Evidence is accumulating that lipids play important roles in permeabilization of the mitochondria outer membrane (MOM) at the early stage of apoptosis. Lamellar phosphatidylcholine (PC) and nonlamellar phosphatidylethanolamine (PE) lipids are the major membrane components of the MOM. Cardiolipin (CL), the characteristic lipid from the mitochondrial inner membrane, is another nonlamellar lipid recently shown to play a role in MOM permeabilization. We investigate the effect of these three key lipids on the gating properties of the voltage-dependent anion channel (VDAC), the major channel in MOM. We find that PE induces voltage asymmetry in VDAC current-voltage characteristics by promoting channel closure at cis negative applied potentials. Significant asymmetry is also induced by CL. The observed differences in VDAC behavior in PC and PE membranes cannot be explained by differences in the insertion orientation of VDAC in these membranes. Rather, it is clear that the two nonlamellar lipids affect VDAC gating. Using gramicidin A channels as a tool to probe bilayer mechanics, we show that VDAC channels are much more sensitive to the presence of CL than could be expected from the experiments with gramicidin channels. We suggest that this is due to the preferential insertion of VDAC into CL-rich domains. We propose that the specific lipid composition of the mitochondria outer membrane and/or of contact sites might influence MOM permeability by regulating VDAC gating.

It is a well established fact that the major form of apoptosis proceeds through the mitochondrial pathway, wherein mitochondria play a controlling role (1–3). Numerous proapoptotic molecules and pathological stimuli converge on mitochondria to induce the permeabilization of the mitochondria outer membrane (MOM),2 which leads to cytochrome c release from the intermembrane space, consequent caspase activation, and irreversible apoptotic cell death. Although it was shown that many proteins could inhibit or prevent MOM permeabilization by acting on mitochondrial membranes, the mechanism of MOM permeabilization during apoptosis remains controversial (4).

The local regulation and execution of MOM permeabilization, predominantly orchestrated by proteins from the Bcl-2 family, involves mitochondrial lipids. For example, a reversible conformational change of the proapoptotic Bcl-2 family protein Bax, which occurs prior to Bax oligomerization, was obtained upon interaction of monomeric Bax with the membrane surface (5). Phospholipids were shown to significantly affect the activity of another proapoptotic BH3 domain-only protein Bid in cell-free assays (6). A caspase-8-cleaved Bid (tBid) was found to display lipid transfer activity (6) and promote negative membrane curvature and, as a result, destabilize bilayer membranes (7). It was proposed (8) that Bax-type apoptotic proteins could form lipidic pore-type nonbilayer structures in the membrane.

It was also shown that cardiolipin (CL), a lipid characteristic of mitochondria membrane, increases binding of tBid to pure lipid vesicles as well as to MOM (9) and promotes formation of large pores by tBid and monomeric Bax (10). CL is a unique phospholipid with four acyl chains. It is found in high concentrations in the inner membrane, where CL is the only major (up to 20 weight % of the total lipids) negatively charged phospholipid (11, 12). In the outer membrane, CL is present in much lower concentrations, but its content is higher in the contact sites, the points of close proximity between the inner and outer mitochondrial membranes (12, 13). CL is strongly bound to various enzymes and protein complexes involved in transport processes across mitochondrial inner membrane (14, 15). For example, CL is required for the cytochrome c oxidase activity (16, 17). It was demonstrated that tBid interacts with CL on functional mitochondria (18). This interaction occurs mainly in the contact sites and might contribute to mitochondrial cristae reorganization and cytochrome c release. Recent experiments with CL-deficient yeast mitochondria provide further evidence of the requirement of this lipid for tBid binding to mitochondria (19, 20). Moreover, the reported inhibition of state-3 respiration and ATP synthesis by tBid also requires the presence of CL (19, 20). These observations, despite sometimes contrasting results (21, 22), have increased current awareness of the role of CL in the mechanism of MOM permeabilization by proapoptotic Bcl-2 proteins.

The major channel in MOM is the voltage-dependent anion channel (VDAC). VDAC is known to be primarily responsible for metabolite flux across the MOM (23, 24). One of the char-
characteristic properties of the VDAC channel reconstituted into planar lipid membranes is its voltage gating (25). VDAC channels can exist in two functional states that differ in their ability to pass nonelectrolytes and to conduct ions (24, 26, 27). Elevated voltages favor “closed” states, the states of smaller conductance. These states are characterized by weak cationic selectivity, as compared with weak anionic selectivity in the open state, and are virtually impermeable for negatively charged metabolites, such as ATP (28). Therefore, VDAC closure greatly diminishes metabolite flux across MOM. However, closed states are able to transport small ions, such as K+ and Cl–. The gating mechanism for VDAC is still under discussion, but most of the experimental and theoretical evidence supports the model proposed by Colombini and co-workers (25, 29), where the existence of a mobile domain in the wall of the channel, called the voltage sensor, is postulated. VDAC responds to the electric field applied to the membrane by moving the sensor to the surface of the membrane, which results in a pore of diminished diameter and inverted selectivity (30–32).

