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Gating and Conductance Changes in BK_{Ca} Channels in Bilayers Are Reciprocal

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Abstract. The energy associated with a mismatch between the hydrocarbon portions of a lipid bilayer and the hydrophobic regions of a transmembrane protein requires that one or both components deform in an attempt to minimize the energy difference. Transmembrane potassium channel subunits are composed of different structural motifs, each responsible for ion-selectivity, conductance and gating capabilities. Each has an inherent degree of flexibility commensurate with its amino acid composition. It is not clear, however, how each structural motif will respond to a fixed amount of distortion applied to the whole structure. We examined the single-channel conductance (G_c) and gating (open probability, P_o) of single BK_{Ca} channels (hslo α-subunits) inserted into planar lipid bilayers containing 1,2-dioleoyl-3-phosphatidylethanolamine (DOPE) or DOPE with either 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) or sphingomyelin (SPM) and 1-palmitoyl-2-oleoyl-3-phosphatidylethanolamine (POPE) with SPM. These latter three binary mixtures formed stable membranes with different distributions of thickness domains as determined by atomic force microscopy. Channels placed in each composition should be exposed to different amounts of distortion. BK_{Ca} channels forced into the DOPE/SPM bilayer containing lipid domains with two different thicknesses showed two distinct levels of G_c and P_o. The alterations in G_c and P_o were reciprocal. A larger conductance was accompanied by a smaller value for gating and vice versa. Channels forced into the POPE/SPM bilayer containing lipid domains with different thicknesses showed more than two distinct levels of G_c and P_o. Channels placed in a uniform bilayer (DOPE/DOPC) showed a uniform distribution of conductance and activation. We conclude that both the inner and outer domains of the channel where these two channel functions are localized respond to deformation and that a fixed amount of distortion results in reciprocal changes in protein function.

Key words: Ion channel — Lipid domain — Conduction — Activation — Atomic force microscopy

Introduction

Large amounts of energy can accumulate in a system whenever there is a significant mismatch between the hydrophobic regions of a transmembrane protein and the surrounding hydrocarbon core of a lipid bilayer. This energy is available to distort the lipid or the protein components of the system until a state of equilibrium is reached where each is distorted sufficiently to provide the best physical match between the two hydrophobic dimensions (Venturoli, Smit & Sperotto, 2005).

All known potassium channels are related members of a single large protein family. Their amino acid sequences are easily recognized as they all contain a highly conserved sequence known as the "K^+ channel signature sequence." This sequence is now known to form part of the outer (extracellular) selectivity filter module of the channel (Doyle et al., 1998). A portion of this structural module (the turret) is hydrophilic, with the remainder buried in the hydrophobic core of the protein. The selectivity filter and its associated pore helices are largely responsible for both the ion-selectivity and conduction properties of the channel (MacKinnon, 2003). Another module, known as the "gating ring," is located near the inner or cytoplasmic portion of the protein and is thought to represent a portion of the channel gate which

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opens and closes in response to various activators (Doyle, 2004b; Jiang et al., 2002a). It is attached to a large hydrophilic region consisting of RCK domains. Thus, the overall "inverted tepee" structure of the protein has both extracellular and intracellular hydrophilic components, which are thought to "float" on the inner and outer surfaces of the bilayer, respectively. They are connected to each other by the hydrophobic transmembrane regions. Forcing this protein structure into a thicker bilayer should have, at the least, the net effect of stretching the protein. Transmembrane structures which have an inherent degree of flexibility commensurate with their amino acid compositions can thus move relative to each other. It is reasonable to suspect that different structural modules within a single protein may differ from each other in their flexibility and how they may accommodate force applied by a thicker bilayer. Potassium channels, in common with other channels, have a variety of other properties, including voltage sensitivity and various modes of activation (Ramu, Xu & Lu, 2006). Individual members of the family have diverse structural domains attached in a modular fashion to the conserved selectivity filter portion of the molecule. Collectively, these assemblies determine the range of properties that distinguish one member of the family from another (Doyle, 2004a).

Large-conductance Ca^{2+}-activated, voltage-gated K^+ (BK_{Ca}) channels represent a functional subtype of K^+ channel that plays an important role in the regulation of neuronal excitability, cell volume regulation, excitation-contraction coupling and hormonal secretion (Gribkoff, Starrett & Dworetzky, 1997; Jakab, Weiger & Hermann, 1997; Vergara et al., 1998). They display a large unitary conductance and can be activated by both membrane depolarization and an elevation in intracellular Ca^{2+} (Vergara et al., 1998). Some of the effects of membrane lipid composition on the conductance of BK_{Ca} channels obtained from different sources have been described. Most of the differences observed were explained initially by differences in membrane surface charge, which derived from the inclusion of negatively charged lipids into the membrane. These, in turn, were thought to affect the local concentrations of Ca^{2+} and K^+ (Bell & Miller, 1984; Lagos et al., 1993; Turnheim et al., 1999). Recent data, however, have revised the conclusion that surface charge is responsible for the shift observed in the conductance in different lipid compositions (Park et al., 2003). Thus, the underlying mechanism for the modulation of the conductance of BK_{Ca} channels in different lipid composition remained largely unknown. Based on our earlier study of a wide variety of lipid compositions in planar bilayers, we hypothesized that simple differences in bilayer thickness in certain compositions could deform functional parts of the channel protein to different degrees and that this deformation of the selectivity pore or the pore helix was responsible for the rather large shifts in conductance we observed (Yuan et al., 2004).

Table 1. Lipids and their Tm

Lipid	Tm (°C)
SPM	45
POPE	26
DOPE	−16
DOPC	−20

Given the modular nature of the channel protein and the possibility of regional differences in the flexibility of the protein and its response to stretch, it is reasonable to ask the following: What are the effects of placing the channel in different bilayer thicknesses and compositions on other aspects of protein function like channel activation? Is the region of the protein responsible for activation also flexible, and how will it respond to changes in stretch?

In membrane reconstitution experiments, it is impossible to know exactly where in the bilayer the inserted protein is located. Thus, it is imperative that comparisons among multiple channel properties are made in the same recording, where the amount of force applied to the protein is fixed. However, we note that the time necessary for these electrophysiological measures places additional restrictions on the membrane compositions that can be used in the reconstitution. Only those with sufficient stability after the insertion of a single protein can be evaluated.

