Regulation of the Gating of BK\textsubscript{Ca} Channel by Lipid Bilayer Thickness\textsuperscript{*}

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Transmembrane segments of ion channels tend to match the hydrophobic thickness of lipid bilayers to minimize mismatch energy and to maintain their proper organization and function. To probe how ion channels respond to mismatch with lipid bilayers of different thicknesses, we examined the single channel activities of BK\textsubscript{Ca} (hSlo \(\alpha\)-subunit) channels in planar bilayers of binary mixtures of DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) with phosphatidylycerolines (PCs) of varying chain lengths, including PC 14:1, PC 18:1, PC 22:1, PC 24:1, and with porcine brain sphingomyelin. Bilayer thickness and structure was measured with small angle x-ray diffraction and atomic force microscopy. The open probability \((P_o)\) of the BK\textsubscript{Ca} channel was finely tuned by bilayer thickness, first decreasing with increases in bilayer thickness from PC 14:1 to PC 22:1 and then increasing from PC 22:1 to PC 24:1 and to porcine brain sphingomyelin. Single channel kinetic analyses revealed that the mean open time of the channel increased monotonically with bilayer thickness and, therefore, could not account for the biphasic changes in \(P_o\). The mean closed time increased with bilayer thickness from PC 14:1 up to PC 22:1 and then decreased with further increases in bilayer thickness to PC 24:1 and sphingomyelin, correlating with changes in \(P_o\). This is consistent with the proposition that bilayer thickness affects channel activity mainly through altering the stability of the closed state. We suggest a simple mechanical model that combines forces of lateral stress within the lipid bilayer with local hydrophobic mismatch between lipids and the protein to account for the biphasic modulation of BK\textsubscript{Ca} gating.

Ion channel proteins play an important role in the regulation of ion fluxes across cell and organelle membranes. They routinely undergo conformational changes during the ion transport process. The opening and closing of ion channels is sensitive to changes in the physical properties of the lipid bilayer, such as lipid composition (1, 2), bilayer thickness (3, 4), surface charge (5), and lateral stress (6), which provide mechanisms through which lipid bilayers regulate the function of resident ion channels. All of these properties of the lipid bilayer are interrelated. For example, changes in lipid composition will likely alter the surface charge, bilayer thickness, and/or the lateral stress forces associated with the membrane bilayer. Increasing the thickness of the lipid bilayer (with the same kind of lipids) has been shown to increase the lateral stress (or stiffness) of the bilayer (3).

The hydrophobic thickness of the lipid bilayer has been shown to be important in lipid-protein interactions in biological membranes (7–9). Specific examples of enzyme activity influenced by bilayer thickness are the Ca\textsuperscript{2+}/Mg\textsuperscript{2+} ATPase (10, 11), diacylglycerol kinase (12), rhodopsin (13), the human erythrocyte hexose transporter (14), and the Na\textsuperscript{+}/K\textsuperscript{+} ATPase (15). Several lines of evidence have established that the parameter of the membrane bilayer also plays an important role in regulating the sorting of proteins between the Golgi complex and the plasma membrane. The length of a protein transmembrane domain tends to conform to the hydrophobic thickness of the lipid bilayer (16, 17). Furthermore, it has been well established that within a single plasma membrane there are a number of lipid microdomains (lipid rafts) that are enriched in sphingolipids and cholesterol. These lipid rafts are thicker and more ordered than the rest of the membrane (18). Some proteins, such as BK\textsubscript{Ca} channels and other voltage-gated K\textsuperscript{+} channels, are targeted to these specific domains. This may influence the properties of these proteins, making them more suitable for particular functions (19–21).

The large conductance Ca\textsuperscript{2+}- and voltage-activated K\textsuperscript{+} (BK\textsubscript{Ca})\textsuperscript{2} channel represents a functional subtype of K\textsuperscript{+} channel that plays an important role in the regulation of neuronal excitability, cell volume regulation, excitation-contraction coupling, and hormonal secretion (22–24). BK\textsubscript{Ca} channels possess many of the common structural features of homotetrameric voltage-gated K\textsuperscript{+} channels, including an ion-selective pore formed by transmembrane segments S5 and S6 and a voltage-sensing module formed by transmembrane segments S1-S4. In addition, BK\textsubscript{Ca} channels have a long intracellular COOH terminus that may form a Ca\textsuperscript{2+}-sensing module. The intracellular COOH terminus of each \(\alpha\) subunit of the BK\textsubscript{Ca} channel contributes two RCK (regulator of K\textsuperscript{+} conductance) domains, and

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\textsuperscript{2}The abbreviations used are: BK\textsubscript{Ca}, large conductance, Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DPPE, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine; SPM, porcine brain sphingomyelin; PC 14:1, 1,2-dimyriristoyl-sn-glycero-3-phosphocholine; PC 18:1, 1,2-dioleoyl-sn-glycero-3-phosphocholine; PC 22:1, 1,2-dierucoyl-sn-glycero-3-phosphocholine; PC 24:1, 1,2-dinervonoyl-sn-glycero-3-phosphocholine; \(P_o\), open probability; HEDTA, N-(2-hydroxyethyl)ethylendiaminetriacetic acid; AFM, atomic force microscopy.
the eight RCK domains from the four α subunits form a gating ring similar to MthK channels (25). The linker that connects the S6 gate to the RCK domains forms a passive spring with the gating ring and is involved in Ca\(^{2+}\)-dependent activation (26).

Although the biophysical properties of voltage and Ca\(^{2+}\) activation of single channel gating have been explored extensively (27–30), the impact of the lipid environment on single channel gating of BK\(_{Ca}\) remains relatively unexplored.

