peak 1' and '*A* for peak 2' are asymmetric with the corresponding range of value re-transformed from the log-value for corresponding peaks as seen in the histogram in Fig. 5. These parameters (asymmetry and range) were calculated as 10^{a} and $10^{a \pm \sigma}$, respectively, where *a* and σ are the mean and standard deviation for the log values. *R* is a relative sampling fraction of peak 1. It is defined as a ratio between the number of channels that belong to peak 1 and peak 2 seen in Fig. 5.

The presented relaxation time constants, τ, are mean values obtained at positive (+) and negative (−) voltage pulses (± STD). The differences between τ obtained at different pH for any peak are statistically unreliable ($P>0.05$). Mean value of the time constants (peak 1 + peak 2) is decreased with decreasing pH because the sampling fraction of peak 1 grew.

A_1 is the mean value of time constant asymmetry (± STD) at the pH noted in column 1. A_2 is the mean value of asymmetry after pH-shift from 5.8 to about 6.5–7.0 and from 7.2 to 5.8–6.0. Solution contained 1 M KCl, 1 mM EDTA, 6 mM $CaCl_2$, 10 mM K-acetate buffer at the desired pH.

[a] Single channel experiments.

[b] Solution contained 1 M KCl, 1 mM EDTA, 10 mM K-acetate buffer at pH 6.5.

conversion between two established structures of VDAC-channels inside the membrane. However, to confirm this, pH-shift experiments were done as follows. VDAC was incorporated in bilayers at pH 7.2 and its kinetic parameters were measured. Appropriate aliquots of 1 M acetic acid then were added in both compartments of the chamber to make a symmetrical pH-shift to about 5.8, and kinetic parameters of VDAC were again examined. A second protocol was also employed in which initially acid pH (5.8) was neutralized with appropriate aliquots of 1 M KOH. We assumed that if VDAC channel structure in bilayers is inconvertible, the asymmetry should not be changed with pH shift.

Thirteen successful experiments were done with the first protocol (acidification, pH 7.2 → pH 5.8) and eight experiments were carried out with the second protocol (alkalization, pH 5.8 → pH 7.2). It was observed that in accordance with existing knowledge of VDAC behavior [36], the numerical values of time constants obtained at +40 and −40 mV decreased with acidification from 5.4 and 12.0 s to 3.4 and 7.0 s, and increased with alkalization from 6.8 and 10.6 s to 9.1 and 14.8 s, respectively, although in each separate experiment the ratio τ_-/τ_+ remained practically invariant. Mean values of the asymmetry were close to 2.0 at pH 5.8 and did not increase (as one would expect if the inter-conversion between two established structures of VDAC-channels inside of the membrane takes place) with pH shift to 7.2 (Table 2). For the second protocol, the mean value of asymmetry does not decrease as a result of the pH shift from 7.2 to 5.8. On the contrary, a tendency towards increase can be seen (Table 2). Thus, the apparent insensitivity of asymmetry of time constants to pH-shift was established. This indicates that VDAC-channel structure in bilayer is essentially not convertible and that kinetic asymmetry is predetermined by differences in VDAC structure in solution. The apparent insensitivity of pre-incorporated VDAC channel structure to pH-shift is not something unusual. It is in accord with the well-known stabilizing effect of bilayer, which makes protein structure resistant to disturbing influences [37–40].

4.3. Role of Ca^{2+}

In experiments with VDACs, researchers have traditionally used buffer solutions containing Ca^{2+} (mM) as one of the salt components, apparently without considering its influence, although Ca^{2+} ions are very important regulators at molecular, cellular and whole-organism levels. The recently established fact that VDAC possesses Ca^{2+}-binding

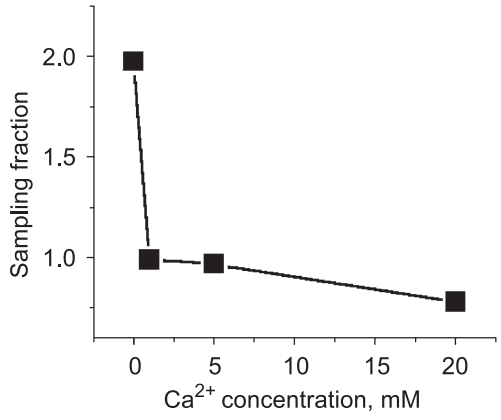

Fig. 6. Influence of Ca^{2+} ions on the relative sampling fraction of the first lowest asymmetry pool of VDAC-modified bilayers. Experiments were done at pH 6.5. Solution contained 1 M KCl, 10 mM K-acetate buffer, and 1 mM EDTA, and the desired concentration of Ca^{2+} ions. Bilayers with more than 50 VDAC-channels were used. Asymmetry for each bilayer was calculated as the ratio between time relaxations of transmembrane current in response to the two applied voltage pulses: τ_- (−40 mV) and τ_+ (+40 mV). The cumulative histograms of that asymmetry in the presence of different Ca^{2+} ions analogous to those presented in Fig. 5 were used to calculate the relative sampling fraction. This was defined as a ratio between the numbers of bilayers that belong to the first and to the second pools.