We explored the question of how different bilayer compositions and thicknesses would modulate channel conductance (G_c) and open probability (P_o) in three relatively stable bilayers, which are binary mixtures of 1,2-dioleoyl-3-phosphatidylethanolamine(DOPE)/sphingomyelin (SPM), 1-palmitoyl-2-oleoyl-3-phosphatidylethanolamine (POPE)/SPM and DOPE/1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC). The advantage of using these three bilayers is that they contain lipid domains with different distributions of thickness, all in proportion to the differences in their lipid main phase transition temperature (Tm) (Table 1). They range from one (DOPE/SPM) in which the thicker and more ordered SPM domain coexists with the thinner DOPE domain to another intermediate composition (POPE/SPM) where the domains are less distinct and more complex to yet another (DOPE/DOPC) in which there is no domain structure at all, as confirmed by imaging the membranes with standard atomic force microscopic (AFM) techniques (Dufrene & Lee, 2000; Rinia et al., 2002; Yuan & Johnston, 2002). In the mixture of DOPE/SPM, we expect that DOPE is in the fluid phase (Tm for DOPE is −16°C [Marsh, 1990]) and SPM is in gel phase (Tm for SPM is 45°C). This bilayer at room temperature is expected to form with

two well-defined thickness domains: a thicker SPM domain and a thinner DOPE domain, both relatively uniform due to their relative immiscibility with each other. In the mixture of POPE/SPM, however, since both POPE and SPM are in the gel phase at room temperature (Tm for POPE is 26°C), the two components may be more miscible with each other. Thus, the SPM domains in a POPE/SPM bilayer should be more complex when compared with the SPM domains in a DOPE/SPM bilayer due to the increased miscibility of SPM with POPE. In the DOPE/DOPC mixture, we expect a uniform thickness as both substances should be in the fluid phase (Tm for DOPC is −20°C) and are quite miscible with each other (Yang, Ding & Huang, 2003). An additional benefit of the study of these three mixtures is that they are all neutral zwitterionic compounds, which should help to rule out differences in surface charge as a factor in affecting the function of the BK_{Ca} channel. We expect that these differences in domain structure will deform the protein in graded ways so that the distribution of electrophysiological measures we obtain will be altered in each mixture.

Collectively, this makes it possible to obtain both slope conduction and channel activation measures in the same channel protein under identical thickness and charge conditions. Recordings from channels forced into the POPE/SPM bilayer show a bimodal distribution of conductance and activation. Interestingly, these two measures are reciprocally related to each other. From this we conclude that both the conductance and activation modules of this protein can be deformed relative to the rest of the protein and that the deformation that reduces conductance also increases activation in the same channel. This is consistent with the model of BK_{Ca} containing a spring that pulls on the activation gate, as proposed by Niu, Qian & Magleby (2004).

Materials and Methods

Materials

DOPE, POPE, DOPC, 1,2-dipalmitoyl-3-phosphatidylethanolamine (DPPE) and brain SPM were obtained from Avanti Polar Lipids (Alabaster, AL). They were used without further purification. Decane and salts were from Aldrich (St. Louis, MO). All aqueous solutions were prepared with 18.3 MΩ/cm Milli-Q water (Millipore Corp., Billerica, MA).

Membrane Preparation

The cDNA encoding *hSlo* α (kindly provided by Dr. P. Ahring, NeuroSearch, Copenhagen, Denmark) was overexpressed in HEK-293 cells. Stably transfected HEK-293 cells were grown in an artificial medium (Ahring et al., 1997), and membrane fragments from them were prepared using a protocol developed for COS cells (Sun, Naini & Miller, 1994), with some slight modifications as described elsewhere (Crowley, Treistman & Dopico, 2003).

Electrophysiology

Single-channel recordings were carried out with standard planar bilayer technology (Alvarez, 1986). Lipid mixtures of DOPE/SPM (3/2), POPE/SPM (3/2) and DOPE/DOPC (1/1) were initially dissolved in chloroform. The solvent was removed by evaporation with an N_2 stream and the dried lipid film resuspended in decane to form a final total lipid concentration of 25 mg/ml. The bilayer was formed by painting the lipid solution over a 100-μm hole (Wonderlin, Finkel & French, 1990) formed in a horizontal plastic coverslip sealed with tackiwax to a Teflon® partition separating upper and lower chambers. Bilayer capacitance was monitored by noting the current across the bilayer in response to a triangle wave (10 m*V*/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 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. Although each of the compositions produced stable bilayers, they differed in their incorporation potential. This was particularly acute in the DOPE/DOPC bilayer, where obtaining recordings with multiple channels was the rule and only infrequently did we produce ones having only a single channel.

The cytoplasmic (*cis*) solution contained 300 mM KCl, 1.05 mM $CaCl_2$, 1.25 mM hydroxyethyl ethylenediaminetetraacetic acid (HEDTA) and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.2). The free Ca^{2+} was estimated to be about 25 μM using the MaxChelator Sliders program (Bers, Patton & Nuccitelli, 1994). The extracellular (*trans*) solution in the lower chamber contained 30 mM KCl, 0.1 mM HEDTA and 10 mM HEPES (pH 7.2). Single-channel currents were recorded with a patch-clamp amplifier (EPC-9; HEKA Elektronik, Lambrecht, Germany) (Gillis, 2000). The *trans* chamber was connected to ground, and all voltages in the *cis* chamber were expressed relative to ground. Single-channel events were sampled at 5 kHz with Pulse software (Gillis, 2000). The data were filtered at 3 kHz for analysis and 500 Hz for display. All experiments were done at room temperature (22°C). Data were analyzed using the TAC and TAC-fit programs (Bruxton, Seattle, WA) and Igor Pro (WaveMetrics, Portland, OR). Slope conductance was calculated from an *I-V* plot in which the voltage ranged from −20 to 20 m*V* in 20-m*V* steps. Each step was 5 s long (Chu et al., 1998). P_o was normally calculated from the same channel with at least 1 min of recording with the membrane held at 0 m*V* (Pietrzykowski et al., 2004).

Microscopy

The lateral organization of supported membranes of the DOPE, POPE, DOPE/SPM, POPE/SPM (3:2 molar ratio) and DOPE/DOPC (1/1 molar ratio) mixtures were examined by AFM. Supported monolayers (Fig. 1) and hybrid bilayers (Fig. 2A, B) were prepared by Langmuir-Blodgett transfer (Yuan et al., 2002). Vesicle deposition, without compression (Fig. 2C, D) as described elsewhere (Yuan & Johnston, 2001) were also prepared from the POPE/SPM and DOPE/DOPC mixtures.

AFM measurements for monolayer samples were carried out on a multimode Nanoscope III atomic force microscope (Digital Instruments, Santa Barbara, CA) in repulsive mode in air. This provides information about the molecular organization of the acyl chains. Bilayer samples were imaged under water with a MAC-mode Picoscan atomic force microscope (Molecular Imaging, Phoenix, AZ) as described elsewhere (Yuan & Johnston, 2001). This provides information about relative bilayer thickness and the bulk structure of the polar headgroups in the outer leaflet.

