Membrane Lipid Composition Regulates Tubulin Interaction with Mitochondrial Voltage-dependent Anion Channel*

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Background: Dimeric αβ-tubulin regulates mitochondrial respiration by blocking VDAC.

Results: The on-rate of tubulin binding to VDAC varies more than 100-fold depending on the lipid type.

Conclusion: VDAC blockage by tubulin involves a crucial step of tubulin interaction with the membrane.

Significance: VDAC-tubulin binding is a new example of lipid-controlled protein-protein interactions that contribute to mitochondrial metabolism regulation.

Elucidating molecular mechanisms by which lipids regulate protein function within biological membranes is critical for understanding the many cellular processes. Recently, we have found that dimeric αβ-tubulin, a subunit of microtubules, regulates mitochondrial respiration by blocking the voltage-dependent anion channel (VDAC) of mitochondrial outer membrane. Here, we show that the mechanism of VDAC blockage by tubulin involves tubulin interaction with the membrane as a critical step. The on-rate of the blockage varies up to 100-fold depending on the lipid composition. Using confocal fluorescence microscopy, we compared tubulin binding to the membranes of giant unilamellar vesicles (GUVs) made from DOPC and DOPC/DOPE mixtures. We found that detectable binding of the fluorescently labeled dimeric tubulin to GUV membranes requires the presence of DOPE. We propose that prior to the characteristic blockage of VDAC, tubulin first binds to the membrane in a lipid-dependent manner. We thus reveal a new potent regulatory role of the mitochondrial lipids in control of the mitochondrial outer membrane permeability and hence mitochondrial respiration through tuning VDAC sensitivity to blockage by tubulin. More generally, our findings give an example of the lipid-controlled protein-protein interaction where the choice of lipid species is able to change the equilibrium binding constant by orders of magnitude.

The ability of lipids to regulate proteins arises from both specific chemical features of lipid molecules and mechanical and structural properties of the lipid bilayer (1–7). Structural, compositional, and elastic parameters of lipid membranes are known to have a strong influence on the function of membrane proteins, such as ion channels, as well as on the interaction of water-soluble proteins with membranes. Hydrophobic mismatch between the acyl chain region of the membrane and the embedded proteins directly influences ion channel behavior (6, 8–11). There is also clear evidence of the strong response of ion channels to the elastic stress within a lipid bilayer or the lipid packing stress (12–14). Phosphatidylcholine (PC)2 and phosphatidylethanolamine (PE) lipids commonly found in cell membranes are the main components of the mitochondrial outer membrane (MOM) (15–17). Most of PC lipids are lamellar lipids that form “flat” bilayers, whereas nonlamellar PE lipids tend to form highly curved nonbilayer phases. When forced into a flat bilayer structure, PE lipids produce a significant stress in the hydrocarbon area of the membrane (13, 18–20). For example, inclusion of PE into a PC bilayer increases the lateral pressure in the hydrophobic core of the bilayer with a compensating decrease of the pressure in the headgroup region. This leads to a redistribution of the lateral pressure in the membrane. Here, we use various PE/PC compositions to manipulate lipid packing stress and to study how this affects the interaction of two proteins: a membrane protein, voltage-dependent anion channel (VDAC) of the outer mitochondrial membrane, and a water-soluble protein, dimeric tubulin. We took advantage of our recent finding that dimeric tubulin reversibly and with high efficiency blocks VDAC reconstituted into planar lipid membranes (21–23). Both proteins, as shown previously, are affected by lipid membrane composition.

The ability of the water-soluble αβ-heterodimer of tubulin to bind to cell membranes with affinity of nM−1 was reported some 30 years ago. Even earlier, it had been shown that tubulin could also bind to liposome membranes, and surprisingly for a water-soluble protein, this tubulin-membrane interaction was proposed to have a hydrophobic component (24–27). Importantly, tubulin, a cytoplasmic protein and the basic structural unit of microtubules, was found to be associated with various