In the present study, we hypothesize that the effect of membrane lipid composition on VDAC voltage gating is related to the elastic stress of lipid packing. The physical mechanisms by which membrane proteins respond to the elastic stress are attracting significant interest (33–39). The general idea is that protein conformational transitions must be coupled to some mechanical displacements that change the elastic stress of surrounding lipids and vice versa. In other words, if a membrane channel switches between two different conformational states, such as open and closed, one of these states might be more energetically favorable due to the protein interaction with the surrounding hydrophobic lipid phase.

In order to test this hypothesis, we investigated the effect of three key mitochondrial lipids: phosphatidylcholine (PC), lamellar lipid with small spontaneous curvature, and two non-lamellar lipids with high negative spontaneous curvature, phosphatidylyethanolamine (PE) and CL. The rationale for our study is that when a nonlamellar lipid, like PE, which spontaneously forms inverted hexagonal phase, is forced into the planar bilayer structure, this results in a significant stress of lipid packing in the region of the acyl chains (34, 37, 39–42). Estimates show that the difference in the corresponding lateral pressure between PE and PC membranes can reach several hundred atmospheres. It is plausible then that such change in the pressure may change VDAC conformational equilibrium.

Here we compare the voltage gating of VDAC channels reconstituted into planar membranes formed from two lipids, PC and PE, whose spontaneous curvature, packing density, and hydrophobic thickness are well known (40, 43–46). We find that the relative probability of the open and closed states changes with lipid substitution. The VDAC gating obtained in the multichannel membranes made of PE, a nonlamellar lipid with high negative spontaneous curvature and high packing stress, is more asymmetric in voltage than in the membranes formed from PC, a lamellar lipid with relaxed bilayer structure. We also demonstrate that CL, which induces negative curvature (47, 48), promotes similar voltage-gating asymmetry. As an internal control of the membrane mechanical properties, we use gramicidin A channels, whose association-dissociation kinetics is known to depend on several bilayer parameters, such as thickness, compression-expansion modulus, bending rigidity, interfacial tension (39), and dipole potential (49), but also on the lipid packing stress. Comparison of VDAC and gramicidin channel behavior in the membranes of the same lipid composition allows us to suggest that VDAC preferably inserts into the CL-rich domains.

**EXPERIMENTAL PROCEDURES**

VDAC from *Neurospora crassa* mitochondrial outer membranes, isolated and purified according to standard methods (50, 51), was a generous gift of Marco Colombini (University of Maryland, College Park). Dioleoylphosphatidylcholine (DOPC), Dioleoylphosphatidylethanolamine (DOPE), and CL were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). BzATP was purchased from Sigma.

The mixtures of DOPC and DOPE or CL were prepared from aliquots of two lipid solutions in chloroform, followed by drying lipid mixtures with nitrogen and then redissolving them in hexane or pentane to a total lipid concentration of 5 mg/ml. Bilayer membranes were formed from monolayers across 70–90-μm diameter orifices in a 15–μm-thick Teflon partition that separated two chambers (52). The experimental Teflon chamber was sonicated for 15 min in a chloroform/methanol (2:1) mixture, and a new partition was used each time when lipid composition was changed in order to avoid any traces of a “foreign” lipid.

The membrane potential was maintained using Ag/AgCl electrodes with 3 m KCl and 15% (w/v) agarose bridges. Aquous solutions of 250 mM or 1 m KCl and 1 mM CaCl2 were buffered by 5 mM HEPES at pH 7.0 or 7.6. VDAC channel insertion was achieved by adding 0.01–0.1 μl of a 1% Triton X-100 solution of purified VDAC in the 1-ml aqueous phase in the cis compartment while stirring. Potential is defined as positive when it is greater at the side of the VDAC addition (cis-side). For more details see Refs. 53 and 54.

The voltage-dependent properties of a VDAC-containing membrane were assessed following the protocol devised by Colombini and colleagues (26, 27, 55) in which gating is inferred from the channel response to a slowly changing periodic transmembrane voltage. In our experiments, we used a symmetrical 5-mHz triangular voltage wave with ±60 mV amplitude from a function waveform generator (model 33120A; Hewlett Packard). Data were acquired with the help of a Digidata 1322A board (Axon Instruments, Inc.) at a sampling frequency of 1 Hz and analyzed using the pClamp 9 software (Axon Instruments).

Voltage-dependent channels respond to the transmembrane voltage ($V$) by changing their conformational equilibrium. For a two-state channel, the equilibrium probabilities of the open and closed states, $P_{\text{open}}(V)$ and $P_{\text{closed}}(V)$, obey the Boltzmann distribution (56),

$$P_{\text{closed}}(V)/P_{\text{open}}(V) = \exp((V_0 - V)/ne/kT) \quad (\text{Eq. 1})$$

where $n$ is the net effective gating charge, which is a measure of the voltage dependence steepness, and $V_0$ is the voltage at which half the channels are open; $e$, $k$, and $T$ have their usual
VDAC Voltage Gating

meanings of the elementary charge, Boltzmann constant, and absolute temperature.