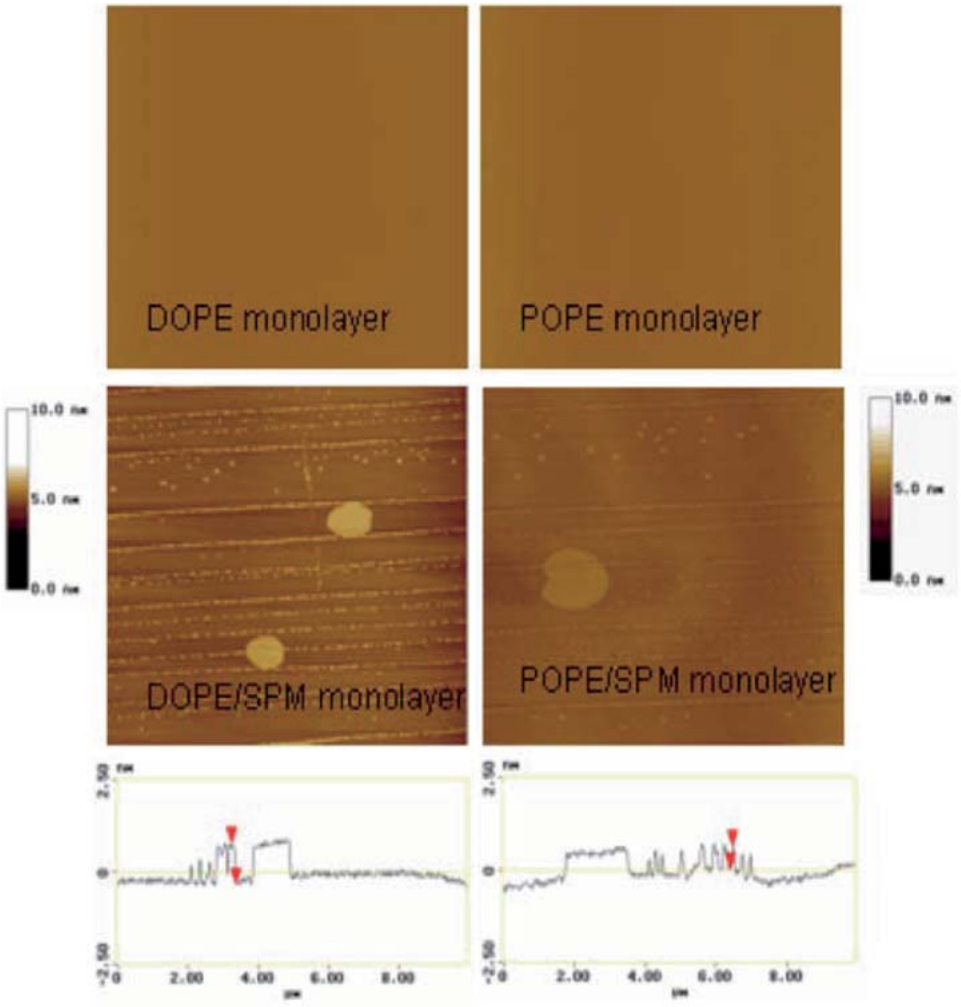

Fig. 1. AFM images of monolayers of DOPE (*upper left*), POPE (*upper right*), DOPE/SPM (*lower left*) and POPE/SPM (*lower right*) transferred onto mica at a surface pressure of 30 mN/m. The z-scale is 10 nm for all images. Section analysis traces for the lines across these images are shown under their respective images. *Arrowheads* mark points of measurement.

Results

Microscopy

Inclusion of SPM forms domains in phospholipid membranes. Monolayer membranes of DOPE and POPE were transferred onto hydrophilic mica to display the hydrophobic surface of acyl chains and imaged with AFM (Fig. 1). The addition of SPM into the DOPE or POPE monolayer resulted in the formation of large, bright, SPM-rich domains, while pure DOPE or POPE alone formed flat, featureless monolayers. The SPM-rich domains are about 1.0 nm thicker than the rest of the monolayer in DOPE and about 0.7 nm thicker in the POPE monolayer. The bright SPM-rich domains are of various sizes and appear to cover more surface area in the DOPE monolayer than in the POPE monolayer, suggesting that SPM is more miscible with POPE than with DOPE, as would be predicted from differences in their respective phase transition temperatures (Table 1). This kind of lipid-lipid immiscibility is likely the origin of raft formation in biological membranes (Brown & London, 2000; Giocondi et al., 2004).

Monolayers of DOPE/SPM and POPE/SPM were also transferred to flat DPPE-coated mica to form hybrid bilayers in order to display the polar head surface of the bilayer. AFM images of these bilayers (Fig. 2A, B) also show the formation of SPM-rich domains in both the DOPE/SPM and POPE/SPM images. The SPM-rich domain (high phase) in the DOPE/SPM bilayer is about 1.5 nm higher than the DOPE phase of the bilayer (low phase). The SPM-rich domain (high phase) in the POPE/SPM bilayer is about 1.3 nm higher than the POPE phase of the bilayer (low phase). Defects (dark holes) in the bilayers allowed us to estimate bilayer thickness. The bilayer thickness is about 5 nm for the SPM-rich domains and 3.4 nm for the DOPE or 3.6 nm for the POPE phase.

The topography of the SPM-rich domain structure in the two bilayers is different from that in the monolayers, indicating that reorganization of SPM-rich domains occurs after bilayer deposition. This

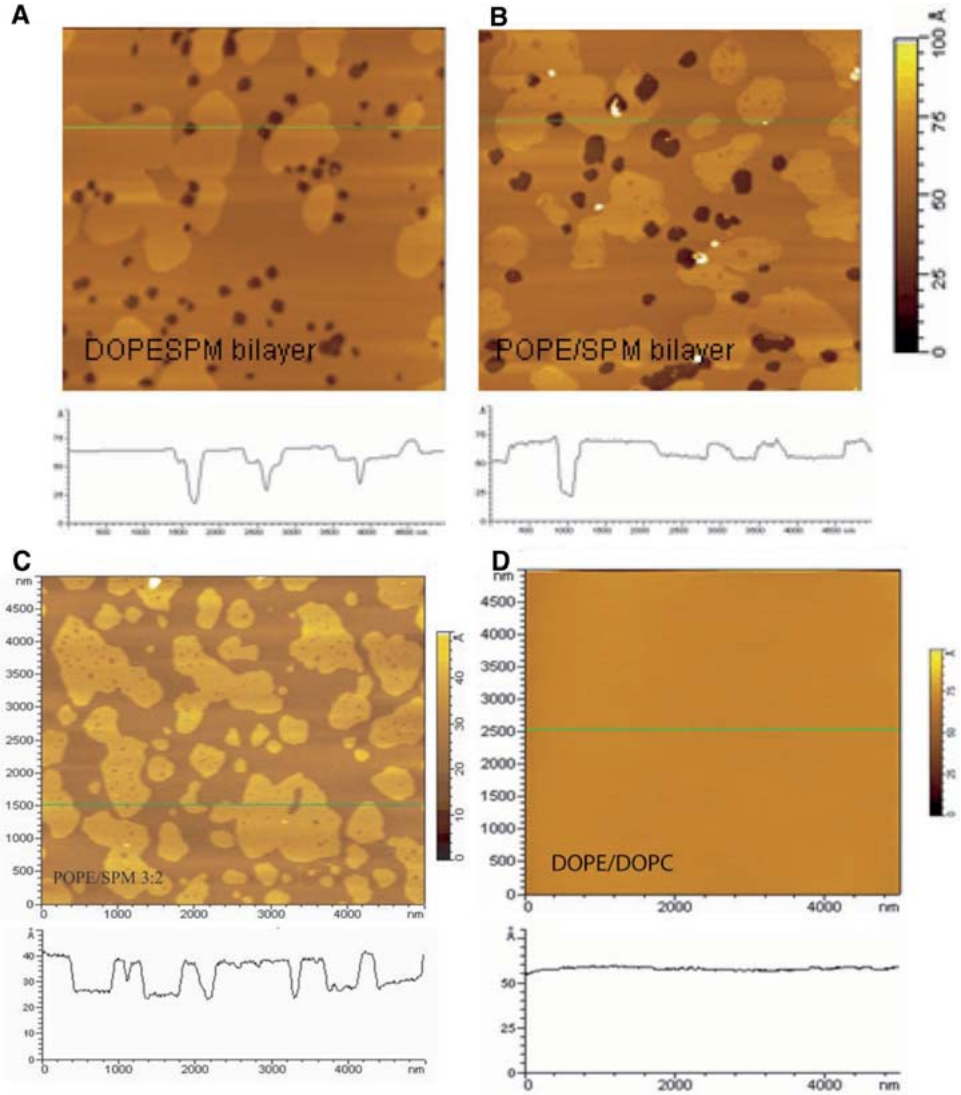

Fig. 2. AFM images of bilayers of DOPE/SPM (***A***) and POPE/SPM (***B***) formed on DPPE-coated mica by Langmuir-Blodgett transfer at a surface pressure of 30 mN/m. Bilayer of POPE/SPM formed by the vesicle fusion technique (***C***). Bilayer of POPE/POPC formed by the vesicle fusion technique (***D***). The z-scale is 10 nm for all images. Section analysis traces for the lines across these images are shown under their respective images.

