The role of lipids in VDAC oligomerization

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Supporting Material

VDAC activity measurements

To check the functionality of VDAC refolded in detergent, electrophysiology measurements on black lipid membranes (BLM) were carried out. BLMs were formed across apertures (70–90 μm in diameter) in a Teflon film with a thickness of 0.025 mm separating two chambers filled with 10 mM Tris, 5 mM $CaCl_2$, 1 M KCl, pH 7.4. VDAC in LDAO was added to the cis-compartment at a concentration of 0.2–0.3 μg/ml. Currents were recorded under the voltage clamp conditions using an Axopatch 200B patch clamp amplifier (Axon Instruments Inc, Union City, CA). Data were acquired with the help of a Digidata 1440A board and analysed using the pClamp 10 software (Axon Instruments Inc, Union City, CA).

After protein insertion, the potential across the membrane was increased in increments of 10 mV from −60 mV to +60 mV, and conductance was determined from the recorded current as a function of the voltage applied across the membrane. The maximum conductance was determined, and the relative conductance was plotted as a function of the applied voltage. The typical bell-shaped curve was obtained both for VDAC and for its fluorescently labeled mutants VDACred and VDACgreen (Fig. S1A).

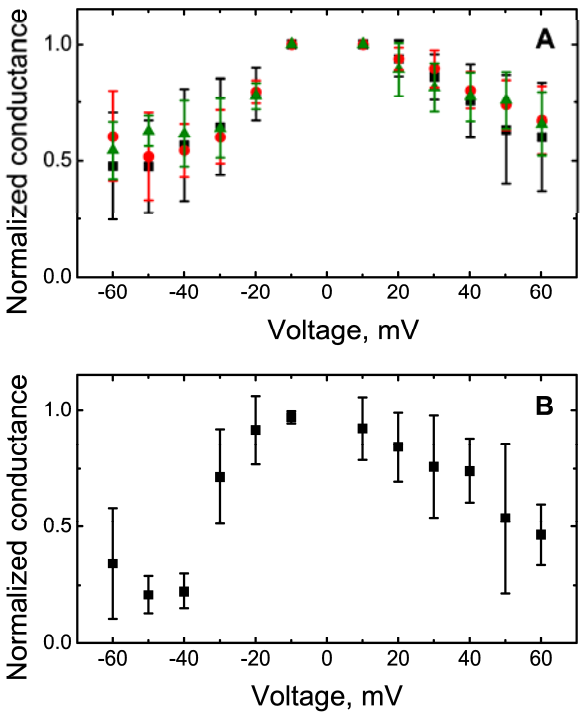

Figure S1. Multi-channel conductance of VDAC in BLM as a function of applied voltage. Panel (A) shows results for VDAC reconstituted from detergent LDAO: wild-type VDAC (black), VDACred (red) and VDACgreen (green) into a DPhPC membrane. Panel (B) shows results for VDAC reconstituted from proteoliposomes into a DOPC membrane. Data are averages from three to eight independent experiments. Error bars represent the standard deviation.

To check whether the protein was functional after the reconstitution procedure, electrophysiology measurements on proteoliposomes were carried out using a Bilayer Explorer (Ionovation GmbH, Osnabruck, Germany). For these studies, proteoliposomes made of DOPC containing VDACred and VDACgreen were fused to the BLM also prepared from DOPC, in presence of a salt gradient. After fusion, the buffer in both compartments was exchanged to 10 mM Mops-Tris, 1 mM CaCl2, 250 mM KCl, pH 7.2. Currents were recorded under voltage clamp using a EPC10 USB Patch clamp system (HEKA Elektornik, Lambreht, Germany). Data was analyzed using the Ephys10 software (Ionovation GmbH, Osnabrück, Germany). As can be seen from Fig. S1B, in this case VDAC also shows the typical voltage-dependent behavior, indicating proper folding of VDAC in proteoliposomes.

Two-focus two-color fluorescence cross-correlation spectroscopy on giant unilamellar vesicles

To determine the concentrations and diffusion coefficients of fluorescently labeled proteins in lipid membranes in the form of giant unilamellar vesicles (GUVs), we used an advanced version of the fluorescence correlation spectroscopy (FCS) technique (for review of this experimental method, see, e.g., (1)). In this technique, time-dependent fluorescence intensity $F(t)$ from a small confocal detection volume is detected, and its fluctuations $\delta F(t) = F(t) - \langle F \rangle$ about the mean value $\langle F \rangle$ are used to calculate the autocorrelation function of intensity fluctuations

$$G(\tau) = \langle \delta F(t) \delta F(t+\tau) \rangle / \langle F \rangle^2 \equiv g(\tau) / \langle F \rangle^2 \qquad \text{(S1)}$$

where $g(\tau)$ is the non-normalized fluorescence autocorrelation function. The above fluorescence autocorrelation function contains information on the diffusion coefficient and concentration of fluorescent species exercising Brownian motion in the surrounding medium, e.g., in a lipid membrane.