In the case of VDAC two distinctly different gating processes take place at negative and positive potentials (25). We use Equation 1 for the gating at negative applied potentials and define \( P_{\text{open}}(V) \) as the ratio,

\[
P_{\text{open}}(V) = (G(V) - G_{\text{min}})/(G_{\text{max}} - G_{\text{min}})
\]

where \( G_{\text{max}} \) and \( G_{\text{min}} \) are the maximum and the minimum conductances, corresponding to almost all channels open (small voltages) and almost all channels closed (high voltages), respectively. The 5-mHz voltage wave is usually slow enough to obtain quasi-equilibrium \( G(V) \) distributions, which allow comparison of VDAC gating under different experimental conditions (for more detailed discussion of this protocol, see Refs. 27, 55, 57, and 58). To visualize the quality of the fitting and to find parameters \( n \) and \( V_0 \), we use the logarithmic version of Equation 1,

\[
\ln((1 - P_{\text{open}}(V))/P_{\text{open}}(V)) = (V_0 - V)n/\epsilon kT
\]

where \( n \) is determined from the slope of the dependence, and \( V_0 \) is determined from the intersection with zero ordinate.

Gramicidin A (a generous gift of O. S. Andersen, Cornell University Medical College) was added from 0.1–1 mM ethanol stock solutions to both aqueous compartments at the amount sufficient to give a single-channel activity. Aqueous solutions of 1 mM KCl were buffered by 5 mM HEPES at pH 7.4. In the experiments with gramicidin channels, data were filtered by a low pass 8-pole Butterworth filter at 5 kHz and saved into the computer memory with a sampling frequency of 10 kHz. Data were analyzed using pClamp 9 software. A digital 8-pole Bessel low pass filter set at 50 Hz was applied to all records, and then single channels were discriminated. Gramicidin lifetimes were calculated by fitting logarithmic single exponentials to logarithmically binned histograms of at least 250 single-channel events (59). Nine different logarithmic probability fits were generated using different fitting procedures, and the mean and S.E. values of the fitted time constants were used as mean and S.E. for the lifetime. All measurements were made at room temperature, \( T = 23 \pm 1.0^\circ C \).

RESULTS

VDAC in PC, PE, and PC/CL Membranes—Voltage gating is one of the characteristic properties of a VDAC channel reconstituted into planar bilayer membranes. Once inserted, the channel remains in a high conducting or “open” state at low applied potentials (\(<30 \text{ mV by modulus})\). However, at relatively high potentials (\(>30 \text{ mV}\)), the channel moves into one of the multiple low conducting or “closed” states (Fig. 1). Sometimes channel conductance fluctuates between one open and multiple closed states. At 0 mV, the channel opens and closes again if high potential (50 mV, as in Fig. 1) is applied to the membrane. Channel closure occurs at both positive and negative potentials.

The most convenient and reliable method to study VDAC channel gating was designed by Colombini and co-workers (26, 27, 55). In this method, the slowly changing periodic voltages applied to the membrane allow the collection of necessary statistics. The channel closure at different potentials is obtained from \( G/V \) plots at increasing (by modulus) transmembrane voltages. Fig. 2A illustrates current responses to a symmetrical triangular voltage wave of 5 mHz and 60-mV amplitude (lower trace) applied to a multichannel membrane formed from PC (upper trace) and PE (middle trace). It is seen that in what concerns voltage polarity, the current trace is symmetric in PC membrane and asymmetric in PE membrane. At negative applied voltages, the current amplitude in PE membrane is lower than at positive voltages, due to more pronounced VDAC closure at negative potentials. Currents in PE membrane also tend to demonstrate a more pronounced hysteretic behavior.

Although the gating is a random phenomenon (note the variability in responses to the repeated identical sweeps of the applied voltage in Fig. 2A), with many channels and sufficient averaging, a clear bell-shaped voltage dependence of conductance is observed (Fig. 2B). To take into account the variable number of channels in each experiment, the \( G/V \) plots in Fig. 2B are expressed as a normalized conductance. Each data point is an average of a few independent experiments performed on individual multichannel membranes. Again, Fig. 2B shows that VDAC gating obtained on the membranes made from pure PE is more asymmetric than on the membranes formed from pure PC. Specifically, the channel closure at the cis negative voltages in PE membranes is more pronounced than in PC membranes.

In order to quantify the observed asymmetry of the voltage dependences with respect to the sign of applied voltage, we introduced an asymmetry factor (Fig. 3A) as a ratio of conductances at positive and negative applied voltages. VDAC gating in PC membranes was symmetrical up to 40 mV, and slight asymmetry appeared at higher potentials with an asymmetry factor of 1.1. The asymmetry factor for PE membranes increased with voltage and reached a steady state level of 1.9 at potentials higher than 40 mV. In membranes made from the 1:1 (mol/mol) mixture of PC and PE, the asymmetry factor was between those obtained for pure PC and PE membranes, as could be expected. A comparison of asymmetries measured at 45 mV for different lipid compositions is given in Fig. 3B. In the
membranes that contained more than 10 channels and up to 130 channels, the asymmetry factor was 1.1/0.2 for PC and 1.9/0.3 for PE membranes (Fig. 3B).