suggests that the top monolayer in the bilayer is highly dynamic compared with the same monolayer deposited on mica. A subtle, but clear, difference between SPM-rich domains in bilayers is that SPM-rich domains in the DOPE/SPM bilayer are relatively homogeneous, given their immiscibility with each other, while SPM-rich domains in the POPE/SPM bilayer are more heterogeneous, with many small POPE islands inside the large SPM-rich domains. This indicates again that SPM is more miscible with POPE than with DOPE. Similar heterogeneous distributions among SPM domains have also been observed in SPM additions to DOPC and POPC bilayers (Lundbaek et al., 2004).

POPE/SPM and DOPE/DOPC bilayers were also prepared by vesicle fusion to provide a better model for the membranes used for the single-channel recording (Fig. 2C, D). Here, bilayers are allowed to self-assemble without the addition of compressive forces, as is the case in the Langmuir-Blodgett assembly. Similar SPM domains 1.5 nm above the surrounding fluid phase are again observed in POPE/SPM, but there are few defects. The SPM domains here also show islands of a lower phase that is similar in height to the POPE phase surrounding the domains. Interestingly, there is again no evidence for domains of an intermediate height due to SPM in one leaflet and POPE in the other, suggesting that SPM domains are coupled in both membrane leaflets. Bilayers of DOPE/DOPC prepared by this method (Fig. 2D) are relatively featureless, with no indication of the formation of

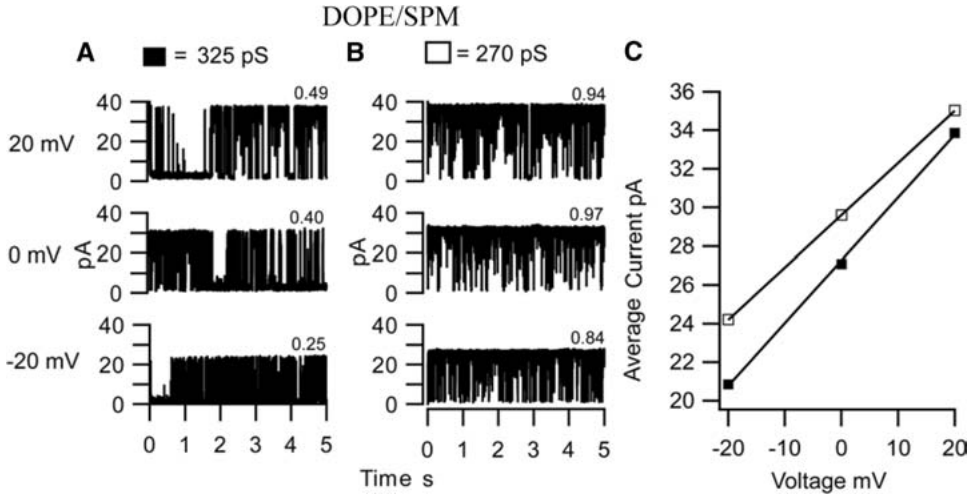

Fig. 3. (*A*) Recordings of a single BK_{Ca} channel measured in a DOPE/SPM bilayer during the first 5 s of recording at each of the indicated voltages. (*B*) Another BK_{Ca} channel also in DOPE/SPM. (*C*) *I-V* curves plotted for each of these recordings. The slope conductance (from *C*) of each set of recordings is shown at the top of each set of traces. Also indicated at the *top right of each trace* is the open probability for the interval shown.

domains. This is entirely consistent with their relative miscibility (Marrink & Mark, 2004). Although we attempted to produce defects by scanning at high force, none was produced, suggesting that both components were in the fluid state, as expected from their Tm values.

Electrophysiology

The conductance of the BK_{Ca} channel in bilayers was measured by recording single-channel currents at various applied voltages and then plotting the current *vs.* voltage (*I-V* curve) to obtain the slope conductance. Open probabilities were also measured in the same recording, with the channel held at 0 *V* for a time sufficient to observe at least 5,000 openings. For most recordings, this totaled an additional minute of activity. Addition of SPM to a bilayer changes the distribution of single-channel properties. One begins to find recordings in which the conductance is lower than expected, and in these recordings the activation of the BK_{Ca} channel is higher than expected. This reciprocal bimodal distribution is easily seen in the pair of recordings from DOPE/SPM bilayers illustrated in Figure 3. It is clear that there are two main levels of G_c and P_o in DOPE/SPM bilayers. When these measures are plotted against each other, as they are in Figure 4, it is clear that they are very well correlated. The correlation coefficient ($R^2 = 0.75$, $F_{1,10} = 33.29$, $P < 0.0002$) for the straight line fit to the DOPE/SPM data is significant. The values of conduction and activation for each channel show a strong reciprocal relationship with a slope close to −1 (−0.88). From this we suggest that channels in thicker regions of the bilayer respond to the applied deformation to the protein with decreased conduction and

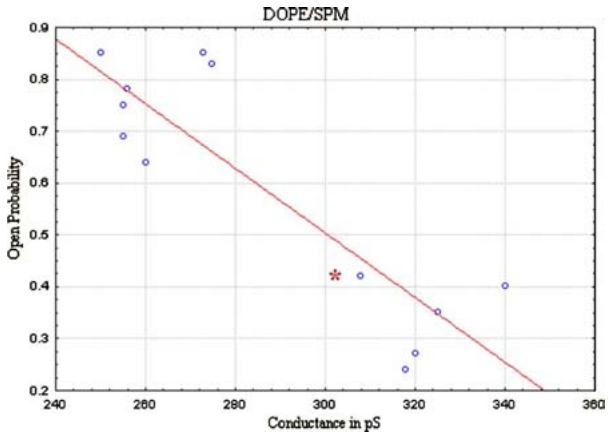

Fig. 4. Each individual conductance measurement is plotted against its companion measurement of activation for single-channel recordings in the DOPE/SPM bilayer. A clear reciprocal relationship is observed when the slope of the regression line is considered. Also included (*) is the average ($n = 4$) value for channels recorded in pure DOPE bilayers.

increased activation. Also included in Fig. 4 is the average conductance and open probability of a few channels ($n = 4$) forced into pure DOPE bilayers. This serves to identify the lower right quadrant of the distribution as the region corresponding to channels in that composition. In a like fashion, the upper left quadrant represents channels forced into an SPM domain.

