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Attenuation of Channel Kinetics and Conductance by Cholesterol: An Interpretation Using Structural Stress as a Unifying Concept

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Abstract. The ubiquity of cholesterol in cell membranes and changes in its concentration during development, aging and in various diseases suggest that it plays an important role in modulating cell function. We examined this possibility by monitoring the effects of cholesterol on the activity of the calcium-activated potassium (BK) channel reconstituted into lipid bilayers from rat brain homogenates. Increasing the cholesterol concentration to 11% of total lipid weight resulted in a 70% reduction in channel mean open time and a reduction of the open probability of the channel by 80%. Channel conductance was reduced by 7%. Cholesterol is known to change the order state and the modulus of compressibility of bilayers. These physico-chemical changes may be translated into an overall increase in the structural stress in the bilayer, and this force may be transmitted to proteins residing therein. By examining the characteristics of the BK channel as a function of temperature, in the presence and absence of cholesterol, we were able to estimate the activation energy based on Arrhenius plots of channel kinetics. Cholesterol reduced the activation energy of the BK channel by 50% for the open to closed transition. This result is consistent with an increased stress energy in the bilayer and favors the channel moving into the closed state. Taken together, these data are consistent with a model in which cholesterol induces structural stress which enhances the transition from the open to the closed state of the channel. We suggest that this is an important mechanism for regulating the activity of membrane-integral proteins and therefore membrane function, and that the concept of structural stress may be relevant to understanding the modulation of ion channel activity in cell membranes.

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Key words: Calcium-activated potassium channel — Cholesterol — Conductance — Lateral elastic stress — Lipid bilayers — Lipid-channel interactions

Introduction

The opening and closing of ion channels is thought to result from conformational changes in channel proteins (Miller & White, 1984; Catterall, 1986; Guy & Conti, 1990) in response to an appropriate energy modality (i.e., electrical, chemical or mechanical). Recent studies have shown that lipids play a significant role in modulating protein conformation in bilayer systems (Fong & McNamee, 1987; Gibson & Brown, 1993; Butler & McNamee, 1993). Since membrane integral proteins, like ion channels, are surrounded by lipids whose composition and concentrations change during development, disease and environmental adaptation (Hitzemann & Johnson, 1983; Hazel & Williams, 1990; Gleason, Medow & Tulenko, 1991), it is likely that interactions between lipids and ion channels affect their function. We investigated these interactions by reconstituting the high-conductance, calcium-activated potassium (BK) channel, isolated from rat brain, into bilayers of known composition. Using the patch clamp technique, we examined single channel properties as a function of cholesterol which was added to POPE/POPS (1-palmitoyl-2-oleoylphosphatidylethanolamine/1-palmitoyl-2-oleoyl-phosphatidylserine) bilayers.

Cholesterol has been shown to affect membrane-integral proteins. For example, it is essential for maintenance of functional integrity of the *Torpedo* nicotinic acetylcholine receptor (nAChR; Fong & McNamee, 1986). Cholesterol also has been shown to modulate the activity of potassium channels in vascular smooth muscle cells. Reducing the cell membrane cholesterol con-

centration, by blocking synthesis, increased the probability of the Ca-activated potassium channel being in the open state (Bolotina et al., 1989). Cholesterol also has a dual effect on the activity of the Na^+-K^+-ATPase of epithelial cells, where at low concentrations (up to 30% by weight) it increases activity and at higher concentrations it inhibits it (Yeagle, Rice & Young, 1988). The former effect was attributed to direct interactions of cholesterol with the protein, while the latter was concluded to be due to changes in membrane structure (Yeagle, 1991). In *Xenopus* myocytes, Lechleiter, Wells and Gruener (1986) showed that while cholesterol, at relatively low concentrations, did not affect the activity of the nAChR directly, it attenuated the inhibitory effects of a volatile anesthetic.

Changing the cholesterol concentration in cell membranes, or in artificial bilayers, has been shown to reduce the order parameter at temperatures below the phase transition temperature (T_c) and to increase it above T_c (Dufourc et al., 1984; Leonard & Dufourc, 1991). Moreover, cholesterol has been shown to increase the modulus of compressibility (K) in lipid bilayers by fivefold as its concentration was increased from 0 to 50% (Evans & Needham, 1987; Needham & Nunn, 1990). This large increase in the K value, increases the lateral elastic stress energy in the bilayer (Helfrich, 1973; Israelachvili, 1991). Adding cholesterol to POPE/POPS bilayers also promotes the formation of nonlamellar hexagonal (H_{II}) structures (Tilcock et al., 1984) which tend to induce a curvature stress that results in a generalized increase in structural stress in planar bilayers (Gruner, 1985). The concept of structural stress is useful in understanding the effects of cholesterol (and other agents) on the lipid structure of membranes and hence on ion channels which undergo conformational changes in this environment.