CL is another lipid that is known to induce high negative spontaneous curvature. We studied voltage gating of VDAC in CL-containing PC membranes. Figs. 2B and 3, A and B, show that in the membranes made from the mixture of PC with 4 mol % of CL, the asymmetry of VDAC gating is similar to that observed in pure PE membranes. At voltages higher than 40 mV, the asymmetry factor reached a steady level of about 2. Thus, lipids with high spontaneous curvature, PE and CL, promote VDAC voltage-gating asymmetry.

A possible explanation of the difference in the voltage-gating asymmetry between PE and PC multichannel membranes could be the difference in the degree of orientation of VDAC channel insertion. Indeed, suppose that insertion is random in PC bilayers and predominantly directional in PE bilayers. Then, if the gating asymmetry is an intrinsic property of VDAC channels, this could explain the observed difference.

Zizi et al. (57) demonstrated that VDAC insertion into planar phospholipid membrane made of asolectin (mixture of soy bean lipids) is an oriented process in the sense that the second and following channels insert in the same direction as the first one. Interestingly, the orientation of the first channel was found to be random. The present data suggest that in pure PE membrane, most of the channels are inserted in the same direction, not only in a particular experiment but in the orientation that persists from experiment to experiment. However, the symmetric gating in PC membranes could be explained by random insertion of VDAC channels into these membranes, wherein the channel asymmetry is compensated for, on average, by the oppositely orientated channels.
VDAC Voltage Gating

To test the orientation of VDAC channel insertion in PC membranes, we used a photoaffinity analog of ATP, BzATP. The idea is based on our finding that the symmetric addition of BzATP to both sides of the membrane generates asymmetric noise in the current through the channel. A typical experiment is demonstrated in Fig. 4A. Trace a represents ion current through four VDAC channels in PC membrane before the BzATP addition. The current is symmetric, and the total conductance at +30 mV is 15.6 nS. This corresponds to a single-channel conductance of 3.9 nS, a typical value in 1 mKCl solutions (23). The addition of BzATP to both sides of the membrane in equal concentration generates current noise whose amplitude depends on the sign of applied voltage. In Fig. 4A, trace b, the current trace is wider at the negative potential. The multichannel conductance is decreased to 12.9 nS. Moreover, after the insertion of three more channels following BzATP addition, the noise asymmetry is maintained (trace c). The exact number of VDAC channels during the experiment was counted by monitoring the stepwise channel insertion (not shown) and by measuring single-channel conductance in the presence of BzATP.

Current noise was analyzed by averaging the low frequency portion of the spectral density between 100 and 1000 Hz (52, 60). The low frequency spectral density, \( S(0) \), calculated for \( \pm 30 \) mV, is plotted in Fig. 4B. The spectral density in the presence of 1 mM BzATP shows a pronounced and easily detectable asymmetry in the applied voltage. The low frequency spectral density of four channels at \(-30 \) mV in the presence of 1 mM BzATP is 100 times higher than in control and 6 times higher than at \(+30 \) mV in the presence of BzATP. It is also seen that the noise spectral density in the BzATP-containing solutions is proportional to the number of channels, but the 6-fold asymmetry persists. The asymmetry of noise with respect to the sign of the applied voltage is definitely coupled with the inherent VDAC asymmetry (see “Discussion”) (60) and its orientation in the bilayer, because the system is symmetric otherwise. Based on these results, we conclude that the difference in VDAC gating in PC and PE membranes cannot be explained by different insertion orientation in these lipids.

It is worth mentioning that the effectiveness of VDAC insertion was also sensitive to lipid composition. When the same volumes of VDAC stock solution in Triton X-100 were added to the cis compartment of the cell, the average number of channels in PC membrane was about 4 times less than in PE membranes and about 40 times less than in PC/PE (0.5:0.5) membranes. As an internal control, we examined VDAC insertion into asymmetrical membranes made from opposed PC and PE monolayers (data not shown). Three experiments were made on the membranes with PC monolayer facing the cis compartment and PE monolayer facing the trans compartment and four experiments on the membranes with the reversed configuration. VDAC was added to the cis compartment in all experiments. The average number of channels in the membranes where VDAC was inserted via PC monolayers was 3 times smaller than in membranes where VDAC was reconstituted from the PE side.

Thus, not only gating but also protein partitioning between the membrane and the bulk depends on membrane lipid composition. This correlates well with the corresponding observations made earlier for the alamethicin channel (40, 61).