Some of the basic effects of membrane lipid composition on BK\(_{Ca}\) channel conductance and activity have been characterized in different tissues (5, 31, 32). The conductance and open probability of BK\(_{Ca}\) channels was shown to be increased in lipid bilayers enriched with negatively charged lipids (31), an effect attributed to differences in surface charges leading to alterations of local concentrations of Ca\(^{2+}\) and K\(^{+}\) (5, 32). This conclusion, however, was revised in a recent study using Ba\(^{2+}\) to block the BK\(_{Ca}\) channel. An enhancement of the Ba\(^{2+}\) block of the BK\(_{Ca}\) channel in negatively charged membranes, which is predicted by the electrostatic mechanism, was not observed (33). The molecular shape of lipid molecules has also been suggested to influence the activity and conductance of BK\(_{Ca}\) channels (31).

In addition, Chang et al. (6) proposed the concept of structural stress to account for the reduction in the conductance and kinetics of BK\(_{Ca}\) channels that accompanies the incorporation of cholesterol into lipid bilayers. Based on our earlier study of a wide variety of lipid compositions in planar bilayers, we hypothesized that simple differences in bilayer thickness as a function of increasing acyl chain length. The inclusion of DOPE in these bilayers was necessary to promote the incorporation of the channel protein. SPM was chosen for comparison with the PC series because SPM has the same polar head group as the PCs but has a very high phase transition temperature (45 °C) (35). At room temperature, SPM membranes are in a more ordered gel phase and should be thicker than bilayers containing only PCs, all of which are in the liquid crystal phase at room temperature. Moreover, SPM is one of the major components of lipid rafts (besides cholesterol), and analyses of bilayers prepared with this lipid should shed some light on how the BK\(_{Ca}\) channel functions in native lipid rafts. Additionally, we present a model based on our results that is consistent with developing structure-function analyses of potassium channels. The model suggests that at least two processes account for the influence of bilayer thickness on BK channel kinetics and open probability; lateral stress seems to predominate in thinner bilayers, whereas hydrophobic mismatch seems to be the primary determinant of the effect in thicker bilayers.

**EXPERIMENTAL PROCEDURES**

*Materials*—The following lipids were obtained from Avanti Polar Lipids (Alabaster, AL) and used without further purification in these experiments: DOPE, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (PC 14:1), 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC 18:1), 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC 22:1), 1,2-dihexanoyl-sn-glycero-3-phosphocholine (PC 24:1), 1,2-dipalmitoyl sn-glycero-3-phosphoethanolamine (DPPE), and porcine brain sphingomyelin. Lipids were stored in chloroform at 10 mg/ml at -20 °C until use. Decane and salts were obtained from Sigma-Aldrich. All aqueous solutions were prepared with 18.3 megaohms/cm Milli-Q water.

*Membrane Preparation*—The cDNA encoding hSlo α, kindly provided by Dr. P. Ahring, NeuroSearch A/S (Copenhagen, Denmark), was overexpressed in HEK-293 cells. Stably transfected HEK-293 cells were grown in artificial medium (36), and membrane fragments were prepared using a protocol developed for COS cells with some slight modifications as described elsewhere (2).

*Electrophysiology Measurements and Data Analyses*—Single-channel recordings were performed with standard planar bilayer technology (37). Lipid mixtures of DOPE with PCs (1:1 mole ratio) or with SPM (3:2 mole ratio) were initially dissolved in chloroform. The solvent was removed by evaporation under N\(_2\), and the dried lipid film was resuspended in decane to form a final total lipid concentration of 25 mg/ml. A bilayer was formed by painting the lipid solution over a 100-μm hole (38) formed in a horizontal plastic coverslip sealed with Tackiwax to a Teflon partition separating an upper and lower chamber. Bilayer capacitance was monitored by noting the current across the bilayer in response to a triangle wave (10 mV/25 ms). Membrane suspensions containing crude membrane fragments (0.2–0.5 μl) were added to the upper cis chamber with a micropipette. In most bilayers this caused the thinned membrane to rupture. Channel incorporation was typically achieved within a few minutes after "brushing" the membrane fraction with fresh lipid solution across the annulus while forming a new bilayer. The cytoplasmic cis solution contained 300 mM KCl, 1.05 mM CaCl\(_2\), 1.25 mM HEDTA, 10 mM HEPES, pH 7.2. The free Ca\(^{2+}\) was estimated to be about 25 μM using the Max-Chelator Sliders program (39). The extracellular (trans) solution in the lower chamber contained 30 mM KCl, 0.1 mM HEDTA, 10 mM HEPES, pH 7.2. Single channel currents were recorded with a patch clamp amplifier (EPC-9, HEKA Elektronik, Lambrecht, Germany) (40). The trans chamber was connected to ground, and all voltages in the cis chamber were expressed relative to ground. Experiments were done at room temperature (22 °C).

Single channel events were sampled at 5 kHz with Pulse software (40). The data were filtered at 1 kHz for analysis and 500 Hz for display. Only recordings containing a single channel were analyzed. This was determined by a long depolarizing step from 0 to +40 mV to ensure that there was only one channel opening in the bilayer. At least 20 s of continuous recording at 0 mV was used to obtain an estimate of P\(_o\) from all-points histo-
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d gram. Durations of open and closed times were measured with half-amplitude threshold analysis. Dwell-time data were plotted with a logarithmic time axis along the abscissa and a square-root ordinate exhibiting the number of events in each bin. The bin density was 20 bins per decade. A lower limit of 0.6 ms was set for the dwell time distribution of open and closed intervals due to the time resolution of sampling and filtering (41). The occasionally long closed periods (shut intervals, > 5 s) were also excluded due to extremely low activity. A maximum-likelihood minimization routine was used to fit the distribution of open and closed times (42). All analyses were done using TAC and TAC-fit programs (Bruxton Corp.).