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2 The abbreviations used are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; MOM, mitochondrial outer membrane; VDAC, voltage-dependent anion channel; DOPE, dioleoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; CTT, C-terminal tail; PLE, polar lipid extract; GUV, giant unilamellar vesicle; CL, cardiolipin; DPHPC, diphytanoyl-phosphatidylcholine; DOTAP, dioleoyl-trimethylammonium-propane; DPHPS, diphytanoyl-phosphatidylserine.
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cell membranes, including mitochondrial membranes (24, 28, 29), and it was even suggested as their inherent component (30). A variety of reported interactions between tubulin and liposomes demonstrated a potential for both integral and surface attachments of tubulin to phospholipid bilayers, although it remains unclear how tubulin becomes an integral component of cell membranes. Tubulin was shown to insert into the bilayers of saturated phosphatidylcholine vesicles and form stable vesicle-tubulin complexes without any requirements of detergent or sonication (24–26). The insertion process is accompanied by structural perturbations of both tubulin and lipid bilayer and involves hydrophobic interactions (24, 25). It was also demonstrated that tubulin could reversibly adsorb to PC or PS membranes above the lipid phase transition (31). There are strong indications that tubulin-lipid interactions are highly lipid-specific. Although tubulin binding to phospholipid membranes has been extensively studied, the physiological role of this interaction remains poorly understood (28).

Voltage gating of VDAC reconstituted into planar membranes was shown to depend on the presence of nonlamellar lipids (21, 32). Previously, we have found that nonlamellar lipids, characteristic for mitochondrial membrane, PE, and cardiolipin (CL), change VDAC conformational equilibrium to promote the low conducting “closed” states at negative potentials, suggesting a coupling between the mechanical pressure in the hydrocarbon region of the lipid bilayer and the voltage-induced conformational transitions of VDAC.

VDAC, the major channel in MOM, not only serves as a principal pathway for ATP, ADP, and other mitochondrial respiratory substrates across MOM but also controls these fluxes, and thus it plays the role of a global regulator of mitochondrial functions and cell metabolism (33–36). Recently, we have found that in the presence of tubulin, the conductance of VDAC reconstituted into a planar lipid membrane fluctuates between the open and tubulin-blocked state (22). Although the tubulin-blocked state is still conductive for small ions (about 40% of the open state conductance in 1 M KCl), it has reduced dimensions compared with the open state, reversed ionic selectivity, and most importantly is virtually impermeable for ATP (37). These data strongly suggest that not only ATP, but ADP and other mitochondrial respiratory substrates, most of which are negatively charged and are close to or larger than ATP by their molecular weight, cannot permeate through the tubulin-blocked state due to the steric restrictions and the electrostatic barrier. It was concluded that by blocking VDAC permeability, tubulin may selectively regulate fluxes of metabolites across MOM and therefore control mitochondrial respiration. Indeed, experiments with isolated mitochondria (22, 38) and with intact cells (39) supported this conjecture.

Earlier, we proposed a model of VDAC-tubulin interaction where the negatively charged C-terminal tail (CTT) of tubulin permeates into the channel lumen, interacting with VDAC and reversibly blocking channel conductance in a highly voltage-dependent manner (22, 23). Here, we report that the mechanism of the VDAC-tubulin interaction is more complex than was initially thought and, what may seem to be unexpected, strongly depends on the specific composition of the lipid membrane. We find that the on-rate of VDAC blockage by tubulin varies up to 100-fold between DOPC and DOPE membranes, increasing with the PE content. VDAC-tubulin interaction depends on both hydrophobic and polar parts of the lipid molecule. Our data imply that the previously shown ability of tubulin to bind to lipid membranes in a saturable, reversible, and specific manner (28, 29) could greatly impact VDAC blockage by tubulin. At a physiologically low salt concentration of 100 mM KCl, we found that the charge of lipid headgroups significantly affects the on-rate of the blockage too. Finally, using confocal microscopy of giant unilamellar vesicles (GUVs) in the presence of fluorescently labeled dimeric tubulin, we demonstrated that measurable adsorption of tubulin requires the presence of PE. Thus, our findings suggest a new regulatory role of the mitochondrial lipids in control of MOM permeability and mitochondrial respiration through the lipid-mediated tuning of VDAC sensitivity to tubulin.