We propose here that the increased structural stress, associated with incorporation of cholesterol into phospholipid bilayers (Evans & Needham, 1987; Needham & Nunn, 1990), favors the closed state of the channel by causing it to close sooner than in the absence of cholesterol. We tested this hypothesis by reconstituting the BK channel from rat brain into planar bilayers in the presence or absence of cholesterol, and monitoring channel kinetics and conductance. Our results are interpreted in the context of the structural stress of the bilayer and are consistent with the hypothesis that lipid-protein interactions provide potent modulation of ion channel activity.

Materials and Methods

Materials

The calcium-activated potassium (BK) channel was prepared from rat brain homogenates and reconstituted into lipid bilayers made from POPS and POPE (Avanti Polar Lipids, Alabaster, AL). Membrane vesicle preparation, lipid bilayer formation and channel incorporation were similar to procedures published previously (Krueger, Worley & French, 1983) with slight modifications as follows. Phospholipids (POPE and POPS) were dissolved in pure decane (at a weight ratio of 55/45), first dehydrated by passage over an alumina gel column. The POPE/POPS mixture was dried in a stream of nitrogen gas and redissolved in decane (with or without cholesterol) to a final phospholipid concentration of 60 mg/ml. Cholesterol (Sigma, St. Louis) was dissolved in warm (*ca.* 50°C) decane at concentrations from 7.5–30 mg/ml. Dry phospholipids were dissolved in the cholesterol solution cooled to room temperature such that the cholesterol concentration ranged from 6 to 33% (percent of total lipid weight). This lipid mixture contained ~5% decane as a constant contaminant.

Ion Channel Reconstitution

Whole brains (10–12 g) from six adult rats were placed in a cold buffer containing 0.34 M sucrose and 10 mM HEPES at pH 7.4. All procedures were performed at 4°C or on ice. The tissue was minced with scissors and rinsed with buffer. The rinsed tissue was homogenized (Polytron; Tekmar TR-10, at 60% full power) for five periods of 20 sec duration interrupted by 10 sec pauses. The resultant suspension was centrifuged at 1,000× g (Sorvall RC5b; SS-34 rotor) for 15 min. The supernatant was saved and the pellet was washed, homogenized in a glass homogenizer and recentrifuged as before. The supernatants were combined and centrifuged at 10,000× g for 20 min. The supernatant was saved, and the pellet was washed in the buffer, homogenized and recentrifuged at 10,000× g as before. The supernatants were again combined and ultracentrifuged at 100,000× g (Beckman, Ti-50.2 rotor) for 45 min. The last centrifugation step was repeated with the resuspended pellet, and the final pellet (P3 preparation) served as the vesicle fraction that was enriched with plasma membranes. The P3 preparation was resuspended in buffer (0.25 mM sucrose, 10 mM HEPES, pH 7.4). Typically, the protein concentration in the resuspended P3 was ~7 mg/ml; it was aliquoted into 100 μl samples and stored at −80°C. Channel activity was measurable, from such aliquots, for up to 18 months without detectable loss of activity. To estimate the enrichment of P3 with plasma membranes, the activity of the Na^+-K^+-ATPase was assayed (Radominska-Pyrek et al., 1982). Typically, P3 showed a 3–5-fold increase in enzyme activity when compared to the crude homogenate.