To study interaction of species labeled with distinct (e.g., green and red) fluorescent labels, one can use an extension of the technique known as fluorescence cross-correlation spectroscopy (FCCS). In this case, the two-color fluorescence cross-correlation function is used, which is defined as follows:

$$G_{rg}(\tau) = \langle \delta F_r(t) \delta F_g(t+\tau) \rangle / \left(\langle F_r \rangle \langle F_g \rangle \right) \equiv g_{rg}(\tau) / \left(\langle F_r \rangle \langle F_g \rangle \right) \qquad \text{(S2)}$$

where $g_{rg}(\tau)$ is the non-normalized two-color fluorescence cross-correlation function. The subscripts *r* and *g* are used here to denote the corresponding (red or green) detection channel. This fluorescence cross-correlation function contains information on the diffusion coefficient and concentration of double-labeled fluorescent species.

In the standard implementation of the FCS or FCCS technique to study diffusion of molecules in giant unilamellar vesicles, the confocal detection volume is parked on the upper pole of a GUV sitting on top of a lower coverslip of a flow chamber. However, as a result of thermal fluctuations of the vesicle shape, the membrane may randomly change its position with respect to the center of the detection volume, which can result in appearance of additional components in FCS correlation functions, as well as false positive cross-correlation between differently labeled fluorescent species (for details, see (1)).

In addition, it should be emphasized that absolute diffusion coefficients and concentration of species can be extracted from FCS and FCCS data only if proper calibration of the setup using a standardized sample is carried out, which is not possible in all cases.

To overcome these drawbacks, an extended advanced version of FCCS has been proposed (2), which combines two-focus and two-color fluorescence cross-correlation with the repeated scanning the two confocal detection volumes through the equator of the GUV. The distance between the two foci (two confocal detection volumes) can be measured independently, which makes this approach calibration-free and allows for determination of absolute diffusion coefficients and concentrations of fluorescent species. The two-color feature obviously allows one to use this technique to study interactions between two differently labeled species. Finally, the repeated scanning of the detection volumes through the GUV equator allows one to correct the fluorescence fluctuation data for possible random displacements of the membrane and thereby to avoid the potential related artifacts.

The theory behind this approach, as well as the aspects of its experimental implementation, are described in detail elsewhere (2). In brief, two-focus two-color scanning FCCS measurements across the GUV equator produce six distinct fluorescence auto- and cross-correlation curves: single-focus single-color red channel autocorrelation, single-focus single-color green channel autocorrelation, two-focus single-color red channel autocorrelation, two-focus single-color green channel autocorrelation, single-focus two-color red–green channel cross-correlation, and two-focus two-color red–green channel cross-correlation.

Assuming that the sample contains two fluorescent species *a* and *b*, all these single- and double-focus, and single- and double-color correlation functions can be described in a

unified manner using the following expression for the single-species correlation function for Gaussian detection volumes:

$$g^{i}_{\mu\nu}(\tau) = (2/\pi)\eta_\mu\eta_\nu C_i \alpha_{\mu\nu}(\tau)\beta_{\mu\nu}(\tau)\exp\left(-2d^2_{\mu\nu}\beta^2_{\mu\nu}(\tau) - 2d^2\alpha^2_{\mu\nu}(\tau)\right) \tag{S3}$$

with

$$\begin{aligned} \alpha_{\mu\nu}(\tau) &= \left(8D_i\tau + w^2_\mu + w^2_\nu\right)^{-1/2}, \\ \beta_{\mu\nu}(\tau) &= \left(8D_i\tau + w^2_\mu S^2_\mu + w^2_\nu S^2_\nu\right)^{-1/2}. \end{aligned} \tag{S4}$$