EXPERIMENTAL PROCEDURES

Protein Purification—Frozen mitochondrial fractions of Neurospora crassa and rat liver were a generous gift of M. Colombini (University of Maryland, College Park). VDAC was isolated from mitochondrial outer membranes according to standard methods (40) and purified on a hydroxyapatite/colcite (2:1) column following the method described previously (41). Bovine brain tubulin and tubulin labeled with HiLyte Fluor 488 with excitation and emission waves of 460 and 520 nm, respectively, were purchased from Cytoskeleton (Denver, CO). Dioleoyl-phosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), diphytanoyl-phosphatidylethanolamine (DPhPC), diphytanoyl-phosphatidylserine (DPhPS), dioleoyl-trimethyl-ammonium-propane (DOTAP), cardiolipin from bovine heart, sodium salt (CL), and soybean polar lipid extract (PLE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol was purchased from Sigma. All other chemicals were analytical grade.

Channel Reconstitution Experiments—The mixtures of lipids were prepared from aliquots of two lipid solutions in chloroform, followed by drying with nitrogen and then re-dissolving them in pentane to a total lipid concentration of 5 mg/ml. Bilayer membranes were formed from monolayers as described previously (32). Prior to membrane formation, the orifices in the Teflon film separating reconstitution cell compartments were pretreated either with a suspension of petroleum jelly in petroleum ether (42) or with a 1% solution of hexadecane in pentane (43). Both approaches ensured formation of stable and virtually nonconductive planar membranes. Aqueous KCl solutions were buffered with 5 mM HEPES at pH 7.4. Potential is defined as positive when it is greater at the side of VDAC addition (cis side). After the VDAC channel was inserted and its conductance was measured at different potentials, tubulin was added to the cis side of the membrane under constant stirring for 2 min. Conductance measurements were performed as described previously (32) using an Axopatch 200B amplifier (Axon Instruments, Inc., Foster City, CA) in the voltage clamp mode. Data were filtered by a low pass 8-pole Butterworth filter (Model 9002, Frequency Devices, Inc., Haverhill, MA) at 15 kHz and directly saved into computer memory with a sampling frequency of 50 kHz. For data analysis by Clampfit 9.2, a digital
8-pole Bessel low pass filter set at 500 Hz was applied to all records, and then individual events of current blockages were discriminated. Lifetimes were calculated by fitting logarithmic single or double exponentials to logarithmically binned histograms (44) as described previously (22).

**GUV Preparation and Confocal Microscopy**—GUVs were prepared by the modified electroswelling method (45), as was described previously (46), from DOPC and a mixture of DOPC/DOPA (7:3 molar ratio). To grow GUVs, a 10 Hz sine wave of 1-V amplitude was applied for 2 h followed by a 4 Hz, 2.5-V sine wave applied overnight. After GUVs were formed in 300 mM sucrose, they were transferred to the so-called “observation chamber” of 150-μl volume containing the fluorescent tubulin (HiLyte 488) in an iso-osmolar solution of 314 mM glucose or 182 mM KCl. All solutions were buffered with 2 mM PIPES/HCl (pH 7.4) and 0.01 mM MgCl₂. The solution osmolarity was measured and adjusted using a Vapro 5520 vapor pressure osmometer (Wescor). 20 μl of the GUV-containing solution was injected into the observation chamber. The higher density of sucrose inside the GUVs caused them to sink to the bottom of the chamber and provided a phase contrast. To prevent GUV and tubulin from adhering to the glass surfaces, they were pre-treated with SigmaCote (Sigma) or BSA.

Confocal images were taken using a Carl Zeiss LSM 700 differential interference contrast microscope with two basic excitation lines (488 nm and 505–550 nm). Images were collected and analyzed using software LSM Image Browser and ImageJ for the analysis of single-channel current events. ImageJ was used to acquire the images taken from the microscope, and ImageJ was used to analyze them.