Small Angle X-ray Diffraction Analyses—Membrane samples consisting of binary lipid mixtures of DOPE with PCs (1:1 mole ratio) and binary mixtures of DOPE with SPM (3:2 mole ratio) were prepared as follows. Component lipids (in chloroform) were transferred to 13 × 100 test tubes and shell-dried under N2 while vortex mixing. Residual solvent was removed by drying for a minimum of 1 h under vacuum. After desiccation, each membrane sample was resuspended in diffraction buffer (0.5 mM HEPES, 154 mM NaCl, pH 7.3) to yield a final phospholipid concentration of 2.5 mg/ml. Multilamellar vesicles were then formed by vortex mixing for 3 min at either 40 °C (for SPM-containing membrane samples) or ambient temperature (for all other samples) (43).

Lipid bilayers were oriented for x-ray diffraction analysis as previously described (44). Briefly, 100-μl aliquots (containing 250 μg of total phospholipid) of each multilamellar vesicle preparation were transferred to custom-designed Lucite® sedimentation cells, each containing an aluminum foil substrate upon which the final membrane sample pellets were collected. Samples were then loaded into a Sorvall AH-629 swinging bucket ultracentrifuge rotor (DuPont) and centrifuged at 35,000 × g for 1 h at 5 °C. After orientation, the supernatants were aspirated, and the aluminum foil substrates, supporting the membrane pellets, were removed from the sedimentation cells and mounted onto curved glass slides. The samples were then placed in hermetically sealed brass canisters in which temperature and relative humidity were regulated before and during x-ray diffraction analyses. Saturated solutions of potassium sulfate (K2SO4) were used to establish a relative humidity level of 97% in these experiments, and samples were incubated at this relative humidity for a minimum of 1 h before experimental analysis.

The oriented membrane fraction samples were aligned at grazing incidence with respect to a collimated, monochromatic x-ray beam (CuKα radiation, λ = 1.54 Å) produced by a Rigaku Rotaflex RU-200, high brilliance rotating anode microfocus generator (Rigaku-MSC, The Woodlands, TX). The fixed geometry beamline utilized a single Franks mirror providing nickel-filtered radiation (Kα1 and Kα2 unresolved) at the detection plane. Diffraction data were collected on a one-dimensional, position-sensitive electronic detector (Hecus x-ray Systems, Graz, Austria) using a sample-to-detector distance of 150 mm. In addition to direct calibration, crystalline cholesterol monohydrate was used to verify the calibration of the detector. This technique allows for precise measurement of the unit cell periodicity, or d-space, of the membrane lipid bilayer, which is defined as the distance from the center of one lipid bilayer to the next, including surface hydration. In a multilamellar system, the amount of water that separates each membrane bilayer can be kept constant by carefully controlling relative humidity (using saturated salt solutions as noted above). Under these conditions, the d-space is directly affected by changes in the conformation of the membrane bilayer and its lipid constituents.

The d-space for any given membrane multibilayer is calculated from Bragg’s Law, $\lambda = 2d\sin \theta$, where $\lambda$ is the diffraction order, $\lambda$ is the wavelength of the x-ray radiation (1.54 Å), $d$ is the membrane lipid bilayer unit cell periodicity, and $\theta$ is the Bragg angle equal to one-half the angle between the incident beam and scattered beam.

Atomic Force Microscopy (AFM)—The lateral organization of supported membranes prepared from DOPE/SPM (3:2 mole ratio) mixtures were examined by AFM. Supported hybrid bilayers were prepared by Langmuir-Blodgett transfer as described elsewhere (45). Briefly, a DPPE monolayer was deposited on freshly cleaved, hydrophilic mica at a surface pressure of 45 millinewtons/m and formed a flat, defect-less single layer membrane. The second layer of DOPE/SPM was then deposited on DPPE-coated mica at a surface pressure of 30 millinewtons/m. The resulting bilayers were kept under water in a preset small container and kept under water during transfer to the AFM liquid cell (Molecular Imaging (MI), Phoenix, AZ). The supported bilayer samples were imaged under water with a MAC-mode Picoscan atomic force microscope (Molecular Imaging, Inc., Phoenix, AZ) as described elsewhere (45). These data provided information about the relative bilayer thickness and the phase separation of the bulk structure of the polar head groups in the outer leaflet.

RESULTS

Measurements of Membrane Bilayer Thickness—Small angle x-ray diffraction analyses of DOPE/PCs and DOPE/SPM membrane samples at 22 °C, 97% relative humidity yielded well-defined, reproducible diffraction orders that are consistent with a single lamellar lipid bilayer phase (Fig. 1). Membrane periodicity (d-space) values were calculated directly from the collected diffraction patterns and are summarized in Table 1. Overall membrane width increased systematically as a function of increasing acyl chain length, with a maximal increase of 26% observed between PC 14:1 and SPM. These data are consistent with previously reported measurements of hydrophobic thickness in pure PC bilayers (46) (Table 1). Differences that exist between comparative d-space and hydrophobic thickness values may be due to the structural contribution of equimolar DOPE, which has a hydrophobic thickness value of 29 Å (47). It is reasonable to expect that DOPE would slightly increase overall membrane width when added to pure PC 14:1 and PC 18:1 bilayers while slightly decreasing membrane width when added to pure PC 22:1 and PC 24:1 bilayers. The porcine brain sphingomyelin, used for preparing the SPM/DOPE bilayer samples in this study, contains hydrocarbon acyl chains of varying length, with the most prominent ones consisting of 22 and 24 carbons. AFM imaging of SPM/DOPE membranes indicated a phase separation in which SPM
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is in a thicker and more ordered gel phase because of its high phase transition temperature (Fig. 2). The bilayer thickness for SPM (C24) has been reported as 54 to 56 Å in the liquid crystal phase and 61 to 63 Å in the gel phase (~7 Å difference in thickness between the two phases, which is equal to roughly 4 carbons in acyl chain length) (48, 49). Therefore, we expect that the hydrophobic thickness for brain SPM membranes in the gel phase is around 44 Å and is thicker than PC 24:1 in the liquid crystal phase.