The reconstitution chamber consisted of a Lexan cup into which a ~100 μm diameter aperture was drilled. The cup was placed into the posterior bicameral chamber and the aperture was visualized, through a glass window, with a dissecting microscope. An Ag/AgCl electrode, embedded in agar (1% weight in the *trans* solution) was placed in each compartment for voltage clamping and current detection. The *trans* chamber was grounded, and the *cis* chamber was connected to the positive input of the patch clamp amplifier (AxoPatch AXP 1A; Axon Instruments; Foster City, CA). The *trans* solution consisted of (in mM): KCl 100, HEPES 10, and $CaCl_2$ 0.1; at pH 7.4. The *cis* solution had the same composition except that the KCl concentration was 300 mM. To form a bilayer, lipid at the end of a micropipette tip was brushed over the aperture in the *trans* chamber cup wall. The thinning of the bilayer was revealed by an increase in the total capacitance of the membrane from *ca.* 25 pF (immediately after lipid application) to 70–120 pF as the membrane thinned spontaneously. After thinning, the noise level of the bilayer was about 1–2 pA and trans-bilayer resistance was 50–100 GΩ. Channel incorporation was achieved by brushing the tip of a micropipette tip, which contained a small bubble made from a diluted P3 solution (50–25%), across the bilayer. The holding potential (E_h)was shifted from 0 to +200 mV after vesicle application to detect channel events. Channel incorporation was usually observed within a

few minutes. Channel incorporation was accomplished from the *trans* chamber, and this side was grounded (to allow chamber perfusion when necessary). In this system, channel characteristics show a voltage dependence which appears reversed when compared to that reported elsewhere (Latorre, Vergara & Hidalgo, 1982; Moczydlowski & Latorre, 1983; Farley & Rudy, 1988; Reinhart, Chung & Levitan, 1989). Channel conductance and reversal potentials were used to distinguish among different channel types. Experiments were performed on bilayers which showed activity from only one, high-conductance, calcium-activated potassium (BK) channel in order to facilitate analysis. Activity was accepted to reflect a BK channel if the conductance was large (>300 pS) and the reversal potential was close to the theoretically expected (–28 mV) value. The mean conductance of the BK channel was 339 ± 4 pS (± SEM; n = 25; in PE/PS = 55/45 bilayers), and the mean reversal potential was –27 ± 2 mV. The channel required the presence of calcium in the *trans* chamber, and was insensitive to EGTA on the *cis* side. Virtually all channels were incorporated with this unidirectional orientation as reported also for Na^+ channels (Krueger et al., 1983), Ca^{2+} channels (Nelson, French & Krueger, 1984), and for BK channels (Latorre & Miller, 1983; Reinhart et al., 1989). To determine the effects of temperature on ion channel properties, water of the desired temperature was circulated (Neslabs Circulator; RTE-4) through the jacket of the chamber. Temperature was monitored in the *trans* side, near the bilayer, and could be changed at a rate of *ca.* 1°C/sec. Control experiments were performed at 22.5 ± 0.5°C.

Data Acquisition and Analysis

Single channel currents were fed from the patch clamp amplifier through an internal Bessel filter, at 10 kHz, to an FM tape recorder (Racal SD-4). Channel activity was recorded at holding potentials (E_h) ranging from –60 to +30 mV. Taped signals were filtered at 3 kHz (Frequency Devices, 901F) then fed through an A/D converter (10 kHz sampling rate) into a computer (IBM-AT PC). Channel activity, typically from 30 sec periods, was analyzed with the aid of the pCLAMP software (ver. 5.5.1; Axon Instruments). In the presence of cholesterol, channel activity was significantly reduced and longer periods (up to 2 min) were required to obtain a sufficient number of opening events for characterizing channel properties. The criterion for the closed-to-open transition was set at 50% of the apparent amplitude. Open channel events with durations shorter than 0.3 msec were discarded from kinetic analysis due to the limitations of the frequency response of the system. For amplitude measurements, events shorter than 1 msec were eliminated. Current-voltage (I-V) relationships were plotted in the range of +30 to –60 mV, and the slope conductance was calculated from linear regression of the curve. Open and closed time histograms were fitted with exponential decay functions using the least squares method (pCLAMP software). The probability of the channel being in the open state (P_o) was calculated from the ratio of the time that the channel was in the open state to the total data acquisition time. The significance of difference between results from different experimental conditions was tested using the two-tailed Student's t-test.