**RESULTS**

**VDAC Blockage by Tubulin Strongly Depends on Membrane Lipid Composition**—We found that in some lipids, such as DOPE or DPhPC, VDAC blockage by tubulin is much stronger than in others, such as DOPC or a polar lipid extract mixture from soy bean (PLE). Because there is abundant information available on the mechanical properties of DOPC and DOPE membranes and their effect on ion channel functioning (19, 32, 47, 48), we first compared VDAC blockage by tubulin using membranes of a variable DOPC/DOPE composition. The representative single-channel current traces of VDAC reconstituted into planar membranes formed from pure DOPE, DOPC, and a 1:1 mixture of these lipids are shown in Fig. 1A. Without tubulin (Fig. 1A, trace a), the current through VDAC at −20 mV of applied voltage is steady, and the channel could stay open for a prolonged (up to a few hours) time regardless of the lipid. Single-channel conductance did not depend on the membrane lipid composition in agreement with previous studies (32). However, in the presence of tubulin, the situation has changed drastically. 10 nM tubulin added to the cis side of the DOPE membrane (Fig. 1A, trace d) induced much more frequent channel blockage events than 60 nM of tubulin added to the pure DOPC membrane (Fig. 1A, trace b) or to the membrane formed from the 1:1 DOPE/DOPC mixture (Fig. 1A, trace c). As can be seen from the traces in Fig. 1A, the conductances of VDAC open and tubulin-blocked states were not affected by lipid composition and were equal to 4.1 ± 0.1 and 1.6 ± 0.1 nanosiemens, respectively, in 1 mM KCl. To separate two voltage-sensitive processes, which is tubulin-induced and voltage-induced VDAC closure, the applied voltage in most experiments did not exceed ±30 mV.

As was shown previously (32), VDAC inserts into the planar membranes formed from either DOPE or DOPC unidirectionally. Straightforward evidence is a pronounced asymmetry of VDAC voltage gating with respect to the polarity of the applied voltage in the bilayers made from synthetic lipids, such as DPhPC (21, 32). Recently (49), we were able to establish VDAC orientation in our reconstitution bilayer system with respect to its orientation in the MOM. We have found that after VDAC phosphorylation, the on-rate of VDAC-tubulin binding increased by orders of magnitude but only from cis side (the side of VDAC addition). Therefore, the effect of tubulin from the cis side that corresponds to the cytosolic side of the channel accessible for cytosolic kinases and tubulin is more physiologically relevant.

Statistical analysis of the open times, τ₁off, i.e. the times between blockage events when the channel stays open, is reasonably well described by a single exponential fitting (Fig. 1B). It can be seen that at the same applied potential (−20 mV) in pure DOPE membranes, τ₁off in the presence of 10 nM tubulin is about 2 orders of magnitude smaller (i.e. the blockage frequency is higher) than in pure DOPC membranes even at 60 nM tubulin, whereas in the DOPC/DOPE (1:1) mixture τ₁off appears to be between the τ₁off values obtained in pure DOPC and DOPE (Fig. 1B). In contrast to the strong lipid dependence of the open times, the two characteristic times in the blocked state, τ₂off and τ₂on, i.e. the times during which the channel stays blocked by tubulin, were independent of the DOPE/DOPE ratio (Fig. 1C). At small tubulin concentrations [C], VDAC blockage could be described by a simple first-order reaction with the on-rate constant, k₁on = 1/(τ₁off[C]), being an exponential function of the applied voltage (22). In this study, we find that voltage dependences of the on-rate constants had similar slopes at all DOPE/DOPE ratios (Fig. 2A) and could be described by exponential dependence with the effective “gating charge” n = 6.4 ± 0.5. The increase in the efficiency of VDAC blockage by tubulin with the increase of the DOPE content is best seen in Fig. 2C, where the k₁on value monotonically increases up to 100 times when DOPE is gradually replaced by DOPE.

Both off-rates, k₁off and k₂off, where k₂off = 1/τ₂off did not depend on lipid composition and collapsed into two linear dependences on the applied voltage (Fig. 2B). Fitting the voltage dependences of the off-rate constants to the above equation yields an “effective gating charge” of 3.9 ± 0.7. This is consistent with translocation of 7–10 negative charges on α- or β-CTT of tubulin, respectively (22), half-way through the transmembrane field inside a pore.