**Bilayer Thickness Tunes BK$_{Ca}$ Channel Activity**—BK$_{Ca}$ channels were reconstituted into planar bilayers prepared as binary mixtures of DOPE with PC 14:1, PC 18:1, PC 22:1, PC 24:1, or SPM. BK$_{Ca}$ channels show a reciprocal bimodal distribution of conductance and activity in the phase separated DOPE/SPM bilayers; i.e. the BK$_{Ca}$ channel has a high conductance and a low $P_o$ in the DOPE phase and a low conductance and a high $P_o$ in the SPM phase (65). Here we present only the single channel recordings of BK$_{Ca}$ channels in the low conductance, SPM phase.

Representative single channel current traces and associated all-point histograms, collected from DOPE/PCs and DOPE/SPM membrane samples, are shown in Fig. 3. The $P_o$ is an index of channel activity obtained from the all-points histograms of these traces. The BK$_{Ca}$ channel $P_o$ (calculated from the traces shown in Fig. 3A) was 0.67, 0.394, 0.067, 0.45, and 0.88 in DOPE membranes containing PC 14:1, PC 18:1, PC 22:1, PC 24:1, and SPM, respectively. The same trend was observed for the mean $P_o$ averaged from five single channel recordings of BK$_{Ca}$ in each bilayer (Fig. 3C). An increase in the bilayer thickness from PC 14:1 to PC 18:1 resulted in a decrease in channel activity from a mean $P_o$ of 0.59 ± 0.09 to 0.39 ± 0.04, respectively; channel activity was further reduced to 0.10 ± 0.02 in membrane bilayers containing PC 22:1. Additional increases in bilayer thickness, however, reversed this trend with an increase in mean $P_o$ to 0.45 ± 0.04 and 0.82 ± 0.06 for DOPE/PC 24:1 and DOPE/SPM bilayers, respectively. The nadir of BK$_{Ca}$ channel activity was observed in DOPE/PC 22:1 membrane bilayers, which suggests that a balancing point in the hydrophobic match interaction of the protein with the lipid bilayer is achieved at or near this bilayer thickness (50). Altering the lipid bilayer thickness with either an increase or decrease from this nadir resulted in activation of the channel. Increasing membrane thickness above this point (i.e. above that achieved with PC 22:1) had a more profound effect on BK$_{Ca}$ channel gating (Fig. 3C) if we consider that the change (5.2 Å) in bilayer thickness from PC 22:1 to PC 24:1 is less than the change (6.5 Å) in bilayer thickness from PC 18:1 to PC 22:1 (Table 1).

*Increases in Bilayer Thickness Stabilize the Open Conformation of BK$_{Ca}$—Channel $P_o$ is measured as the amount of time that the channel spends in the open state versus the sum of both open and closed states. A kinetic analysis from single channel dwell-time histograms of BK$_{Ca}$ channels in different bilayers illustrates how lipid bilayer thickness affects the stability of the open and closed conformations. The distribution of the open and closed times of the BK$_{Ca}$ channels in each of the bilayers is shown in Fig. 4. The mean open and closed times averaged from three single channel analyses are shown in Fig. 5. The mean open and closed times (Fig. 4A) in the DOPE/PC 14:1 and DOPE/PC 18:1 bilayers are well fitted by a single exponential, showing that BK$_{Ca}$ channel activity in these two relatively thin bilayers is defined by a single open state. In addition, an increase in bilayer thickness from PC 14:1 to PC 18:1 shifted the open intervals to the right, which indicates an increase in the channel mean open time (from 2.4 ± 1.0 to 7.3 ± 1.8 ms; see Fig. 5). In DOPE/PC

![Graph of x-ray diffraction patterns](Image)

**FIGURE 1.** Representative x-ray diffraction patterns obtained from DOPE/PC and DOPE/SPM bilayer samples. Data were collected on a 1-dimensional, position-sensitive electronic detector at 22 °C and 97% relative humidity. Sample diffraction orders can be observed to shift to the left going from back to front, consistent with an increase in d-space associated with increasing acyl chain length. The d-space value calculated from these diffraction patterns is summarized in Table 1.

**TABLE 1**

Membrane d-space and hydrophobic thickness values

| d-Space for DOPE/PC binary mixtures (mean ± S.D.) | Hydrophobic thickness reported for PCs or SPM alone |
|-----------------------------------------------|-----------------------------------------------|
| PC 14:1                                       | 55.9 ± 3.0                                   |
| PC 18:1                                       | 58.0 ± 3.6                                   |
| PC 22:1                                       | 64.5 ± 3.9                                   |
| PC 24:1                                       | 69.7 ± 0.5                                   |
| SPM                                           | 71.1 ± 1.2                                   |

* X-ray diffraction measurements were conducted in triplicate.
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*FIGURE 2. Phase separation in DOPE/SPM membrane imaged by AFM. Three features can be seen in the image: 1) bright lipid patches (marked by white arrows), which are assigned as SPM-rich domains; 2) darker regions, which are assigned as DOPE-rich domains; 3, the darkest holes (marked by dark arrows), which are bilayer defects. The section analysis gives the height difference between the two domains (about 12 Å). The thickness of the bilayer can be measured from the bilayer defects, which gives roughly 40 Å for the DOPE-rich phase and 52 Å for the SPM-rich phase.*

During gated activity but also stabilizes the channel open conformation, thus increasing mean open time.