Similar results were obtained in the second composition, POPE/SPM (Fig. 5). Here, the data have a complex distribution, which is less clearly bimodal, confirming the presence of additional compositions and thicknesses in the POPE/SPM bilayer. This is probably due to the increased miscibility of

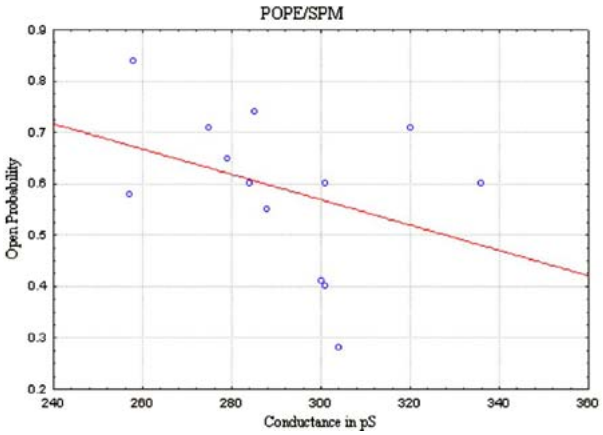

Fig. 5. Each individual conductance measurement is plotted against its companion measurement of activation in the POPE/SPM bilayer. A reciprocal relationship is again observed, although the slope of the regression line fitted to the data is much reduced, indicating more variability among the measures.

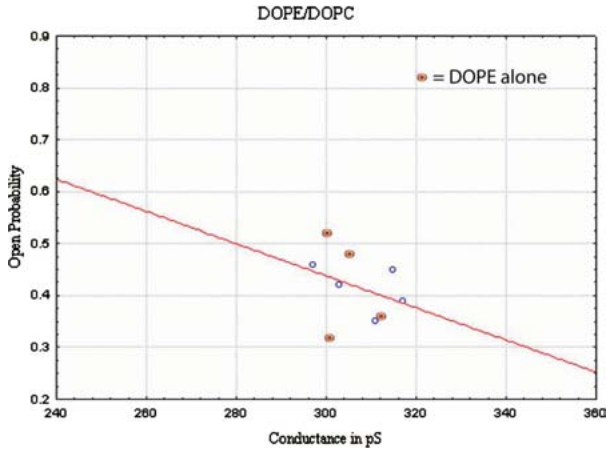

Fig. 6. Each individual conductance measurement is plotted against its companion measurement of activation in the DOPE/DOPC bilayer. Also included (■) are individual values for channels recorded in pure DOPE bilayers. It is clear that the amount of variability among the measures is much reduced.

POPE with SPM. The reciprocal relationship between the measures still holds, but the regression line is not a significant fit to the data ($R^2 = 0.05$, $F_{1,11} = 1.64$, $P < 0.23$) and has a much reduced (−0.36) slope.

We note here that there is much more variability in this composition. Based on our earlier study (Yuan et al., 2004), we expected that the potential for additional thicknesses in this composition as confirmed by AFM would have simply filled in points on the plotted line. Instead, we found channels in which the amount of activation was disproportionate to the shift in conductance. This suggests to us that there must be other variables in addition to thickness that affect these measures. Variables such as membrane elasticity (Lundbaek et al., 2004) or alterations in the lateral pressure profile (Cantor, 1999) should be considered.

The correlation we have seen between domain structure and electrophysiological distributions observed in the previous two SPM-containing bilayers is again seen in the third composition, DOPE/DOPC. Here, AFM shows that there is no domain structure, so the bilayer is relatively uniform in thickness (Fig. 2D). As expected from this, the distribution of electrophysiological results (Fig. 6) is very uniform, with all of the measures occurring in a restricted portion of the electrophysiological space. We have also included in this figure the few single-channel recordings we were able to obtain in pure DOPE bilayers. First, this again identifies the lower right quadrant of the space that represents channels forced into the DOPE thickness and shows that there is relatively little difference between channels forced into the two compositions (DOPE/DOPC *vs.* DOPE alone). Interestingly, the resulting data, although tightly clustered, still appear vaguely reciprocal, suggesting that the channel in these simple bilayers remains under stress.

Although we earlier suggested several simple ways in which stretch of the selectivity pore by increased bilayer thickness could result in reduced conductance (Yuan et al., 2004), it was less clear to us how this same stretch could increase channel activation. However, the reciprocal relationship we observed in these recordings is readily explained if one considers the presence of a simple spring-like component in the protein somewhere between the pore and the activation gate, as suggested by Niu et al. (2004). In this model, the hydrophilic portions of both the extracellular and cytosolic regions of the protein will resist being pulled into the hydrocarbon core of the thicker bilayer. This will apply a stretch to the selectivity pore region, which reduces its conduction and could also increase the stretch on the spring attached to or formed by the activation gate, pulling it open and thus reciprocally increasing P_o. Although there is still much uncertainty about the general mechanism of gating, the reciprocal relationship we observed is consistent with the presence of a protein "spring" in the process (Krishnamoorthy et al., 2005).

Discussion

At physiological temperatures, both lipids and proteins are in the fluid phase (Fenimore et al., 2002; Zaccai, 2000). In this state, both components should be flexible. There is every reason to believe that conformational flexibility must be an essential feature of most proteins, including channels, transporters and enzymes (Fenimore et al., 2002; Yamashita et al.,

2005; Zaccai, 2000). Transmembrane protein structures must have an inherent degree of flexibility, each commensurate with its amino acid composition. Different structural modules within a single protein may differ in flexibility (Réat et al., 1998). We have shown that the conductivity of the BK_{Ca} channel protein can be modulated by alterations in membrane thickness (Yuan et al., 2004). We assumed that the diameter of the selectivity filter portion of the protein or the angle of the pore helices or the spatial relationship among each of the subunits is deformed by the resolution of the energy that arises from the hydrophobic mismatch between the lipid membrane and the protein. Further, we have concluded that this deformation is sufficient to reduce the throughput of ions through the selectivity pore of the channel (Yuan et al., 2004). We believe that alterations in thickness can affect channels directly by deforming their relatively flexible transmembrane structures. Similar ideas have been suggested by others (Horrigan, Heinemann & Hoshi, 2005; Jiang et al., 2001; Niu et al., 2004). Changes in membrane thickness have also been shown, by others, to alter many of the functional aspects of a transmembrane protein (Duong-Ly et al., 2005). For example, such changes are known to change the ion selectivity of some channel proteins without altering channel conductance (Garavaglia et al., 2004). Since we know that different aspects of channel function, i.e., ion selectivity, conduction and activation, arise from structural modules that are assembled in various ways to produce channels with specific properties (MacKinnon, 2003), we asked here if changes in thickness would also affect other aspects of BK_{Ca} channel function, such as channel gating. Are all functional domains within a protein equally flexible (Réat et al., 1998)? In some cases, the cytosolic portion of a transmembrane protein may have a different degree of flexibility than the extracellular portion (Zaccai, 2000). Thus, it was not surprising to find that alterations in thickness had effects on functions that are localized in different portions of the protein.

We also note that in membrane reconstitution experiments, such as the one reported here, it is impossible to know exactly where in the bilayer the protein may reside. Thus, it is imperative that functional comparisons are only made in the same channel recording to insure that all aspects of the protein receive the same strain. Further, the time necessary for these measures places additional restrictions on the membrane composition that may be used in the reconstitution. Only those compositions with well-defined thickness domains that may distort the constituents in a regular manner and with sufficient bilayer stability can be evaluated. In fact, it could be argued that the observed changes we have seen in the function of BK_{Ca} provide a proof of principal that functional parts of proteins are flexible in bilayers and can be deformed by alteration in bilayer thickness (Williamson et al., 2002).