To get insight into the mechanism of the observed lipid dependence of VDAC blockage by tubulin, we first examined the effects of lipid hydrocarbon acyl chains and polar headgroups on the VDAC blockage kinetics. When oleoyl chains C18:1 in DOPC were replaced with phytanoyl chains C16:0(CH₃)₄ in DPhPC, VDAC blockage by tubulin increased by about 70 times. The on-rate constant of the blockage in DPhPC membranes (at orifice for membrane formation pretreated with petroleum jelly) at −25 mV of applied voltage was 348 ± 60
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A natural lipid composition of MOM is a mixture of predominantly PC/PE/PI lipids and cholesterol (15, 17). Therefore, we conducted experiments using planar bilayers made of soy bean polar lipid extract (PLE) of which the composition, PC/PE/PI/PA (4.6:2.2:1.8:0.7), closely resembles the lipid composition of the rat liver MOM (15, 16). In PLE membranes, tubulin blocked VDAC 200 times less effectively than in DOPE membranes and even less effectively than in pure DOPC (Fig. 2A). Addition of 10% (w/w) cholesterol to PLE did not affect the on-rate of VDAC blockage. Addition of 8% (w/w) of cardiolipin (CL) to DOPC also did not change the on-rate (Fig. 2A). Both characteristic off-rates, $k_{\text{off}}^1$ and $k_{\text{off}}^2$, were virtually the same in all tested lipids (Fig. 2B). Logarithms of the voltage dependences of these times collapsed on the same linear regressions as those obtained in the DOPE/DOPC mixtures. Another unexpected finding was that the on-rate of VDAC blockage by tubulin depends on the pretreatment of the partition orifice across which planar membranes are formed (see under “Experimental Procedures” for the description). When bilayers were formed using hexadecane, the $k_{\text{on}}$ was about 10 times higher than when bilayers were formed using petroleum jelly for the pretreatment (data not shown). At the same time, VDAC basic channel properties, such as single-channel conductance and ionic selectivity, did not depend on the method of membrane formation. The off-rates of VDAC blockage by tubulin also did not depend on the solvent used for the pretreatment. Our explanation is that different partitioning of hexadecane and petroleum jelly to the hydrophobic core of the bilayer influences VDAC blockage by tubulin, thus supporting our conjecture that not only lipid polar headgroups but also the hydrophobic region of the lipid bilayer are involved in VDAC-tubulin interaction.
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In the range of low tubulin concentrations, the blockage could be adequately represented by a simple binding reaction with the on-rate of VDAC blockage by tubulin, $1/\tau_{on}$, increasing linearly with the tubulin concentration (22). However, at higher tubulin concentrations, the on-rate demonstrated a typical saturation behavior that strongly depended on the lipid composition.

FIGURE 2. Voltage dependences of the on- and off-rate constants of VDAC blockage by tubulin in the membranes of different lipid composition. A, on-rate increases with the increasing DOPE content in the membranes formed from the DOPE/DOPC mixtures. Addition of cardiolipin to DOPC or cholesterol to PLE did not affect VDAC-tubulin binding. The voltage dependences of the on-rate constants, $k_{on}^1$, for different lipids could be fitted to an exponential dependence (dashed lines) $k_{on}^1 = k_{on} \exp[\delta V / (F / R T)]$, where $V$ is the applied voltage and $F$, $R$, and $T$ have their usual meaning, with the effective "gating charge" $\delta = 6.4 \pm 0.5$. B, both off-rates, $k_{off}^1$ and $k_{off}^2$, of the tubulin blockage do not depend on the lipid composition. The dashed lines are fits to the equation as in A, with effective gating charge equal to 3.9 ± 0.7. Membranes were formed from pure DOPC and DOPE; from mixtures of DOPC/DOPE of 3:1, 1:1, and 1:3 mole ratios, respectively; from DOPC with cardiolipin, DOPC/CL (1:0.08); and from PLE without and with cholesterol (10:1). C, $k_{on}$ obtained at −25 mV of applied voltage in different DOPE/DOPC mixtures. Data are mean values obtained at different tubulin concentrations ± S.E. Experimental conditions are as in Fig. 1.

In the range of low tubulin concentrations, the blockage could be adequately represented by a simple binding reaction with the on-rate of VDAC blockage by tubulin, $1/\tau_{on}$, increasing linearly with the tubulin concentration (22). However, at higher tubulin concentrations, the on-rate demonstrated a typical saturation behavior that strongly depended on the lipid composition. The on-rate, $\tau_{on}$, in DPhPC membranes linearly increases with tubulin concentration up to 30 nm and becomes saturated at about 50 nm. In DOPC/DOPE (3:1) membranes the on-rate is lower than in DPhPC membranes and does not reach saturation up to 200 nm (shown in a finer scale in the inset). The applied voltage was −20 mV. VDAC was isolated from rat liver mitochondria. Salt concentration was 1 M KCl.