**Effect of Bilayer Thickness on the Closed Conformation of $\text{BK}_{\text{Ca}}$—**The closed time distributions for the $\text{BK}_{\text{Ca}}$ channel in all of the DOPE/PC and DOPE/SPM lipid bilayers were best fitted by a mixture of four exponential components (Fig. 4B), indicating that changes in lipid bilayer thickness do not alter the number of closed states that the $\text{BK}_{\text{Ca}}$ channel enters during gated activity. However, changes in bilayer thickness do have a complex effect on the closed time distributions of the channel. First, an increase in bilayer thickness from PC 14:1 to PC 18:1 and PC 22:1 shifted the closed time distribution to the right while also causing the channel to spend less time in short closed states and more time in longer closed states. With further increases in bilayer thickness (to PC 24:1 and SPM), however, the closed time distribution was shifted back to the left. As shown in Fig. 5B, the mean channel closed time increased with increasing acyl chain length, from PC 14:1 to 22:1, but then decreased with further increases in acyl chain length (PC 24:1 and SPM). These changes mirror those observed for $P_{\text{op}}$, indicating a dominant contribution of the closed time to the changes in channel open probability. In addition, these changes indicate that the closed state conformation of the $\text{BK}_{\text{Ca}}$ channel (similar to the open conformation) is stabilized by increases in bilayer thickness up to the membrane width achieved with PC 22:1; further increases in bilayer thickness (PC 24:1 and SPM), however, dramatically shift the distribution of closed intervals to the left, consistent with a destabilization of the closed state conformation in thicker membrane bilayers.

**Voltage and Ca$^{2+}$ Dependence of $\text{BK}_{\text{Ca}}$ Gating in SPM Membranes—**The robust activation of the $\text{BK}_{\text{Ca}}$ channel in SPM membranes is of great interest since $\text{BK}_{\text{Ca}}$ channels have been thought to be specifically targeted to lipid rafts. $\text{BK}_{\text{Ca}}$ channels are activated by membrane depolarization and/or elevations in intracellular Ca$^{2+}$ concentrations. Thus, the activation of $\text{BK}_{\text{Ca}}$ channels in SPM membranes could arise from 1) increased sensitivity of the voltage sensors, 2) increased Ca$^{2+}$ sensitivity, 3) increased Ca$^{2+}$ concentration at $\text{BK}_{\text{Ca}}$ channel binding sites, or 4) other influences such as lateral stress or hydrophobic mismatch resulting from the introduction of the $\text{BK}_{\text{Ca}}$ channel into thicker membrane bilayers.

We examined the voltage dependence of $\text{BK}_{\text{Ca}}$ channel gating in SPM membranes and compared it with similar measurements conducted in PC membrane bilayers (Fig. 6). The $P_{\text{op}}$, membrane potential (V) curve was left-shifted for $\text{BK}_{\text{Ca}}$ channels in SPM membranes compared with those measured in PC membranes; however, changes in lipid bilayer thickness, from PC 14:1 to SPM, had little apparent effect on the limiting slope (28) over the range of $P_{\text{op}}$ values examined in this study, suggesting that altering the thickness of the bilayer does not significantly affect the voltage sensitivity of the $\text{BK}_{\text{Ca}}$ channel (defined as the change in membrane potential required for an e-fold change in $P_{\text{op}}$) (28). The numerical slope value is 0.038 mV$^{-1}$ for PC 14:1, 0.048 mV$^{-1}$ for PC 18:1, 0.065 mV$^{-1}$ for PC 22:1, 0.052 for PC 24:1, and 0.031 mV$^{-1}$ for SPM, respectively. The $\text{BK}_{\text{Ca}}$ channel in SPM membranes is the least sensitive to voltage changes while it has the greatest $P_{\text{op}}$, indicating that the activation of the $\text{BK}_{\text{Ca}}$ channel in SPM membranes cannot be attributed to increased voltage sensitivity.
The shift in BK$_{Ca}$ channel gating in SPM membranes (as indicated by a leftward shift in the $P_o$-membrane voltage curve, Fig. 6) is consistent with an increase in Ca$^{2+}$/H$^{1+}$ sensitivity of the channel or an increase in Ca$^{2+}$/H$^{1+}$ concentration at membrane sites adjacent to the channel, which have also been shown to shift the $P_o$-V leftward (27, 51, 52). To evaluate whether or not the activation of the BK$_{Ca}$ channel in SPM membranes is due to increased Ca$^{2+}$/H$^{1+}$ sensitivity, we examined the effects of Ca$^{2+}$/H$^{1+}$ on BK$_{Ca}$ channel gating in DOPE/SPM and DOPE/PC 18:1 membrane bilayers (Fig. 7). Calcium sensitivity is defined as the change in calcium concentration required for an e-fold change in $P_o$ and can be assessed by the slope of the curves of log $P_o$ versus log [Ca$^{2+}$] in Fig. 7C. These data show that BK$_{Ca}$ channels are actually less sensitive to changes in Ca$^{2+}$ concentration in SPM membranes when compared with PC 18:1 membranes. Thus, the enhanced activation of BK$_{Ca}$ channel in SPM membranes appear not to result from increased Ca$^{2+}$ sensitivity.