sites [41] makes it reasonable to suppose that Ca^{2+} might modify VDAC structure in solution and consequently modify VDAC channel structure in membranes. As was shown above (Tables 1 and 2), the presence of 5 mM Ca^{2+} augments the quantity of bilayers with larger VDAC asymmetry. So, the mean values of the time constants (s) of VDAC-current relaxations were found to be smaller at positive voltage (5.5 ± 3.9, $N=25$; 6.6 ± 5.6, $N=36$) than at negative voltage (12.9 ± 6.0, $N=25$; 10.8 ± 4.6, $N=36$) both with and without Ca^{2+}, respectively. Analogous experiments were done at several other Ca^{2+} concentrations. Analysis of the asymmetry distributions shows considerable decrease in the relative sampling fraction of the first pool of membranes with increasing Ca^{2+} (Fig. 6). As a result, it was also found that the mean asymmetry of time constants increases with Ca^{2+} concentration (Fig. 7A). It seems that the occupation of Ca^{2+} binding sites favors such structure of VDAC in solution, which forms the channel with larger asymmetry.

Analysis of Ca^{2+} influence on the time constants reveals that τ^+ and τ_- have different sensitivities to Ca^{2+}: τ_+ is almost invariant while τ_- increases with Ca^{2+} (Fig. 7B). Hence, Ca^{2+} apparently can affect only one gating process. Such results support assumptions about the oriented insertion of VDAC in membranes, and indicate asymmetry in VDAC channel structure.

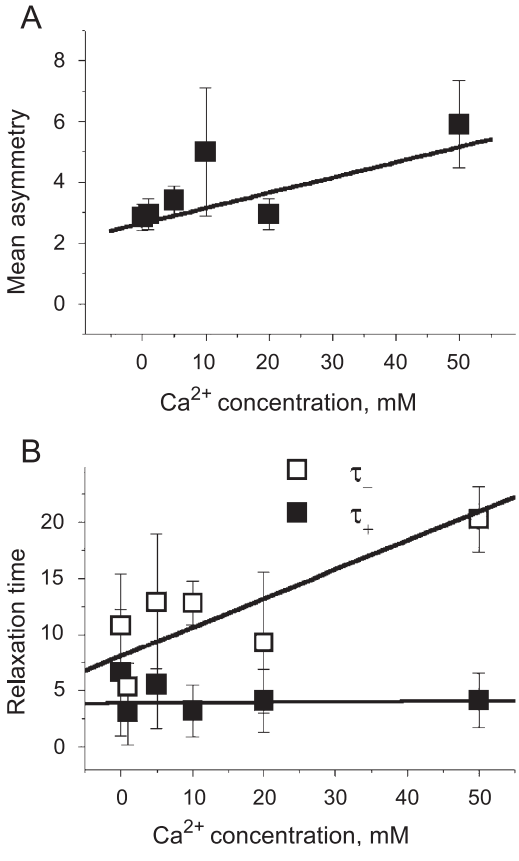

Fig. 7. Influence of Ca^{2+} ions on the mean asymmetry (A) and on the relaxation times (s) at -40 mV (τ_-) and $+40$ mV (τ_+) (B) of VDAC-modified bilayers. All other conditions are as described in the legend to Fig. 6.

5. Concluding remarks

Taken altogether, our results appear to demonstrate that there are two main conformations of wild-type VDAC inserted in lipid bilayers. Both conformations are predetermined (programmed) in solution. Neutral pH and high Ca^{2+} favor the channel structure having greater asymmetry in the sensitivity to different polarities of transmembrane voltages. Acid pH and low Ca^{2+} concentration favor a VDAC structure that forms channels with largely equal responses to voltages of different polarities. The VDAC structure of channels already incorporated into bilayers is not altered by changes in pH of the bathing solution. The incorporation of wild-type VDAC in lipid bilayers is oriented and is a self-directed process in which the properties of the first channel seem to determine the properties of subsequent channels. Until this point, all our results analyzed in qualitative manner fall well in the frame of the generally accepted view of VDAC behaviors. The apparent disagreement appears with application of quantitative analysis. We found that a relatively small amount of the channels with asymmetry smaller than 1 just belong to the left "wing" of the first pool of VDAC with the mean value of the asymmetry larger than 1, in all conditions used. This is consistent with the hypothesis that wild-type VDACs are homogeneously oriented in membranes. At the same time, it points out that the two established conformations of the channel could result from relatively small differences of topologically similar inserted VDAC and indicates the relatively large dispersion in voltage sensor localization in VDAC structure.