Our attempts to examine the asymmetry of voltage gating on a single channel level were less conclusive due to limited statistics. Indeed, because the channel response to the applied voltage is rather slow, the voltage ramp period had to be chosen to last several minutes. Only one or two open/closed transitions took place during that time, so obtaining reliable statistics required several h of continuous measurement. Single-channel
experiments of this duration proved to be problematic. To aggravate the situation even more, some of the single VDAC
channels on the time scales of hours showed time-dependent
 gating properties. This time dependence was never observed in
multichannel experiments. Fig. 5 compares results obtained
from a multichannel membrane during the first 20 min and
more than 1 h later.

The difference between VDAC multichannel and single-
channel gating behaviors in reconstitution experiments was
observed in our earlier study on the role of tBid in VDAC reg-
ulation (54). The reasons for this discrepancy are not clear at
the moment. However, based on the idea that VDAC molecules
in the multichannel membranes are organized in clusters (57),
we can speculate that the clusters, due to their larger size, are
less sensitive to the local irregularities in the membrane struc-
ture. Therefore, channel clusters are better detectors of the
integral properties of the lipid bilayers than single channels
whose gating characteristics may depend on the particular
localization of the channel in the membrane and even change in
time if the channel diffuses in the membrane plane.

**Gramicidin A Channels in PC, PE, and PC/CL Membranes—**
There are no experimental methods available so far that would
allow measuring lipid packing stress directly. All information
about mechanical properties of PC and PE bilayers comes from
experiments with spontaneous lipid structures (38, 40, 44 – 46,
62, 63). In an attempt to characterize the mechanical properties
of PE versus PC planar bilayers, we used gramicidin A channels
reconstituted into these membranes. Although the mecha-
nisms of modulation of gramicidin channel parameters by
membrane lipids are still under vivid discussion, this channel
has been proven to be a useful tool in probing bilayer mechan-
ic properties (68, 69), which can be related to bilayer thick-
ness, compression modulus, bending rigidity, etc. (for a review,
see Ref. 39). Bilayer packing stress is among several dominants
that determine its absolute value (41, 70, 71).

We examined gramicidin lifetime in the membranes of the
same lipid compositions as in the experiments with VDAC.
Representative current traces of gramicidin channels in the
membranes made from PC, PE, PC/PE (0.5:0.5), and PC/CL
(0.96:0.04) are shown in Fig. 6, A–D. It is seen that lipid com-
position significantly affects gramicidin channel lifetime. The
lifetimes were collected only from individual single-channel
events, and the corresponding time constants were determined
from logarithmic single-exponential fits (59) shown on the
right. The results are summarized in Table 1. In PE membranes,
gramicidin lifetime was about 13 times shorter than in PC
membranes. In membranes made from a PC/PE (0.5:0.5) mix-
ture, the lifetime was 6 times shorter than in pure PC mem-
brellas (Table 1). The effect of the addition of CL to a PC membrane on the
gramicidin channel lifetime turned out to be much smaller than
in the case of VDAC. At 4 mol % of CL in PC, the gating asym-
metry of VDAC was similar to that obtained in pure PE mem-
branes (Figs. 2B and 3A and B). In contrast, gramicidin lifetime
in the presence of 4 mol % of CL was reduced in comparison
with pure PC membranes only by a factor of 1.2 (Fig. 6D and
Table 1). In the membranes with 7 mol % CL in PC, gramicidin
channel lifetime was 1.7 times shorter than in pure PC mem-
branes (Table 1). Thus, although both lipids of high spontane-
ous curvature, PE and CL, reduce gramicidin channel lifetime,
the effect of CL is rather moderate (Table 1).

The experiments with the VDAC channel were performed in
the presence of 1 mM Ca^{2+} in the bathing solutions to enhance
VDAC insertion into the membranes, whereas experiments
with gramicidin were done without the Ca^{2+} addition. Previ-
ously, it was demonstrated that 6–8 mM Ca^{2+} induced mild
leakage in liposomes made of the membranes with high CL
content, namely CL/DOPC in a 1:2 molar ratio (7). In the 1–3
mM concentration range, Ca^{2+} induces a hexagonal phase in
aqueous dispersion of sodium salt of bovine cardiolipin due to
the 1:1 binding of Ca^{2+} to the negatively charged CL mole-
cule (47, 48). In order to test the possible effect of Ca^{2+} on PC/CL
membranes, we performed experiments with gramicidin channels
in PC/CL (4 mol %) in the presence of 1 mM CaCl_2. We did
not find any effects of Ca^{2+} on the gramicidin channel lifetime
(data not shown). Also, in a specially designed experiment, we
made sure that the presence of 1 mM Ca^{2+} in 250 mM KCl
changed neither VDAC conductance nor its voltage gating
(data not shown).