To determine whether or not the enhanced activation of BK$_{Ca}$ channels in SPM membranes is mediated through increased Ca$^{2+}$ concentrations at membrane sites, we measured the effects of Ca$^{2+}$ on the kinetic properties of BK$_{Ca}$ and compared those results with changes that occur by modulating

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**Figure 3. Bilayer thickness tunes the activity of the BK$_{Ca}$ channel.** A, representative single channel current traces of BK$_{Ca}$ channels reconstituted into planar bilayers of equal molar lipid mixtures of DOPE with PCs of different acyl chain lengths and with SPM (3:2, molar ratio), shown on two time scales. B, all-point histograms used to compute $P_o$. C, mean $P_o$ computed from five single channel recordings collected from each lipid bilayer group. Data were recorded at room temperature at a holding potential of 0 mV. The arrows show the position where the traces are shown on an expanded time scale.
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For these analyses, we compared the gating properties of the BK\textsubscript{Ca} channel in SPM membranes exposed to 25 \(\mu\text{M} \text{Ca}^{2+}\) (Fig. 7E) with those in PC 18:1 membranes exposed to 25 \(\mu\text{M} \text{Ca}^{2+}\) (Fig. 7D). These \(\text{Ca}^{2+}\) levels produced similar \(P_o\) values in SPM and PC 18:1 membranes (\(P_o = 0.385\) and 0.394 in SPM and PC 18:1 bilayers, respectively); however, the open and closed dwell-time distributions of the BK\textsubscript{Ca} channel in these two membranes were very different from each other. BK\textsubscript{Ca} channels in SPM membranes have two open states with a longer open time constant; in PC 18:1 bilayers, BK\textsubscript{Ca} channels have only one open state with a much shorter open time constant. In the closed time distributions, BK\textsubscript{Ca} channels in both membrane preparations have four closed states, but the time constants for the closed states and the frequency of entry into each closed state are drastically different. The mean open and closed times are much longer in SPM membranes (11.3 and 40.2 ms, respectively) compared with those in PC 18:1 membranes (7.3 and 5.6 ms, respectively). If SPM activation of the BK\textsubscript{Ca} channel occurs through an alteration in \(\text{Ca}^{2+}\) concentration at the binding site, we would expect that reducing the \(\text{Ca}^{2+}\) concentration in SPM membranes to obtain the same level of \(P_o\) as in PC 18:1 membranes would result in comparable gating kinetics. The marked differences in single channel gating kinetics observed in these membrane samples indicates that the activation of BK\textsubscript{Ca} channel in SPM membranes does not occur as a result of changes in intracellular \(\text{Ca}^{2+}\) concentration at the binding sites. Taken together, these data suggest that the enhanced activation of the BK\textsubscript{Ca} channel in SPM is most likely the result of membrane structural influences such as lateral stress or hydrophobic mismatch.

**DISCUSSION**

The data presented in this study clearly show that the activity of the BK\textsubscript{Ca} channel is modulated by changes in bilayer thickness. The shape of the activation curve for the channel (Fig. 3C) is inverted from that usually seen in membrane-bound enzymes (13), where the enzyme activity is normally reduced when placed in thicker bilayers, presumably by...
the increased rigidity of thicker bilayers. The robust activation of the BK<sub>Ca</sub> channel in SPM membranes is not due to alterations in voltage or Ca<sup>2+</sup>/H<sub>11001</sub>sensitivity nor is it influenced by changes in Ca<sup>2+</sup>/H<sub>11001</sub> concentration at the binding sites. All of the lipids used in this study are zwitterionic at physiologic pH, which rules out the influence of surface charge in these modulations (5, 33). Changes in the thickness of the lipid bilayer are likely accompanied by global changes in other membrane properties, such as lateral stress and membrane tension (53). In our study, the lateral stress (or stiffness) associated with the lipid bilayer probably increases monotonically with increases in membrane thickness, since lipid bilayers become more ordered with an increase in thickness, as reflected by an increase in the main phase transition temperature (11). An increase in lateral stress would be expected to hinder the closed-open conformation transition (gating) of the BK<sub>Ca</sub> channel by raising the energy barrier of the transition, resulting in decreased channel activity (P<sub>o</sub>).

In addition, increases in bilayer thickness can lead to local changes in hydrophobic mismatch between the channel protein and the lipid bilayer. The potassium channel, K<sub>c</sub>S<sub>A</sub>, has been reported to be best matched with PC 22:1 lipid bilayers, but by tilting its transmembrane α-helices, K<sub>c</sub>S<sub>A</sub> can efficiently induce a hydrophobic match with thin bilayers, such as those comprised of PC 14:1 and PC 18:1 (50). In thick membranes like PC 24:1 and SPM, the local hydrophobic mismatch between the channel protein and lipid bilayer could become significant. The modulation of BK<sub>Ca</sub> channel activity by altered bilayer thickness shown in Fig. 4 likely reflects the collective effect of both global changes in membrane properties, such as lateral stress, and local structural aspects of hydrophobic mismatch between the protein and the lipid bilayer.

With an increase in bilayer thickness, as observed from DOPE/PC 14:1 to DOPE/PC 18:1 and DOPE/PC 22:1 lipid bilayers, the activity of the BK<sub>Ca</sub> channel is decreased from a mean P<sub>o</sub> of 0.59 ± 0.085 to 0.39 ± 0.041 and 0.104 ± 0.02, respectively, showing that the BK<sub>Ca</sub> channels are more active in thinner bilayers. Similar gating properties have been observed for a mechanosensitive channel, MscL (3), which is gated by lateral tension transmitted through the bilayer. We suggest that the gating of the BK<sub>Ca</sub> channels in these thin bilayers is similar to that seen in the MscL channel and is regulated primarily by changes in lateral stress within the lipid bilayer. The BK<sub>Ca</sub> channel is a stretch-sensitive ion channel (54–56), and the mechanosensitivity of BK<sub>Ca</sub> channels plays an important role in many physiological processes involving mechanical signal transduction (57, 58). Decreasing bilayer thickness would reduce the lateral stress applied to the membrane protein by surrounding lipids, thus lowering the activation energy of the channel (3). This is supported by the kinetic data of BK<sub>Ca</sub> channel opening and closing in the thin DOPE/PC 14:1 bilayers. BK<sub>Ca</sub> channels in this bilayer open and close very rapidly, with the shortest mean open time (T<sub>o</sub> = 2.4 ± 1.0 ms) and closed time (T<sub>c</sub> = 4.2 ± 1.2 ms) observed in this study. This phenomenon suggests that there is only a small energy barrier separating the open and closed conformation of the channel in this lipid system. In contrast, BK<sub>Ca</sub> channels are likely exposed to greater lateral stress in the thicker, more ordered lipid bilayers, which would be expected to raise the activation energy level and impede the closed-open conformation transition (P<sub>o</sub>-gating).