Although there are other physical changes that could occur as lipid composition is changed, resolution of hydrophobic mismatch by the simultaneous accommodation of both the lipid and the protein seems to us the most parsimonious explanation. Although we favor this direct model, it is clear that there are many other indirect interactions between lipid and protein that could alter channel function. For example, alterations in lipid composition could alter the symmetry of the subunit assembly as it is known that lipid is an important structural component of the channel (Valiyaveetil, Zhou & MacKinnon, 2002). Shifts in symmetry like those suggested for a thinner bilayer can be expected to alter channel function. Less clear is the possibility that alterations in lipids surrounding the voltage sensor could alter its ability to move and activate the channel (Tombola, Pathak & Isacoff, 2005).

The AFM images we present here clearly show that the incorporation of SPM into DOPE and POPE monolayers results in the formation of SPM-rich domains that are thicker than the DOPE and POPE membranes themselves (Fig. 1). Note that this difference in thickness is observed in both monolayers and single leaflets of a bilayer. Thus, the difference in thickness between symmetrical bilayers containing SPM and DOPE domains, as are used in these recording experiments, is expected to be more than the 1.5 nm observed for single leaflets. The height difference for the domains in the vesicle fusion bilayer is $\sim$1.5 nm, which is in good agreement with previous reports that bilayer thickness is 5.2–5.6 nm for SPM (C24) and 3.5 nm for PC (C16:0/C18:1) (Maulik & Shipley, 1995; Nezil & Bloom, 1992). It is also worth noting that the lack of miscibility between SPM and DOPE at room temperature likely results in SPM domains that are more uniform in the DOPE-containing bilayer than those found in the POPE-containing membrane. This also results in more confined SPM domains in DOPE-containing bilayers. The increased miscibility of POPE with SPM likely accounts for the increased complexity in composition of POPE/SPM bilayers. We believe this difference accounts, in part, for the additional variability in the distribution of measures seen in the recordings from this mixture. Similar observations and conclusions have been made in bilayers containing SPM and DOPC or POPC (Giocondi et al., 2004). In a like fashion, we interpret the uniformity of the recordings from DOPE/DOPC bilayers to be reflective of their general miscibility with each other and the uniformity of the bilayers seen in AFM images (Fig. 2D).

Although the thickness hypothesis we have advanced here is consistent with all of the data we have observed, it must be reiterated that we do not yet have a direct and independent method to determine exactly

where a particular protein molecule might be in a bilayer. However, by inference, it seems likely that recordings from channels that have a smaller conductance and a larger P_o than others represent channels residing in the thicker (SPM) domain. The reciprocal characteristics likely represent channels in the thinner (DOPE) domain. However, since there are a great number of isomorphisms among the various physical properties of a bilayer, it may ultimately prove, when techniques arise to manipulate them, that variations in some other set of mechanical parameters are responsible for our observations. In any case, the comparisons made among BK_{Ca} channel properties in different mixtures have been made in constant conditions.

The increase in the activity of the BK_{Ca} channel in the thicker SPM domains was initially surprising. SPM domains, when compared with DOPE domains, are thicker and should be in a more ordered gel phase. We initially assumed that this would increase the lateral stress or pressure within the bilayer, as has been proposed by others (Cantor, 1997, 1999). Thus, we assumed that the increased pressure would lower the activity of the BK_{Ca} channel by increasing the amount of energy necessary for gate movement. However, the increase in activity (P_o) of the BK_{Ca} channel we observed suggests that another mechanism beyond bilayer stiffness must predominate.

The decreased conduction we observed in response to stretching the protein is consistent with the structural model of the K^+ channel described by MacKinnon and colleagues (Doyle et al., 1998; Jiang et al., 2002a, 2002b; Morais-Cabral, Zhou & MacKinnon, 2001). Any shift in the optimal spatial relationships among the subunits or the various modules that make up the selectivity pore will reduce conduction. However, the effect of stretching the protein on activation is only predictable if one assumes that there is a spring-like component in the protein which, when stretched by a thicker bilayer, pulls the activation gate open. We assume that in a thicker bilayer the hydrophilic intracellular portions of the channel will resist the tendency to be pulled into the lipid bilayer. This provides the energy to stretch the spring component and to pull on the gating ring and activate the channel. Similar models have been proposed for K (Krishnamoorthy et al., 2005; Niu et al., 2004) and other (Corey et al., 2004; Gillespie & Walker, 2001; Howard & Bechstedt, 2004) channels.

Although we continue to emphasize the importance of bilayer thickness in modulating the BK_{Ca} channel protein, it must be noted that thickness alone merely sets the magnitude of the energy associated with the hydrophobic mismatch (de Planque et al., 2001). The relative portioning of the available energy of this interaction between altering the physical dimensions of the protein and the surrounding lipid structure depends closely on the nature of their interaction (Weiss et al., 2003). It is possible that insertion of the relatively bulky BK_{Ca} protein into the bilayer causes a local curvature in the neighboring bilayer structure in an attempt to resolve the mismatch. Some fraction of the available energy may also be spent in altering the dimensions of the protein or in stretching the spring. Clearly then, the deformability of the bilayer and the flexibility of each portion of the protein will determine where the equilibrium position of each will be reached. Thus, it is impossible to predict which of the changes in physical properties of the bilayer are the cause and which are the effect of the mismatch (Lundbaek & Andersen, 1994). It seems reasonable to assume that bilayers that are more easily deformable, perhaps because of the shape of their headgroups or the lengths of their acyl chains, will curve and resolve more of the mismatch, leaving less energy available for distortion of the embedded protein (van den Brink-van der Laan et al., 2001).

Our finding that the conductance and gating of a BK_{Ca} channel can be significantly altered simply by placing it in different lipid compositions gives considerable weight to the notion that lipids exert powerful direct effects on channel structure and function (Park et al., 2003). It is now recognized that cell plasma membranes contain a mosaic of different lipid domains (Maxfield, 2002). The location of some ion channels in these lipid rafts has been observed (Martens et al., 2000, 2001). The BK_{Ca} channel itself may be selectively targeted to particular kinds of domains in certain cells (Bravo-Zehnder et al., 2000). This may, in turn, finely tune the channel properties in each raft composition. Further studies will be necessary to elucidate the functional role of lipid raft composition and distribution on the natural neuronal signal properties of BK_{Ca} and other types of channels.

Why should the changes seen in BK_{Ca} channel properties seen here be ascribed to deformations in the protein and not to second-order effects related to changes in lipid structure? Primarily, we see a simple mechanical model that posits changes in protein structure to account for all of the changes we have seen in protein function. It further suggests a direct mechanical mechanism for the alterations in protein function. It is more difficult to see how lipid changes alone could elicit these changes if the protein remains rigid and fixed in space. Mutagenesis studies in which the flexibility of the protein structure is deliberately manipulated will provide important conformations for our assumptions and conclusions.