Indices $(\mu,\nu) = \{r, g\}$ denote the spectral detection channel (red or green). Index $i = \{a, b, ab\}$ denotes the species (a or b) or the inter-species complex (ab). The quantity d_{rg} describes the overlap between the red and green detection volumes, and equals zero for single-color auto- and cross-correlation curves $d_{rr} = d_{gg} = 0$. Parameter d is the distance between the two lines along which the two foci are scanned; for single-focus auto- and cross-correlation curves $d = 0$. $w_{r,g}$ are the lateral dimensions of the detection volumes, and $S_{r,g}$ are their axial-to-lateral aspect ratios. D_i is the diffusion coefficient of species i. The quantities η_r and η_g denote the so-called channel brightness introduced in (2) as follows:

$$\begin{aligned} \eta_r &= \left(\langle F_r\rangle - \kappa\langle F_g\rangle\right)\Big/\left(C_a + C_{ab}\right), \\ \eta_g &= \langle F_g\rangle\Big/\left(C_b + C_{ab}\right). \end{aligned} \tag{S5}$$

Parameter κ is the crosstalk coefficient between the red and green detection channels.

The non-normalized correlation functions are given by the following expressions:

$$\begin{aligned} g_{rr}(\tau) &= g^{a}_{rr}(\tau) + g^{ab}_{rr}(\tau) + \kappa^2 g^{b}_{gg}(\tau) + \kappa^2 g^{ab}_{gg}(\tau) + 2\kappa g^{ab}_{rg}(\tau), \\ g_{gg}(\tau) &= g^{b}_{gg}(\tau) + g^{ab}_{gg}(\tau), \\ g_{rg}(\tau) &= g^{ab}_{rg}(\tau) + \kappa g^{b}_{gg}(\tau) + \kappa g^{ab}_{gg}(\tau). \end{aligned} \tag{S6}$$

Notice that each of the above expressions for auto- and cross-correlation functions describes either single- or two-focus measurements, depending on whether d is set to zero or to the measured value between the two foci, and therefore this set of equations provides a complete description of the shape of all six curves measured in the two-color two-focus FCCS experiment.

Finally, by using the following expressions for the mean fluorescence intensities detected in the red and green channels:

$$\begin{aligned} \langle F_r\rangle &= \eta_r\left(C_a + C_{ab}\right) + \kappa\eta_b\left(C_b + C_{ab}\right), \\ \langle F_g\rangle &= \eta_g\left(C_b + C_{ab}\right), \end{aligned} \tag{S7}$$

one can construct the set of normalized auto- and cross-correlation functions

$$G_{\mu\nu}(\tau) = g_{\mu\nu}(\tau) \Big/ \left(\langle F_\mu \rangle \langle F_\nu \rangle \right) \tag{S8}$$

which can be directly used to analyze experimental data.

In our experiments, the amplitude of the two-focus two-color red–green channel cross-correlation was on the level of the experimental noise, and it was not included in the data sets subjected to quantitative analysis.

To carry out quantitative analysis of the measured sets of auto- and cross-correlation curves, the distance d between the foci has to be measured and the spectral cross-talk parameter κ should be estimated. Additionally, the data analysis becomes much more stable if the detection volume aspect ratios are determined in an independent experiment and fixed during the data analysis. Notice that because the inter-foci distance d serves as an internal length reference, the lateral dimensions of the detection volumes $w_{r,g}$ do not need to be determined in independent measurements, but rather are obtained as a by-product of the data analysis.

The distance between the lines along which two foci were scanned was determined to be $d = 0.56$ μm. The axial-to-lateral aspect ratios of the red and green detection volumes $S_{r,g}$ were determined independently by calibration single-focus FCS measurements in solutions of Alexa 488 and ATTO 655 fluorophores. The spectral cross-talk between fluorescence emission of these fluorophores was found to be below the detection limit, and therefore during the analysis of data we set $\kappa = 0$.

In the analysis, all diffusion coefficients for red and green particles and their complexes (D_i, $i = \{a, b, ab\}$) were assumed to have the same value, as discussed in the main text of the article.

As a result, the global analysis of the set of auto- and cross-correlation curves allows one to determine the concentrations of particles carrying labels of a single color (C_r and C_g) and two colors (C_{rg}), as well as their diffusion coefficient D.

Supporting References

1. Petrov, E. P. and Schwille, P. (2008). State of the art and novel trends in fluorescence correlation spectroscopy. *In* Standardization and Quality Assurance in Fluorescence Measurements II. Springer Series on Fluorescence, Vol. 6. U. Resch-Genger, editor. Springer, Berlin Heidelberg New York, pp. 145–197. http://dx.doi.org/10.1007/4243_2008_032

2. Ries, J., Petrášek, Z., García-Sáez, A. J., and Schwille, P. (2010). A comprehensive framework for fluorescence cross-correlation spectroscopy. *New J. Phys.* 12, 113009–113041.