Small Angle X-Ray Diffraction

Oriented membrane "multilayer" samples of POPE/POPS in the absence and presence of cholesterol were prepared for small angle x-ray diffraction as follows. Phospholipid and/or cholesterol dissolved in chloroform were dried in a stream of nitrogen gas to a thin film on the sides and bottom of a glass 13 × 100 mm test tube while vortexing. Residual solvent was removed under vacuum overnight. A specified volume of buffer (0.5 mM HEPES, 2.0 mM NaCl, pH 7.3) was added to the dried lipids while vortexing, yielding a final phospholipid concentration of 5 mg/ml. Multilamellar vesicles were formed by vortexing the buffer and lipids for 3 min at ambient temperature (Bangham, Standish & Watkins, 1965). Oriented membrane multilayer samples for small angle x-ray scattering were prepared from the vesicles as described previously (Chester et al., 1987). The membrane multilayer samples composed of 250 μg phospholipid were equilibrated overnight in glass vials containing a saturated salt solution which served to define a specific relative humidity of 95% at 5°C. Oriented membrane samples were then placed in sealed brass canisters containing aluminum foil windows in which temperature and relative humidity were precisely controlled.

Small angle x-ray scattering was carried out by aligning the samples at near-grazing incidence with respect to the x-ray beam. The radiation source was a collimated, monochromatic x-ray beam (CuK_α, λ = 1.54 Å) from an Elliot GX-18 rotating anode microfocus generator (Enraf Nonius, Bohemia, NY) operated at 40 kV and 30 mA in the Biomolecular Structure Analysis Center at the University of Connecticut Health Center (Farmington, CT). A helium tunnel was positioned between the sample and detector to reduce scattering from the air. The fixed geometry beamline utilized a single Franks mirror providing nickel-filtered radiation ($K_{\alpha 1}$ and $K_{\alpha 2}$ unresolved) at the detection plane. The beam height at the sample was ~1 mm.

A measurement of the overall lipid bilayer width, including surface water, is called the d-space or the unit cell periodicity. When the amount of water between the stacked membranes in the multilamellar system is kept constant, the d-space is directly affected by changes in the conformation of lipid molecules. The d-space of the membrane lipid bilayer was the measured distance from the center of one lipid bilayer to the next, including surface hydration. The d-space for the membrane multilayer sample was calculated using Bragg's Law:

$$n\lambda = 2d\sin\Theta \tag{1}$$

in which n is the diffraction order number, λ is the wavelength of the x-ray radiation (1.54 Å), d is the membrane lipid bilayer unit cell periodicity, and Θ is the Bragg angle equal to one-half of the angle between the incident beam and scattered beam. Bragg's diffraction data from the oriented membrane multilayer samples were recorded on a one-dimensional position-sensitive electronic detector (Innovative Technologies, Newburyport, MA) then converted into the Bragg angle. In addition to direct calibration of the detector system, lead stearate was used to verify the calibration (Mason, Trumbore & Pettegrew, 1994).

Calculations of Activation Energy

The activation energy of channel transitions, from the open to the closed state, was calculated according to Eisenberg and Crothers (1979), using the following equations:

$$\ln(k) = (-E_a/RT) + \ln B \tag{2}$$

where k is the transition rate, E_a the activation energy, R the gas constant (1.99 cal mol^{-1} $°K^{-1}$), T the absolute temperature and B is a constant. The change in Gibb's free energy was calculated from

$$\Delta G = -RT \ln (k) + RT \ln (k_b T/h) \tag{3}$$

where k_b (1.38×10^{-23} $J°K^{-1}$) is the Boltzmann constant and h is the Plank constant (6.63×10^{-34} Js). The entropy change (ΔS) was calculated from:

$$\Delta G = \Delta H - T\Delta S \tag{4}$$

where the enthalpy change $\Delta H = E_a - RT$.