FIGURE 3. Dependence of VDAC blockage on-rate on tubulin concentration. The on-rate, $\tau_{on}^{-1}$, in DPhPC membranes linearly increases with tubulin concentration up to 30 nm and becomes saturated at about 50 nm. In DOPC/DOPE (3:1) membranes the on-rate is lower than in DPhPC membranes and does not reach saturation up to 200 nm (shown in a finer scale in the inset). The applied voltage was −20 mV. VDAC was isolated from rat liver mitochondria. Salt concentration was 1 M KCl.

If the above hypothesis is correct, the lipid headgroup charge should affect binding of the net negatively charged tubulin to the membrane and consequently change the on-rate of VDAC blockage by tubulin. Indeed, addition of the negatively charged DPhPS to the neutral DPhPC in a 4:1 ratio resulted in a decrease in the on-rate (Fig. 4A). The similarity between the concentration dependence of the on-rate of VDAC blockage by tubulin and tubulin binding to the membrane suggests that the latter process is responsible for the observed lipid dependence of the VDAC-tubulin interaction.

In high salt concentration of 1.5 M KCl, where the electric fields of membrane and protein surface charges are salted out, there was no measurable effect of either negatively or positively charged lipids on $k_{on}$. In 0.1 M KCl,ug, the addition of the positively charged synthetic lipid DOTAP to DPhPC also caused a decrease in the blockage on-rate; the membranes formed from DOTAP/DPhPC (2:1) mixture demonstrated an order of magnitude reduction in $k_{on}$ (Fig. 4A).

Salt concentration affected the on-rate constant of the blockage in both neutral and charged membranes. In 0.1 M KCl, $k_{on}$ was ~5 times higher than in 1.5 M KCl in the neutral DPhPC membranes and ~50 times higher in the negatively charged DPhPS/DPhPC membranes (Fig. 4). These results confirm a significance of the long range electrostatic component in the
VDAC-tubulin interaction. Thus, VDAC blockage by tubulin depends on both hydrophobic and polar parts of the phospholipid molecule. Our findings suggest that at the physiologically relevant low salts, the charge of the lipid headgroup may be crucially important, changing the equilibrium constant of the blockage by 2 orders of magnitude. Clearly, all these factors should be taken into account in modeling VDAC-tubulin interaction.

Tubulin Binding to the PE-containing GUV Membranes—To directly test tubulin binding to the DOPE and DOPC membranes, we used a system of giant unilamellar vesicles (GUVs) and the fluorescently labeled tubulin-488. GUVs displayed a rather uniform appearance with an average diameter of around 25 μm. Representative images in Fig. 5A show accumulation of fluorescent tubulin on the surface of the GUVs made of DOPC/DOPE (7:3) (panels a and c). By contrast, when liposomes were made of pure DOPC, there was no detectable adsorption of tubulin-488 on their membranes (Fig. 5A, panels b and d). There was no detectable difference in the tubulin-488 adsorption on GUVs when sucrose in the observation chamber was replaced by 182 mM KCl.

To quantify tubulin adsorption, the fluorescence signal was measured as the height-averaged fluorescence over the length of the chosen rectangular window as illustrated in Fig. 5B. The corresponding fluorescence profile obtained on a DOPC/DOPE GUV in the presence of 50 nM tubulin-488 is shown in FIGURE 5A, confocal images of GUVs prepared from DOPC/DOPE in a 7:3 molar ratio (left column, panels a, c, and e) and pure DOPC (right column, panels b, d, and f) in the presence of 25 nM (panels a and b) and 50 nM (panels c and d) of tubulin (Tub) in the observation chambers. It can be seen that tubulin adsorbs on the surface of DOPE-containing GUV membranes although its adsorption on the GUVs formed from DOPC is not detectable. The GUVs presented in panels c and d are shown in white light in panels e and f, respectively. Scale bars, 10 μm.