**DISCUSSION**

The elastic properties of biological membranes are recog-
nized as important functional modulators of ion channels,
receptors, and other integral proteins (38–41, 62, 72–75).
These proteins are mechanically coupled to the membrane
environment, and therefore, their conformational transitions
are differently constrained, depending on the membrane lipid
composition. Recent studies on OmpA protein from the bacte-
rial outer membrane suggest that nonspecific bilayer interac-


**FIGURE 5.** Asymmetry in voltage gating in multichannel membranes per-
sists in time. The asymmetry factor does not change during 100 min of
recording on multichannel membrane. After about 150 VDAC channels were
reconstituted into the membrane, five consequent G/V plots were collected
and averaged during the first 20 min (open symbols); the same procedure was
repeated 100 min later (closed symbols). The bilayer membrane was made
from a 1:1 mixture of PE and PC. The asymmetry factor was calculated as
described in the legend to Fig. 2. Experimental conditions were as in Fig. 2.
tions and lipid packing could be more important factors in stabilizing membrane proteins than specific chemical lipid-protein interactions (75). It has been shown (40, 41) that the model channel, alamethicin, has different properties in the PC and PE membranes. Indirect protein-protein interactions involving membrane lipids were recently demonstrated for a model system of gramicidin channels (33). It was also proposed that inhibition of stretch-activated cation channel by neuroactive amphipatic peptide involves not only the protein itself but also surrounding lipid (33).

When a planar membrane is formed by two monolayers of lamellar lipids (lipids with a small spontaneous curvature), the elastic stress in the region of hydrophobic lipid chains is relatively small. However, when nonlamellar, inverted hexagonal phase-forming lipids are forced into a planar bilayer structure, there is a significant lateral pressure in the hydrophobic core of the membrane (34, 44). The lipid with a higher (negative) spontaneous curvature induces a higher lateral pressure in the hydrocarbon chain area. By x-ray diffraction and NMR spectroscopy, it was shown that switching from lamellar DOPC to nonlamellar DOPE reduces the repulsive forces between the headgroups, changing the cross-section area per lipid molecule from 72 Å² (45) to 64 Å² (43). The decrease in the repulsion between lipid headgroups is compensated for by the increase in the lateral pressure in hydrophobic lipid chains and the corresponding increase in the bilayer thickness.

Here we have shown that two nonlamellar lipids with higher lipid packing stress, PE and CL, facilitate VDAC closure at negative applied

**FIGURE 6. Lipid composition affects gramicidin A channel lifetime.** Shown are representative current traces and the corresponding logarithmic lifetime histograms of gramicidin A channels in bilayer membranes formed from PC (A), PE (B), PC/PE (0.5:0.5) (C), and PC/CL (0.96:0.04) (D). Lipid content in parentheses is given as mol fractions. The lifetime histograms were collected from 250–500 single-channel events for each membrane composition indicated on the left. The medium consisted of 1 M KCl buffered with 5 mM HEPES at pH 7.4. The applied voltage was 150 mV. Current records were filtered using an averaging time of 30 ms.

**TABLE 1**

Nonlamellar lipids, PE and CL, change gramicidin A channel lifetime

Bilayer membranes of the indicated lipid composition were formed in 1 M KCl solutions buffered with 5 mM HEPES at pH 7.4. The applied voltage was 150 mV.

| Lipid Composition | Lifetimea ($) | DOPC | DOPE | DOPC/DOPE (0.5:0.5)b | DOPC/CL (0.96:0.04) | DOPC/CL (0.94:0.06) | DOPC/CL (0.93:0.07) |
|-------------------|---------------|------|------|----------------------|---------------------|---------------------|---------------------|
|                   | s             |      |      | s                    | s                   | s                   |
| 6.6 ± 0.5         | 0.5 ± 0.2     | 1.1 ± 0.1 | 5.4 ± 0.6 | 4.2 ± 0.22 | 4.0 ± 0.4 |

a Lifetime is calculated as described in the legend to Fig. 6. Each value presents the mean lifetime of nine different log probability-fitting procedures ± S.E.
b Lipid content is expressed in mol fractions.
potentials. This is seen in the multichannel membranes as an extra (in comparison with lamellar PC) reduction in conductance at negative potentials (Fig. 2B). The asymmetry of current-voltage characteristics induced by nonlamellar lipids cannot be attributed to rectifying properties of the open or closed states of the VDAC channel because of their nearly perfect Ohmic behavior (27). Therefore, this asymmetry is an inherent property of the channel gating, which can be amplified by the nonlamellar lipid. However, one of the hypothetical possibilities to account for the symmetric gating in PC membranes (and, thus, for the differences in gating in PC and PE) is to attribute it to the randomness of VDAC insertion into these membranes. Indeed, randomly inserted channels would compensate for each other’s intrinsic asymmetry and result, on average, in symmetric G/V plots.