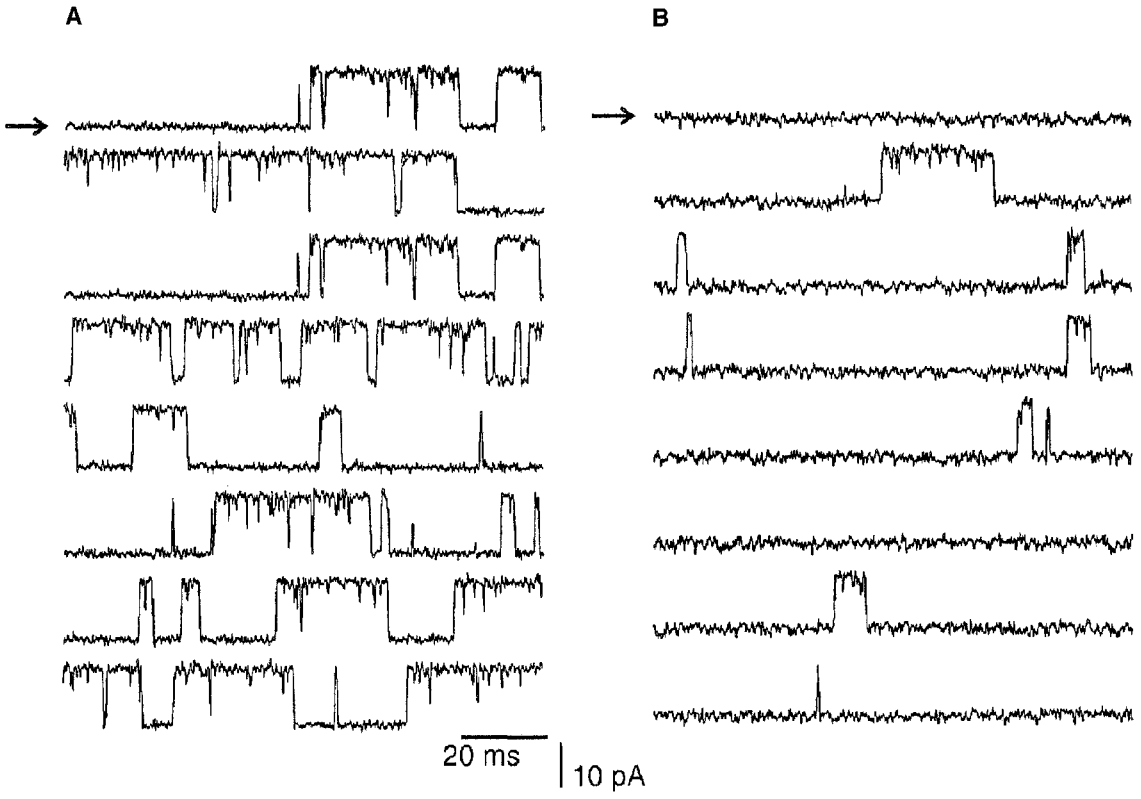

Fig. 1. Single channel activity of a high-conductance, calcium-activated potassium (BK) channel reconstituted into a POPE/POPS = 55/45 bilayer (*A*), and into a similar bilayer containing 11% (by weight) cholesterol (*B*). The holding potential E_h was +10 mV in both cases. Arrows indicate the closed state of the channel. The *trans* chamber solution contained (in mM) KCl = 100, $CaCl_2$ = 0.1, HEPES = 10, pH = 7.4. The *cis* solution had the same composition except for KCl = 300. The temperature was 22.5 ± 0.5°C. The *trans* chamber was connected to the ground terminal of the patch clamp amplifier.

Results

Figure 1 shows the behavior of a calcium-activated potassium (BK) channel in a control (cholesterol-free) and in a cholesterol-containing bilayer (11% by weight). In the control bilayer (PE/PS = 55/45; *A*), open events were frequent, and their duration had a mean open time (t_o) of 11.2 ± 0.3 msec (±SEM; 1,282 events). Event amplitude, at a holding potential (E_h) of +10 mV, was 12.8 ± 0.4 pA. In the cholesterol-containing bilayer (*B*), channel open time durations were markedly shorter resulting in a mean open time of 4.3 ± 0.3 msec (219 events). Channel amplitudes were also reduced to a mean of 11.8 ± 0.4 pA. For these two channels, the corresponding conductances (g) were 356 pS for the control and 332 pS (a significant difference; $P < 0.05$). Examination of the open time histograms (Fig. 2), reveals a marked reduction in channel open time durations in the presence of cholesterol. Both histograms were best fit with a two exponential decay function. In the control bilayer (*A*), the time constants were τ_1 = 1.3 msec (8% of total events) and τ_2 = 11.3 msec (92%). In the cholesterol-containing bilayer (*B*), the corresponding parameters were: τ_1 = 0.51 msec (65% of total events) and τ_2 = 2.21 msec (35%). A calculation of the probability that these channels are in the open state further documents the reduction in channel kinetics from a control value P_o = 47% to an almost complete shutdown of channel activity in the presence of cholesterol where P_o = 3%.

Figures 3–5 summarize channel characteristics as a function of cholesterol concentration. Figure 3 shows that as the cholesterol concentration increases, the channel mean open time decreased with the sharpest decline, at about 10% cholesterol, from a mean open time value of 11 to 2.5 msec. The sigmoidal shape of this relationship appears for all parameters examined. The decrease in mean open time is mirrored in the probability of the channel being in the open state (P_o) as shown in Fig. 4. P_o was relatively independent of the cholesterol concentration between 0–8% and between 12–33%, with a steep decline at about 11% cholesterol. The responses of open time constants and mean closed times, as a function of cholesterol concentration are summarized in Table 1.