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References

Ahring, P.K., Strobaek, D., Christophersen, P., Olesen, S.P., Johansen, T.E. 1997. Stable expression of the human large-conductance Ca^{2+}-activated K^+ channel. *FEBS Lett.* **415:**67–70

Alvarez, O. 1986. How to set up a bilayer system. *In:* Miller, C., editor. Ion Channel Reconstitution. pp 115–139, Plenum Press, New York

Bell, J.E., Miller, C. 1984. Effects of phospholipid surface charge on ion conduction in the K^+ channel of sarcoplasmic reticulum. *Biophys. J.* **45:**279–287

Bers, D.M., Patton, C.W., Nuccitelli, R. 1994. A practical guide to the preparation of Ca^{2+} buffers. *In:* Nuccitelli, R., editor. A Practical Guide to the Study of Calcium in Living Cells. pp 3–29, Academic Press, New York

Bravo-Zehnder, M., Orio, P., Norambuena, A., Wallner, M., Meera, P., Toro, L., Latorre, R., Gonzalez, A. 2000. Apical sorting of a voltage- and Ca^{2+}-activated K^+ channel α-subunit in Madin-Darby canine kidney cells is independent of *N*-glycosylation. *Proc. Natl. Acad. Sci. USA* **97:**13114–13119

Brown, D.A., London, E. 2000. Structure and function of sphingolipid- and cholesterol-rich membrane rafts. *J. Biol. Chem.* **275:**17221–17224

Cantor, R.S.. 1997. The lateral pressure profile in membranes: a physical mechanism of general anesthesia. *Biochemistry* **36:**2339–2344

Cantor, R.S.. 1999. Lipid composition and the lateral pressure profile in bilayers. *Biophys. J.* **76:**2625–2639

Chu, B., Dopico, A.M., Lemos, J.R., Treistman, S.N. 1998. Ethanol potentiation of calcium-activated potassium channels reconstituted into planar lipid bilayers. *Mol. Pharmacol.* **54:**397–406

Corey, D.P., Garcia-Anoveros, J., Holt, J.R., Kwan, K.Y., Lin, S.Y., Vollrath, M.A., Amalfitano, A., Cheung, E.L., Derfler, B.H., Duggan, A., Geleoc, G.S., Gray, P.A., Hoffman, M.P., Rehm, H.L., Tamasauskas, D., Zhang, D.S. 2004. TRPA1 is a candidate for the mechanosensitive transduction channel of vertebrate hair cells. *Nature* **432:**723–730

Crowley, J.J., Treistman, S.N., Dopico, A.M. 2003. Cholesterol antagonizes ethanol potentiation of human brain BK_{Ca} channels reconstituted into phospholipid bilayers. *Mol. Pharmacol.* **64:**365–372

de Planque, M.R., Goormaghtigh, E., Greathouse, D.V., Koeppe, R.E., Kruijtzer, J.A., Liskamp, R.M., de Kruijff, B., Killian, J.A. 2001. Sensitivity of single membrane-spanning α-helical peptides to hydrophobic mismatch with a lipid bilayer: Effects on backbone structure, orientation, and extent of membrane incorporation. *Biochemistry* **40:**5000–5010

Doyle, D.A.. 2004a. Structural changes during ion channel gating. *Trends Neurosci.* **27:**298–302

Doyle, D.A.. 2004b. Structural themes in ion channels. *Eur. Biophys. J.* **33:**175–179

Doyle, D.A., Morais, C.J., Pfuetzner, R.A., Kuo, A., Gulbis, J.M., Cohen, S.L., Chait, B.T., MacKinnon, R. 1998. The structure of the potassium channel: Molecular basis of K^+ conduction and selectivity. *Science* **280:**69–77

Dufrene, Y.F., Lee, G.U.. 2000. Advances in the characterization of supported lipid films with the atomic force microscope. *Biochim. Biophys. Acta* **1509:**14–41

Duong-Ly, K.C., Nanda, V., Degrado, W.F., Howard, K.P. 2005. The conformation of the pore region of the M2 proton channel depends on lipid bilayer environment. *Protein Sci.* **14:**856–861

Fenimore, P.W., Frauenfelder, H., McMahon, B.H., Parak, F.G. 2002. Slaving: Solvent fluctuations dominate protein dynamics and functions. *Proc. Natl. Acad. Sci. USA* **99:**16047–16051

Garavaglia, M., Dopinto, S., Ritter, M., Furst, J., Saino, S., Guizzardi, F., Jakab, M., Bazzini, C., Vezzoli, V., Dossena, S., Rodighiero, S., Sironi, C., Botta, G., Meyer, G., Henderson, R., Paulmichl, M. 2004. Membrane thickness changes ion-selectivity of channel-proteins. *Cell. Physiol. Biochem.* **14:**231–240

Gillespie, P.G., Walker, R.G. 2001. Molecular basis of mechanosensory transduction. *Nature* **413:**194–202

Gillis, K.D.. 2000. Admittance-based measurement of membrane capacitance using the EPC-9 patch-clamp amplifier. *Pfluegers Arch.* **439:**655–664

Giocondi, M.C., Boichot, S., Plenat, T., Le Grimellec, C.C. 2004. Structural diversity of sphingomyelin microdomains. *Ultramicroscopy* **100:**135–143

Gribkoff, V.K., Starrett, J.E., Jr., Dworetzky, S.I. 1997. The pharmacology and molecular biology of large-conductance calcium-activated (BK) potassium channels. *Adv. Pharmacol.* **37:**319–348

Horrigan, F.T., Heinemann, S.H., Hoshi, T. 2005. Heme regulates allosteric activation of the Slo1 BK channel. *J. Gen. Physiol.* **126:**7–21

Howard, J., Bechstedt, S. 2004. Hypothesis: A helix of ankyrin repeats of the NOMPC-TRP ion channel is the gating spring of mechanoreceptors. *Curr. Biol.* **14:**R224–R226

Jakab, M., Weiger, T.M., Hermann, A. 1997. Ethanol activates maxi Ca^{2+}-activated K^+ channels of clonal pituitary (GH3) cells. *J. Membr. Biol.* **157:**237–245

Jiang, Y., Lee, A., Chen, J., Cadene, M., Chait, B.T., MacKinnon, R. 2002a. Crystal structure and mechanism of a calcium-gated potassium channel. *Nature* **417:**515–522

Jiang, Y., Lee, A., Chen, J., Cadene, M., Chait, B.T., MacKinnon, R. 2002b. The open pore conformation of potassium channels. *Nature* **417:**523–526

Jiang, Y., Pico, A., Cadene, M., Chait, B.T., MacKinnon, R. 2001. Structure of the RCK domain from the *E. coli* K^+ channel and demonstration of its presence in the human BK channel. *Neuron* **29:**593–601

Krishnamoorthy, G., Shi, J., Sept, D., Cui, J. 2005. The NH_2 terminus of RCK1 domain regulates Ca^{2+}-dependent BK_{Ca} channel gating. *J. Gen. Physiol.* **126:**227–241

Lagos, R., Wilkens, M., Vergara, C., Cecchi, X., Monasterio, O. 1993. Microcin E492 forms ion channels in phospholipid bilayer membrane. *FEBS Lett.* **321:**145–148

Lundbaek, J.A., Andersen, O.S. 1994. Lysophospholipids modulate channel function by altering the mechanical properties of lipid bilayers. *J. Gen. Physiol.* **104:**645–673