The increased energy barrier that separates the open and closed conformation would also stabilize both the open and closed state conformations, as reflected in the much longer mean open

FIGURE 5. Mean open time (A) and closed time (B) from kinetic analyses of at least three single channel recordings of BK<sub>Ca</sub> channels in each of the indicated lipid compositions.

FIGURE 6. The voltage dependence of BK<sub>Ca</sub> channel gating in SPM and PC membranes. The BK channel currents were recorded for 10 s at holding potentials between −40 mV and +40 mV, with incremental steps of 10 mV in each bilayer. P<sub>o</sub>-membrane potential curves were plotted on logarithmic coordinates. At least three separate channels were evaluated in each lipid bilayer.
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**FIGURE 7. The Ca\textsuperscript{2+} dependence of BK\textsubscript{Ca} channel gating in SPM and PC 18:1 membranes.** Single channel currents are recorded for BK\textsubscript{Ca} channels at various Ca\textsuperscript{2+} concentrations in SPM membranes (A) and in PC 18:1 membranes (B). C, plots of log P\textsubscript{o} against log [Ca\textsuperscript{2+}] for BK\textsubscript{Ca} channels in SPM and PC 18:1 membranes. Each data point is averaged from two channel recordings for each bilayer. Shown are the open time (D) and closed time (E) distributions for the BK\textsubscript{Ca} channels recorded at 15 μM Ca\textsuperscript{2+} in SPM membranes, which has a same level of activity (P\textsubscript{o}, 0.385) as the BK\textsubscript{Ca} channel in PC 18:1 membranes at 25 μM Ca\textsuperscript{2+} (P\textsubscript{o}, 0.394). The time constant for each fit and their relative contribution to total fit are listed on the figure.

Further increasing bilayer thickness from PC 22:1 to PC 24:1 and then to SPM leads to increases in channel activity (Fig. 3C), which suggests that another factor besides lateral stress is likely involved. Lateral stress changes monotonically with membrane thickness, but BK\textsubscript{Ca} channel activation does not (Fig. 3C). The resulting V-shaped relationship between channel activity and bilayer thickness indicates a balancing point in the forces acting on the BK\textsubscript{Ca} channel and the lipid bilayer at or near the bilayer thickness associated with DOPE/PC 22:1. The KcsA potassium channel, as recently shown in fluorescence quenching studies of its interactions with various PCs, shows a similar dependence on membrane bilayer thickness (50).

As previously noted, a dramatic increase in channel activity in SPM membranes was unexpected since one would assume that the lateral stress in SPM, gel phase membranes would be much higher than that in thinner PC, liquid crystalline phase membranes. Increased lateral stress would be expected to restrict channel opening. Examination of the voltage and Ca\textsuperscript{2+} dependence gating of BK\textsubscript{Ca} channels in SPM membranes demonstrated that the increased activation is not due to either increased voltage sensitivity (Fig. 6) or increased Ca\textsuperscript{2+} sensitivity (Fig. 7C). We propose instead that placing BK\textsubscript{Ca} channels in thicker SPM membranes results in a dramatic increase in the hydrophobic mismatch between the BK\textsubscript{Ca} channel and the surrounding lipids and that this effect dominates other channel gating influences, such as lateral stress, that may be more pronounced in thinner membranes where there is less mismatch energy.

We considered a recent mechanical model proposed for BK\textsubscript{Ca} channel gating by Niu et al. (26) to account for the activation of BK\textsubscript{Ca} channels by local hydrophobic mismatch in thicker bilayers. In the proposed model the gating ring formed from eight RCK (regulator of K\textsuperscript{+} conductance) domains and the linker that connects the S6 gates with RCK1 form a passive spring, which when stretched can apply force to the gates, pulling them open (see Fig. 8). In their study, Niu et al. (26) demonstrated that a decrease in the linker length by even a single amino acid residue (0.35 nm if the linker is an extended polypeptide chain) increases channel activity substantially. The channel protein is anchored firmly into the lipid bilayer by aromatic residues at the ends of transmembrane α-helices, acting as “floats” at the interface (50). When BK\textsubscript{Ca} channels are placed into a thicker bilayer like a membrane containing SPM, the hydrophobic thickness of the bilayer is larger than the hydrophobic thickness of the transmembrane segments of the BK\textsubscript{Ca} channel (by about 7–10 Å if we assume that they do match in the pure PC 22:1 bilayer (50)). The negative hydrophobic mismatch (47) between the BK\textsubscript{Ca} channel and the thick lipid bilayer could conceivably stretch the protein, pulling the linker inward toward the hydrophobic core of the bilayer and increasing the tension on the spring (Fig. 8). The tension on the spring linker will pull the gate open and increase the activity of the BK\textsubscript{Ca} channel. In other words, the tension on the spring connected to the S6 gates caused by the hydrophobic mismatch will destabilize the closed state conformation and favor the open state conformation of the BK\textsubscript{Ca} channel. Once the channel opens, the tension on the spring is partially relieved, and the mismatch has less effect on the open state conformation. This model would predict that the major effect of the hydrophobic mismatch in thick bilayers is on the closed state conformation. This is supported by our observation that the mean open time of the BK\textsubscript{Ca} channel increases from 8.6 ± 2.1 ms in PC 22:1 membranes to 25.2 ± 6.2 ms (about a 3-fold increase) in SPM membranes, whereas the mean closed time decreases dramatically from 79.7 ± 18.3 ms in PC 22:1 membranes to 4.5 ± 1.5 ms (an 18-fold decrease) in SPM membranes. In thin bilayers like DOPE/PC 14:1 and DOPE/PC 18:1, hydrophobic matching for
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\( BK_{\text{Ca}} \) channels with the bilayer can be efficiently achieved by tilting the whole protein away from the bilayer normal at different angles (Fig. 8), as suggested by others for the \( K_{\text{csA}} \) potassium channel (50). Therefore, we postulate that the progressive increase in lateral stress associated with the increase in thickness of the PC 14:1-PC 22:1 range accounts for most of the reduction seen in this portion of the activity curve (Fig. 4), whereas in thicker bilayers (above PC 22:1), the increase in activity is dominated by the increase in hydrophobic mismatch, although lateral stress is also involved the SPM bilayer.