When we examined the effects of cholesterol on channel conductance (Fig. 5), we found a similar sigmoidal relationship with a significant ($P < 0.05$) reduction

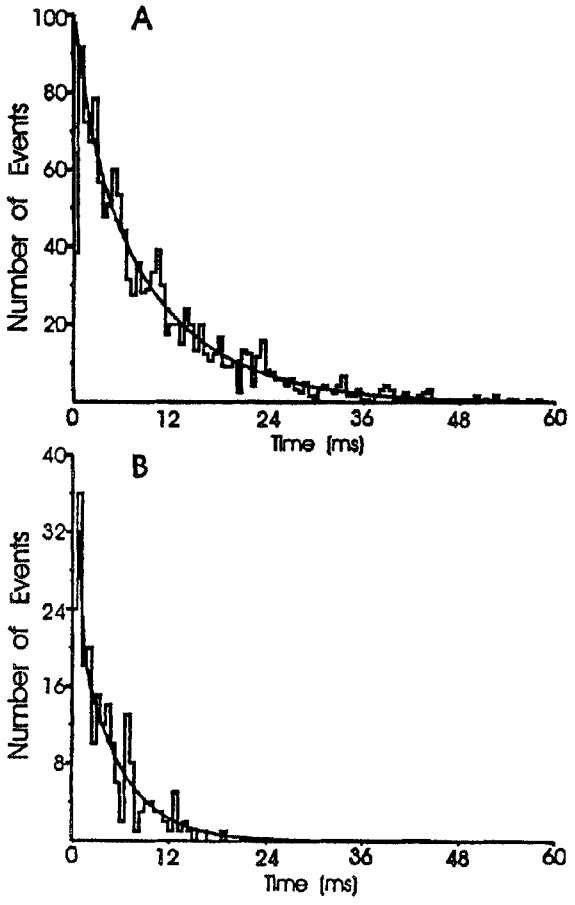

Fig. 2. Open time histograms of single BK channels from a control (POPE/POPS) bilayer (*A*) and from a bilayer containing 11% cholesterol (*B*); data from experiments shown in Fig. 1). Both histograms were best fit with two exponentially decaying functions. The time constants for the control (*A*) were $\tau_1 = 1.34$ msec (8% of total events), $\tau_2 = 11.27$ msec (92% of total events), the mean open time $t_o = 11.24 \pm 0.33$ (±SEM) msec. The number of events $n = 1{,}282$ and the probability of the channel being in the open state $P_o = 0.47$. In the cholesterol-containing bilayer (*B*), $\tau_1 = 0.17$ msec (65%), $\tau_2 = 5.06$ msec (35%), $t_o = 4.34 \pm 0.29$ msec, $n = 219$, $P_o = 0.03$.

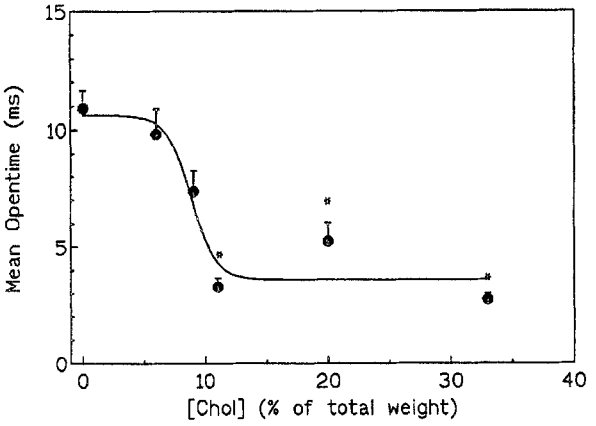

Fig. 3. Effects of cholesterol on single channel mean open times. The data were best fit to a sigmoidal curve described by: $y = 3.59 + 7.04/[1 + \exp(x - 8.84)]$. Each point is the mean ± SEM from 2 to 14 experiments. (*) Values which differ significantly from control (no cholesterol). Significance was tested with the two-tailed Student's *t*-test and considered significant when $P < 0.05$.