B

25 nM Tub

A

DOPC/DOPE

DOPC

50 nM Tub

C

D

E

F

GUVs made from DOPC/DOPE in 7:3 molar ratio (panel a) and pure DOPC (panel b) in the presence of 50 nM tubulin-488. Fluorescence profiles measured across the yellow boxes indicated in panels a and b are shown in panels c and d, respectively. Relative fluorescence was calculated as (I - I_{inside})/I_{bulk} - I_{inside}, where I_{inside} and I_{bulk} are the fluorescent signals inside the liposome and in the bulk, respectively.

FIGURE 4. Effect of charged lipids on VDAC blockage by tubulin depends on salt concentration. A, in 0.1 M KCl the on-rate constant of the blockage, $k_{on}$, is strongly modified by the presence of charged lipids in their mixture with DPhPC. B, in 1.5 M KCl, $k_{on}$ does not depend on the presence of either negatively or positively charged lipids. Membranes were formed from pure DPhPC, DOTAP/DPhPC (2:1), and DPhPS/DPhPC (4:1). The orifice for membrane formation was pretreated with hexadecane. The applied voltage was −25 mV. VDAC was isolated from rat liver mitochondria.
DISCUSSION

The model of VDAC blockage by tubulin proposed earlier (22, 50) took into account only direct interaction between the two proteins and did not imply any involvement of lipids. The remarkable sensitivity of the blockage to the phosphorylation state of VDAC reported later (49) gave additional support for the model. In this respect, the strong effect of the membrane lipid composition reported here was quite unexpected. This finding suggested that the original model was missing an important step of tubulin adsorption to the membrane, and this step of the reaction is the main cause for the observed sensitivity of the blockage to the lipid composition. Such a conclusion is supported by the preferential adsorption of the fluorescently labeled tubulin-488 to the PE-containing GUV membranes as compared with the GUV membranes made of pure DOPC (Fig. 5). The apparent paradox of tubulin, a soluble protein, interacting with neutral lipid membranes has been recognized much earlier (24–26). It was proposed that this interaction has a hydrophobic nature, in which both tubulin and lipid undergo significant conformational changes upon binding (24–26). Importantly, it was shown that the membrane-bound tubulin is not functionally “denatured” because it was still able to bind colchicine and microtubule-associated proteins with an unaltered rate (24).

In this study, we find that another functionally important property of tubulin is not compromised in the membrane-bound state, namely its ability to regulate VDAC. In what seems to be most relevant to this study, it was found that CTTs of liposome-associated tubulin were accessible to proteolysis (51), which indicates that tubulin CTTs are not directly involved in the interaction with membranes and, in our case, could be available for blocking VDAC pore following a proposed earlier model where tubulin CTTs are required for VDAC blockage (22).

Although the mechanism of tubulin interaction with membranes is complex and yet not understood (28), one of the possibilities is that lipid packing stress could influence tubulin-membrane association. From x-ray diffraction, it was inferred that the repulsive forces between DOPE headgroups are significantly smaller than those between DOPC headgroups (47). We hypothesize that the decrease in the repulsive forces between DOPE headgroups provides for a more flexible arrangement of the groups on the membrane surface, thus making the hydrophobic region of the membrane more accessible for its interaction with tubulin. This may explain the differences in tubulin binding to DOPC and DOPE membranes (Fig. 5) and the corresponding differences in the VDAC blockage on-rates (Fig. 2, A and C). Preferential association of tubulin with PE-containing membranes results in the increased effective concentration of tubulin on the surface of these membranes, which is seen as the increased on-rate of the blockage.