Although activation of the \( BK_{\text{Ca}} \) channel in SPM membranes is not due directly to changes in \( Ca^{2+} \) sensitivity or intracellular \( Ca^{2+} \) concentration at the binding sites, the mechanical spring model we employed here suggests that the hydrophobic mismatch in thick SPM membranes has an effect on \( BK_{\text{Ca}} \) gating similar to that produced by increasing intracellular \( Ca^{2+} \). This is actually predictable from our model, since the spring-like linker-gating ring complex is also involved in \( Ca^{2+} \) activation; binding of \( Ca^{2+} \) expands the gating ring and pulls the gate open (26, 59). Tension on the spring, caused by either hydrophobic mismatch or by the binding of \( Ca^{2+} \), would destabilize the closed conformation and favor the open conformation (Fig. 8).

Reducing \( Ca^{2+} \) concentration should have the opposite effect of stabilizing the closed conformation and destabilizing the open conformation. Again, the spring model predicts that the major effect of increasing or reducing \( Ca^{2+} \) concentration will be on the closed state conformation, not the open state conformation. This is supported by the single channel kinetics of \( BK_{\text{Ca}} \) gating obtained at two different \( Ca^{2+} \) concentrations in SPM membranes (Fig. 7, D and E). Reducing \( Ca^{2+} \) from 25 to 15 \( \mu M \) shifted the open interval distribution toward short-opening states and the closed interval toward long-closed states (compare with SPM dwell-time data in Fig. 4). The mean open time decreased from 25 to 11.3 ms (2-fold decrease), whereas the mean closed time increased almost 10-fold from 4.5 to 40.2 ms.

We want to emphasize that although the spring model we applied here provides a simple and reasonable explanation for the increased activation of the \( BK_{\text{Ca}} \) channel in SPM membranes observed in this study, other mechanisms could contribute to the activation of the \( BK_{\text{Ca}} \) channel in SPM membranes, such as direct hydrogen-bonding interactions between SPM and \( BK_{\text{Ca}} \) channel proteins. When compared with PCs, which have only hydrocarbon bond-accepting features (two ester carbonyls) in the interfacial region, SPM has amide and hydroxyl groups that can serve as hydrogen bond donors and acceptors; thus, SPM is capable of forming complex intermolecular hydrogen bonds with membrane proteins (60). Further investigations are under way in our laboratory to explore the potential activation of \( BK_{\text{Ca}} \) channels in SPM membranes by direct hydrogen-bonding interactions between SPM and \( BK_{\text{Ca}} \) channel proteins.

In addition to hydrogen-bonding interactions between \( BK_{\text{Ca}} \) and membrane lipids, it is possible that other transmembrane segments of the \( BK_{\text{Ca}} \) channel, such as \( S4 \), are also involved in channel gating (61). The fact that the gating activity of the \( BK_{\text{Ca}} \) channel can be tuned by lipid bilayer thickness points to the general importance of the lipid environment. Thus, changes in the molecular organization of the cell membrane bilayer, such as those that would occur in the formation and dissociation of lipid rafts, are likely to play an important role in modulating \( BK_{\text{Ca}} \) channel activity. Lipid rafts found in biological membranes are dynamic and highly transitory (18). It is, thus, conceivable that the activity of \( BK_{\text{Ca}} \) channels could be modulated by their dynamic association with lipid rafts. Indeed, we previously reported data collected from a single channel that underwent dramatic, quantal changes in conductance, consistent with its movement between lipid domains (34).

Most biological membranes contain lipids with an average chain length near C18 (35). The activity of a number of membrane proteins has been shown to be dependent on the chain length of the surrounding phospholipids (62), with highest activity seen at a chain length of approximately C18 (35, 50). For the \( BK_{\text{Ca}} \) channel, however, the chain length at which we see maximum activity is in the SPM phase. This may help to explain why many potassium channels locate preferentially to thicker lipid domains (rafts), which are enriched in sphingomyelin and cholesterol (19–21). In an earlier study, we showed that the inclusion of various amounts of cholesterol in lipid bilayers also has a profound effect on \( BK_{\text{Ca}} \) channel gating (2). The presence of cholesterol not only regulates the basal activity of the \( BK_{\text{Ca}} \) channel but also reduces its sensitivity to the stimulatory effect.
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of ethyl alcohol (2). Exposure to environmental stressors such as temperature or alcohol in turn can also alter the concentration and distribution of membrane lipid species (63), thereby regulating the activity of the $\text{BK}_{\text{Ca}}$ channel (63, 64).

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