Lundbaek, J.A., Birn, P., Hansen, A.J., Sogaard, R., Nielsen, C., Girshman, J., Bruno, M.J., Tape, S.E., Egebjerg, J., Greathouse, D.V., Mattice, G.L., Koeppe, R.E., Andersen, O.S. 2004. Regulation of sodium channel function by bilayer elasticity: The importance of hydrophobic coupling. Effects of micelle-forming amphiphiles and cholesterol. *J. Gen. Physiol.* **123:**599–621

MacKinnon, R.. 2003. Potassium channels. *FEBS Lett.* **555:**62–65

Marrink, S.J., Mark, A.E. 2004. Molecular view of hexagonal phase formation in phospholipid membranes. *Biophys. J.* **87:**3894–3900

Marsh, D. 1990. Calorimetric data. In: Handbook of Lipid Bilayers, pp. 135–159. CRC Press, Boca Raton, FL

Martens, J.R., Navarro-Polanco, R., Coppock, E.A., Nishiyama, A., Parshley, L., Grobaski, T.D., Tamkun, M.M. 2000. Differential targeting of Shaker-like potassium channels to lipid rafts. *J. Biol. Chem.* **275:**7443–7446

Martens, J.R., Sakamoto, N., Sullivan, S.A., Grobaski, T.D., Tamkun, M.M. 2001. Isoform-specific localization of voltage-gated K^+ channels to distinct lipid raft populations. Targeting of Kv1.5 to caveolae. *J. Biol. Chem.* **276:**8409–8414

Maulik, P.R., Shipley, G.G. 1995. X-ray diffraction and calorimetric study of *N*-lignoceryl sphingomyelin membranes. *Biophys. J.* **69:**1909–1916

Maxfield, F.R.. 2002. Plasma membrane microdomains. *Curr. Opin. Cell Biol.* **14:**483–487

Morais-Cabral, J.H., Zhou, Y., MacKinnon, R. 2001. Energetic optimization of ion conduction rate by the K^+ selectivity filter. *Nature* **414:**37–42

Nezil, F.A., Bloom, M. 1992. Combined influence of cholesterol and synthetic amphiphillic peptides upon bilayer thickness in model membranes. *Biophys. J.* **61:**1176–1183

Niu, X., Qian, X., Magleby, K.L. 2004. Linker-gating ring complex as passive spring and Ca^{2+}-dependent machine for a voltage- and Ca^{2+}-activated potassium channel. *Neuron* **42:**745–756

Park, J.B., Kim, H.J., Ryu, P.D., Moczydlowski, E. 2003. Effect of phosphatidylserine on unitary conductance and Ba^{2+} block of the BK Ca^{2+}-activated K^+ channel: Re-examination of the surface charge hypothesis. *J. Gen. Physiol.* **121:**375–398

Pietrzykowski, A.Z., Martin, G.E., Puig, S.I., Knott, T.K., Lemos, J.R., Treistman, S.N. 2004. Alcohol tolerance in large-conductance, calcium-activated potassium channels of CNS terminals is intrinsic and includes two components: Decreased ethanol potentiation and decreased channel density. *J. Neurosci.* **24:**8322–8332

Ramu, Y., Xu, Y., Lu, Z. 2006. Enzymatic activation of voltage-gated potassium channels. Nature **442:**696–699

Réat, V., Patzelt, H., Ferrand, M., Pfister, C., Oesterhelt, D., Zaccai, G. 1998. Dynamics of different functional parts of bacteriorhodopsin: H-^{2}H labeling and neutron scattering. *Proc. Natl. Acad. Sci. USA* **95:**4970–4975

Rinia, H.A., Boots, J.W., Rijkers, D.T., Kik, R.A., Snel, M.M., Demel, R.A., Killian, J.A., van der Eerden, J.P., de Kruijff, B. 2002. Domain formation in phosphatidylcholine bilayers containing transmembrane peptides: Specific effects of flanking residues. *Biochemistry* **41:**2814–2824

Sun, T., Naini, A.A., Miller, C. 1994. High-level expression and functional reconstitution of *Shaker* K^+ channels. *Biochemistry* **33:**9992–9999

Tombola, F., Pathak, M.M., Isacoff, E.Y. 2005. How far will you go to sense voltage?. *Neuron* **48:**719–725

Turnheim, K., Gruber, J., Wachter, C., Ruiz-Gutierrez, V. 1999. Membrane phospholipid composition affects function of potassium channels from rabbit colon epithelium. *Am. J. Physiol.* **277:**C83–C90

Valiyaveetil, F.I., Zhou, Y., MacKinnon, R. 2002. Lipids in the structure, folding, and function of the KcsA K^+ channel. *Biochemistry* **41:**10771–10777

van den Brink-van der Laan E., Dalbey R.E., Demel R.A., Killian J.A., de Kruijff B. 2001. Effect of nonbilayer lipids on membrane binding and insertion of the catalytic domain of leader peptidase. Biochemistry **40**:9677–9684

Venturoli, M., Smit, B., Sperotto, M.M. 2005. Simulation studies of protein-induced bilayer deformations, and lipid-induced protein tilting, on a mesoscopic model for lipid bilayers with embedded proteins. *Biophys. J.* **88:**1778–1798

Vergara, C., Latorre, R., Marrion, N.V., Adelman, J.P. 1998. Calcium-activated potassium channels. *Curr. Opin. Neurobiol.* **8:**321–329

Weiss, T.M., Van Der Wel, P.C., Killian, J.A., Koeppe, R.E., Huang, H.W. 2003. Hydrophobic mismatch between helices and lipid bilayers. *Biophys. J.* **84:**379–385

Williamson, I.M., Alvis, S.J., East, J.M., Lee, A.G. 2002. Interactions of phospholipids with the potassium channel KcsA. *Biophys. J.* **83:**2026–2038

Wonderlin, W.F., Finkel, A., French, R.J. 1990. Optimizing planar lipid bilayer single-channel recordings for high resolution with rapid voltage steps. *Biophys. J.* **58:**289–297

Yamashita, A., Singh, S.K., Kawate, T., Jin, Y., Gouaux, E. 2005. Crystal structure of a bacterial homologue of Na^+/Cl^-dependent neurotransmitter transporters. *Nature* **437:**215–223

Yang, L., Ding, L., Huang, H.W. 2003. New phases of phospholipids and implications to the membrane fusion problem. *Biochemistry* **42:**6631–6635

Yuan, C., Furlong, J., Burgos, P., Johnston, L.J. 2002. The size of lipid rafts: An atomic force microscopy study of ganglioside GM_1 domains in sphingomyelin/DOPC/cholesterol membranes. *Biophys. J.* **82:**2526–2535

Yuan, C., Johnston, L.J. 2001. Atomic force microscopy studies of ganglioside GM_1 domains in phosphatidylcholine and phosphatidylcholine/cholesterol bilayers. *Biophys. J.* **81:**1059–1069

Yuan, C., Johnston, L.J. 2002. Phase evolution in cholesterol/DPPC monolayers: Atomic force microscopy and near field scanning optical microscopy studies. *J. Microsc.* **205:**136–146

Yuan, C., O'Connell, R.J., Feinberg-Zadek, P.L., Johnston, L.J., Treistman, S.N. 2004. Bilayer thickness modulates the conductance of the BK channel in model membranes. *Biophys. J.* **86:**3620–3633

Zaccai, G.. 2000. How soft is a protein? A protein dynamics force constant measured by neutron scattering. *Science* **288:**1604–1607