The hydrophobic component of tubulin interaction with the membrane might involve a partial insertion of tubulin hydrophobic domains into the membrane (25). Thus, the effect of lipid on the VDAC blockage by tubulin could be described by a tentative model shown in Fig. 6, where the first step is a saturable lipid-dependent tubulin binding to the membrane. Some lipids such as DPhPC and DOPE, are “tubulin-sticky” and thus increase its concentration around VDAC, whereas other lipids, such as DOPC or PLE, are not. The second step is interaction of tubulin with the cytosolic loops of VDAC, which depends on their phosphorylation state (49). The final step is a partial block of the VDAC pore by tubulin CTT. The on-rate of this final step of blockage should depend on the effective concentration of tubulin tails at the membrane surface in a close proximity to the VDAC entrance and consequently on tubulin binding to the membrane surface and to the cytosolic loops of VDAC. In principle, both step 1 and step 2 can be voltage-dependent. We do not show this dependence in Fig. 6 because, at the moment, we do not have any strong support for such a conjecture. Regarding step 3, it is definitely voltage-dependent (Fig. 2A). Although the on-rate voltage dependence can be explained by hypothetical voltage dependences of step 1 and/or step 2, the off-rate shows profound voltage sensitivity (Fig. 2B). It is important that the off-rate is defined by the applied voltage and interactions between CTT and the VDAC pore and is independent of the VDAC phosphorylation state (49), tubulin concentration in the bulk (22, 23), and tubulin interaction with the membrane (Fig. 2B). Further refinement of the model, as well as quantitative analysis of the kinetics involved in the first two steps, will require specially designed relaxation experiments.

The distribution of times characterizing the on-rate of VDAC blockage by tubulin is well described by a single exponential fitting (Fig. 1B). There is no indication of the presence of
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the second time constant to account for the tubulin-membrane binding kinetics. Therefore, the steps of tubulin binding to the membrane and/or to the VDAC cytosolic loops should be either much slower or much faster than the characteristic VDAC open time found to be in a range of 0.1–10 s (Fig. 1B). Slow kinetics of tubulin binding to the membrane, in the range of more than 100 s, seems to be the most likely scenario, because usually about 5–10 min after tubulin addition are required for blockage events to reach a steady-state frequency. Indeed, even slower kinetics, up to 40 min, were reported for the 125I-labeled tubulin binding to the liposomes formed from plasma membrane fractions (29). Experiments presented in Fig. 3 were carried out following a “quasi-stationary” protocol where \( \tau_{on} \) was measured ~5 min after each tubulin addition to the same membrane. Five minutes appeared to be sufficient for tubulin binding to reach equilibrium when membranes were made from a “sticky” DPhPC lipid, because a steady saturation concentration and lipid type, one of the components of the tubulin-membrane interaction could suppress or synergistically reinforce another. For instance, electrostatic repulsion between acidic tubulin and negatively charged PS in DPhPS/DPhPC membranes at low salt concentrations is expected to suppress the hydrophobic interactions; accordingly, the presence of PS results in a 100 times reduced \( k_{on} \) in comparison with neutral DPhPC membranes (Fig. 4A). In the DOTAP-containing membranes, the positively charged lipid could compete for the negatively charged CTT thus making them less available for VDAC blockage. At the same time, hydrophobic forces could compensate and overcome electrostatic repulsion between tubulin and DOTAP at high salt concentrations (Fig. 4B). It has to be noted here that nonlamellar tendency of charged lipids is a function of salt concentration (19), which further complicates the comparison of the results obtained under different salt conditions. Indeed, there is a possibility that low salts change the lipid packing stress of the DPhPS/DPhPC membranes, which synergistically with electrostatic repulsion reduces \( k_{on} \) in comparison with the DPhPC membranes.

In any case, our results demonstrate that the lipid-dependent tubulin binding to the membrane greatly impacts VDAC blockage by tubulin. It seems reasonable to suggest that hydrophobic interactions between the tubulin membrane-embedded domain and the nonpolar part of the membrane represent an essential component of the multistep VDAC blockage by tubulin. These interactions are sensitive to the bilayer mechanical parameters such as the hydrophobic thickness and lipid packing stress that depend on both the polar headgroup and hydrophobic acyl chains of the lipid. At physiologically low salt concentrations, tubulin-membrane association and consequently VDAC-tubulin interaction may also be modified by Coulomb forces between the lipid headgroup and tubulin charges.

To conclude, our findings suggest a new regulatory role of mitochondrial lipids in control of MOM permeability, and hence mitochondrial respiration, by tuning of VDAC sensitivity to blockage by tubulin. More generally, they give a clear example of lipid-assisted protein-protein interaction which, quantified by the equilibrium binding constant, can be varied by orders of magnitude through the choice of lipid species.

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