To rule out this possibility, we used our finding that the symmetric addition of the ATP analog BzATP induces a pronouncedly asymmetric (in the applied voltage) noise in the current through the open VDAC channel (Fig. 4). Importantly, all conditions except for the VDAC addition were symmetric. Therefore, the current noise asymmetry must arise from the asymmetry of the VDAC channel. In multichannel membranes, the BzATP-induced noise maintained the same asymmetry in the applied voltage as in a single channel (Fig. 4B). Previously, we have shown that symmetrically added adenine di- and mononucleotides induced current noise, whose level depended on the sign of the applied potential (60). We concluded that an asymmetrically located adenine nucleotide binding site(s) could explain the dependence of noise on polarity of the applied potentials. In 95% of experiments, the asymmetry in current noise with respect to the sign of the applied potential (60). We concluded that an asymmetrically located adenine nucleotide binding site(s) could explain the dependence of noise on polarity of the applied potentials. In 95% of experiments, the asymmetry in current noise with respect to the sign of the applied potential was the same. Thus, the VDAC channel is inserted into the membrane essentially in the same direction.

The cooperativity in VDAC insertion into planar lipid membranes was first demonstrated by Zizi et al. (57), where the voltage-gating asymmetry was the same for the first and subsequently inserting channels. However, contrary to our observations, these authors have shown that the direction of the first channel insertion was random. This discrepancy could be due to a number of reasons. In Ref. 57 membranes were formed from asolectin, the soy bean lipid mixture, whereas we used only synthetic pure lipids. The random insertion of the first VDAC channel was demonstrated for two VDAC mutants isolated from yeast. It seems likely that different VDAC species may insert differently into the lipid bilayers of different lipid composition. Besides, experiments in Ref. 57 were performed in 1 M LiCl solutions, which contained dextran sulfate (500 kDa). Both Li⁺ and high molecular weight dextran interact with lipid membrane and protein interfaces (76, 77); this interaction could change VDAC insertion behavior. It is worth mentioning that unidirectional insertion in the “solvent-free” PC membranes was demonstrated for a number of different large channels, which include the α-hemolysin channel (78), the LamB (maltoopin) channel (79), bacterial porins OmpF (80) and OprF (81), and the anthrax PA63 channel (82). Apparently, the VDAC channel from N. crassa is not an exception.

To analyze the data in terms of gating charge, we use the average G/V plots (Fig. 2B). We first calculate the probability of the VDAC channel being open at different voltages using Equation 2. Then we plot the ratio (1 − Popen(V))/Popen(V) in semi-logarithmic coordinates to compare it with the Boltzmann distribution written in the form of Equation 3. It should be noted here that this equation implies conformational equilibrium, whereas the response to the slow triangular wave used here yields only “quasithermodynamical equilibrium” (55) data. In many practical instances (e.g. Ref. 83) that include our results for PE in Fig. 2A, the current response exhibits significant hysteresis, which is a signature of deviation from true equilibrium. This circumstance and the simplifying assumptions discussed under “Experimental Procedures” make the following analysis more qualitative than quantitative in character.

Fig. 7 shows the results of open probability calculations for PC and PE membranes at negative applied potentials. The values of n and V0 (Table 2) have been obtained from the regression analysis with both parameters set free. The regression lines in Fig. 7 for PC and PE membranes are almost parallel, suggesting that the net effective gating charge, n, is very similar for these membranes and is equal to 3.3 ± 0.2. What is different is V0, the intersection with ln((1 − Popen(V))/Popen(V)) = 0, corresponding to the voltage at which half of the channels are closed. For PE membranes, it is shifted by 10 mV toward less negative potentials.

![Figure 7](https://example.com/fig7.png)

**TABLE 2**

| Lipid composition | −V0 | n |
|-------------------|-----|---|
| PC (4)            | 26.8 ± 1.5 | 3.2 ± 0.6 |
| PE (8)            | 16.7 ± 4.4 | 4.0 ± 0.9 |
| PC + PE (1:1) (4) | 17.1 ± 2.6 | 3.2 ± 0.5 |
| PC + 4% CL (3)    | 22.5 ± 4.6 | 5.0 ± 0.2 |
| Low voltage (0−20 mV) | 26.9 ± 6.8 | 2.4 ± 0.3 |
| High voltage (20−45 mV) | 22.5 ± 4.6 | 5.0 ± 0.2 |

The asymmetry of current potentials in VDAC membranes, which include the α-hemolysin channel, the LamB (maltoopin) channel, bacterial porins OmpF and OprF, and the anthrax PA63 channel. Apparently, the VDAC channel from N. crassa is not an exception.

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![Figures and tables](https://example.com/fig7.png)

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In the membranes formed from the PC/PE 1:1 mixture, $V_0$ is between that of pure PE and PC membranes but closer to that of pure PE (Table 2). This suggests that the lipid packing stress in the nonlamellar bilayer (PE) promotes VDAC closure at negative voltages but leaves the gating charge unaffected. The gating charge of about +3.5 for VDAC from N. crassa in neutral lipids was reported previously (84). It was shown that VDAC gating is sensitive to the electrostatic environment, such as salt concentration, pH, and the presence of charge lipid (85). Here, for the first time, we demonstrate an example of coupling between a mechanical pressure in the hydrocarbon lipid region and VDAC channel gating. However, this coupling takes place not through a change in the gating charge but through the other parameter describing gating (see Equations 1 and 3), the characteristic voltage $V_0$. VDAC gating in the presence of CL turned out to be a more complex process than in PC and PE membranes or in their mixture. The best fit of the semilogarithmic probability plot of PC/CL membranes is biphasic (not shown). The gating charge is different at low and high applied voltages. At high voltages, the gating charge is about 2 times higher than at low voltages and 1.4 times higher than in PC and PE membranes (Table 2). The $V_0$ at low voltages is almost the same as in PC membranes, and that at high voltages is between that in PC and PE membranes (Table 2). Such a biphasic behavior was observed previously and attributed to the mobile charge consisting of two parts that could be put in motion at different voltages (85) and influenced by salt concentration or electrostatic environment. This may suggest that CL has a dual effect on VDAC gating: by the excess lipid packing stress and CL headgroup charge.