This conclusion accords with established asymmetry in lumen geometry, recently demonstrated for bovine muscle VDAC [3], that is only possible if VDAC channels are uniformly oriented. Taking into account that the structure and electrophysiological properties are conserved in VDAC from different sources [5,9,10], one can expect that all types of VDACs become inserted in the same manner. Indirect evidence for this conclusion comes from several recent studies where the authors did not focus their study on investigating the orientation of the reconstructed porins (from different sources: human lymphocytes, yeast; the gram-positive bacterium *Mycobacterium phlei* and *Nocardia asteroides*), however, they noted that in response to positive and negative voltage pulses "the current decreased in a somewhat asymmetric fashion which suggested asymmetric insertion of the channels into the membrane" [7,24–26].

It needs to be noted here that in the present work, we reproduce all conditions utilized in the earlier studies where the opposite orientation of VDAC channels in lipid bilayer was suggested [27,28]. In all cases, lipid bilayers were

formed from azolectin, and aliquots of VDAC in detergent solution were added at one side of the experimental chamber. Hence, we need to explain how results obtained under the same experimental conditions (including the possible asymmetry in bilayer membranes) can lead to such contradictory conclusions. What is the reason of this apparent discrepancy?

The hypothesis that VDAC inserts randomly in membranes is based on studies employing site-directed VDAC mutants [27,32,33] and has support from the study of the PorB Class 3 porin of Gram-negative bacteria *Neisseria meningitidis* [42] and, perhaps, the related porin of *N. gonorrhoeae* [43,44]. In the cases of E145K and E152K mutants of yeast VDAC, the mutations made the channels behave asymmetrically, increasing the voltage dependence of one gating process, but not the other. The stressed finding was that the asymmetry varied from membrane to membrane, as if the direction of the channel insertion varied. Additional support for random VDAC orientation also appears to come from *N. crassa* VDAC probed by attaching biotin at specific locations and determining its accessibility to streptavidin. In this system, when biotinylated N38C (and perhaps S7C)-mutated VDAC-channels were incorporated in bilayers, streptavidin was able to interact with biotinylated VDAC only on one side of membrane, although the effective side varied from membrane to membrane. As a result, the hypothesis of random insertion of VDAC with opposite topology seems quite reasonable, but it would represent a significantly anomalous event in light of the general knowledge of the behavior of other membrane proteins.

We believe that the most likely explanation for the accessibility or lack of accessibility of any given residue in the biotinylation studies results from the plasticity of VDAC. This plasticity is much more pronounced in comparison with plasma membrane channels such as really large portions of the VDAC protein, which represents several transmembrane chains, may move through the membrane during the gating process [34]. Our data show relatively wide distribution of asymmetry of VDAC in planar lipid bilayers (Fig. 5). Therefore, there is a wide dispersion in the position of the voltage sensor in VDAC channel structure that also supports the plasticity VDAC presumption. Hence, it is not unreasonable to assume that certain amino acid residues might appear on different sides of the bilayer in slightly different VDAC topologies, representing similarly inserted VDAC. The difference between these two VDAC topologies may lie in the position of a small portion of the protein due to perturbations in the balance of power in the channel structure on account of the mutation and the plasticity of VDAC in solution and/or in membrane. The reported change in the structural organization and conformational flexibility of several proteins (see for example, Refs. [45–48]) evoked by even single point mutations may (in the case of VDAC) interfere with VDAC features also. Thus, experiments with biotinylated VDAC showing the different localization of separate amino acid residues with regard to lipid bilayer could be utilized with caution in the estimation of VDAC orientation.

In our view, the assumption of a relatively wide dispersion of the voltage sensor position in homogeneously oriented VDAC channels in membranes allows us to group VDAC with other membrane proteins in regard to its insertion in membranes, and resolves the apparent contradiction between earlier published and our data. We hope that these findings will help to resolve questions regarding VDAC structure.

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