Our major finding is that the asymmetry of VDAC gating, an intrinsic property of the channel, can be either catalyzed or suppressed by the membrane lipid composition. According to the working model, transitions between the open and closed states of VDAC are associated with relatively large conformational rearrangements of the protein (25, 29, 30, 32). This motion accounts for 50% reduction in pore diameter and decreases the pore volume by 20–40 nm$^3$ (86). It is proposed that, depending on the applied voltage polarity, VDAC gates by two distinctly different processes, wherein a part of the protein is displaced toward one or the other side of the membrane (25). This conjecture is strongly supported by the fact that the closed states can differ by their average conductance for different signs of the applied voltage (Fig. 2B, especially data for PE and PC/CL membrane protein to the changing pressure in the hydrocarbon area of the membrane can be rationalized in a model wherein the transition changes the shape of the protein molecule in a way that either relieves or increases the pressure. In the case of VDAC gating, one of the possibilities is that the protein surface that is in contact with the hydrocarbon area can be concave or hourglass-shaped in the closed state at the negative applied voltages but nearly cylindrical in the open state and at the closed state at the positive voltages (Fig. 8).

For a crude estimate, we take an outer channel radius as $r \approx 1.5$ nm and the average linear deviation from a cylindrical shape as $\Delta r \approx 0.1$ nm. Then, if $h \approx 2$ nm is the height of the protein surface exposed to the lateral pressure, the extra volume that becomes available for hydrocarbon chains upon closure of the channel at the negative voltages (as compared with that at the positive voltages) is about $\Delta V = 2\pi rh\Delta r \approx 2$ nm$^3$. The increase in the lateral pressure in the hydrocarbon area upon the transition from DOPC to DOPE can be estimated as $\Delta P_c \approx 100$ atm or $10^7$ pascals (41, 42). Substitution of PC for PE is expected to introduce a free energy contribution of $\Delta E = \Delta V\Delta P_c$, which is about 5 kT per VDAC molecule or 3 kcal/mol. This is a significant contribution that should be easily seen in conformational transitions.

Thus, our hypothesis that VDAC gating is sensitive to the lipid packing stress in PE membranes is quite plausible. The surprising finding is that 4 mol % of CL in PC membrane causes quantitatively similar asymmetry in VDAC gating (Figs. 2 and 3). At the same time, the addition of 4 mol % of CL to PC membranes affects the gramicidin lifetime only slightly. The presence of 4 mol % CL reduces the gramicidin lifetime by 1.2 times, which has to be compared with the 13-fold reduction caused by PC to PE substitution (Table 1). Although the direct comparison of the VDAC and gramicidin data is problematic for the many reasons discussed above, the gramicidin probe seems to suggest that the effect of CL on VDAC gating cannot be entirely accounted for by the change in the lipid elastic stress unless VDAC predominantly inserts in CL-rich membrane domains. Indeed, it is also plausible that CL has a specific effect on VDAC channel gating.

CL is found in high concentrations in the points of contact between the inner and outer mitochondrial membranes (12, 13). VDAC is also believed to be localized in these contact sites (87, 88). Therefore, CL affinity for VDAC might be physiologically relevant. We suggest that the VDAC channel is more sen-
sitive to the presence of CL than gramicidin A channels, because VDAC inserts into CL-rich domains. An ability of CL to induce formation of CL-enriched clusters was shown with submitochondrial particles (inverted liposomes from the mitochondrion inner membrane) in the presence of millimolar concentrations of Ca\(^{2+}\) (89). In a model system of a 1:1 mixture of lamellar DOPC with CL in the presence of Ca\(^{2+}\), the formation of a new nonbilayer lipid structure, the “lipid particle,” was reported (47). The lipid microdomains in mitochondria and their contribution to apoptosis-associated modification of mitochondria were recently described for T cells (90). It was shown that these microdomains are enriched with VDAC.

Closure of VDAC would greatly reduce the ability of anionic metabolites to diffuse between the cytosol and the mitochondrion intermembrane space. Previously, it was shown that in its closed state VDAC is essentially impermeable to ATP (28). The diminution of the reduced pore size and reversed selectivity of closed state VDAC is essentially impermeable to ATP (28). The lipid microdomains to induce formation of CL-enriched clusters was shown with

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