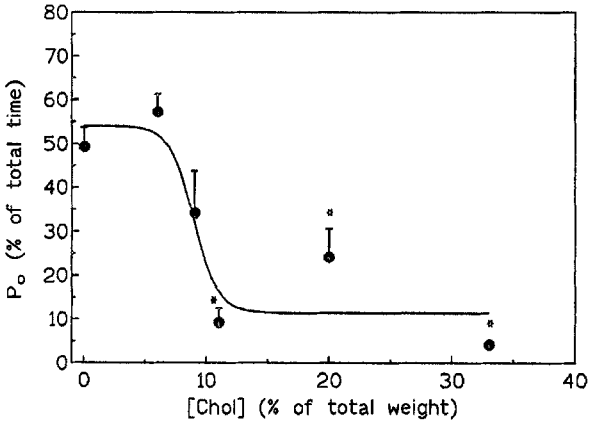

Fig. 4. Effects of cholesterol on the open probability (P_o) of single BK channels. The data were best fit to a sigmoidal curve with $y = 11.40 + 42.66/[1 + \exp(x - 8.94)]$. All other details are as in Fig. 3.

from an average of 335 pS to about 310 pS at high concentrations of cholesterol. The sharpest reduction in conductance was found at the same cholesterol concentration that produced the sharpest decrease in channel kinetics.

The physical structure and phase behavior of the POPE/POPS lipid bilayer in the absence and presence of cholesterol were examined using small angle x-ray diffraction. The oriented membrane "multilayer" samples gave clearly defined, reproducible diffraction orders at the temperatures studied (5, 25 and 35°C). X-ray scattering from POPE/POPS samples in the absence of cholesterol indicated that two separated lamellar phases coexisted: a gel phase (*d*-space = 62 &) and a liquid-crystalline phase (*d*-space = 54 &), as shown in Fig. 6*A*.

In the presence of cholesterol (11% of total weight), however, there was no evidence for the existence of the gel phase under identical experimental conditions (25°C). A single lamellar phase was observed with a *d*-space of 55 &, corresponding to the liquid crystalline phase (Fig. 6*B*). The effects of temperature on phase behavior are summarized in Table 2. Even as low as 5°C, a liquid-crystalline phase was observed for samples containing cholesterol but not POPE/POPS alone. Only at 35°C was a single, liquid-crystalline phase (*d*-space = 52 &) observed for the control POPE/POPS sample. The *d*-space of the control membrane liquid-crystalline phase was affected by cholesterol and the thermal energy in the sample (i.e., 54 & at 25°C *vs.* 52 & at 35°C for control samples) as shown in Table 2.

To examine in more detail the effects of cholesterol

Table 1. Effects of cholesterol on BK channel kinetic properties

[Chol]	0%	6%	11%
τ_1	1.7 ± 0.4 (13)	2.5 ± 0.3 (3)	1.1 ± 0.3 (9)
τ_2	13.4 ± 2.5 (14)	10.5 ± 1.4 (4)	4.3 ± 0.7 (10)*
C_t	20 ± 11 (14)	7.3 ± 0.6 (4)	74 ± 17 (10)*

Values are the mean ± SEM in msec. The number of experiments is shown in parentheses.
(*) Indicates a value that is significantly different from control (0% cholesterol). [Chol] = cholesterol as % of total lipid weight. τ = open time constants. C_t = mean closed time.

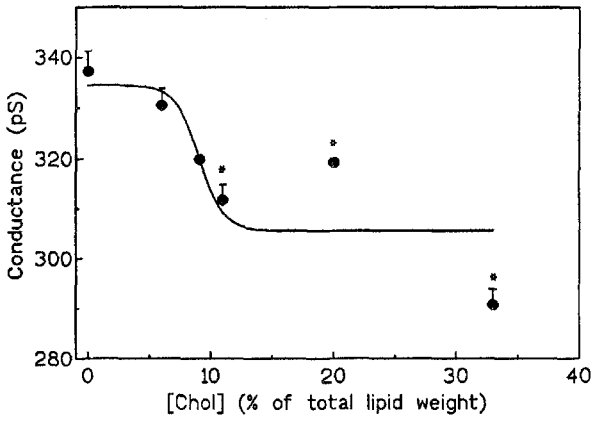

Fig. 5. Effects of cholesterol on BK channel conductance. The data were best fit with a sigmoidal curve with $y = 305.62 + 28.88/[1 + \exp(x - 9.09)]$. Other details as in Fig. 3.

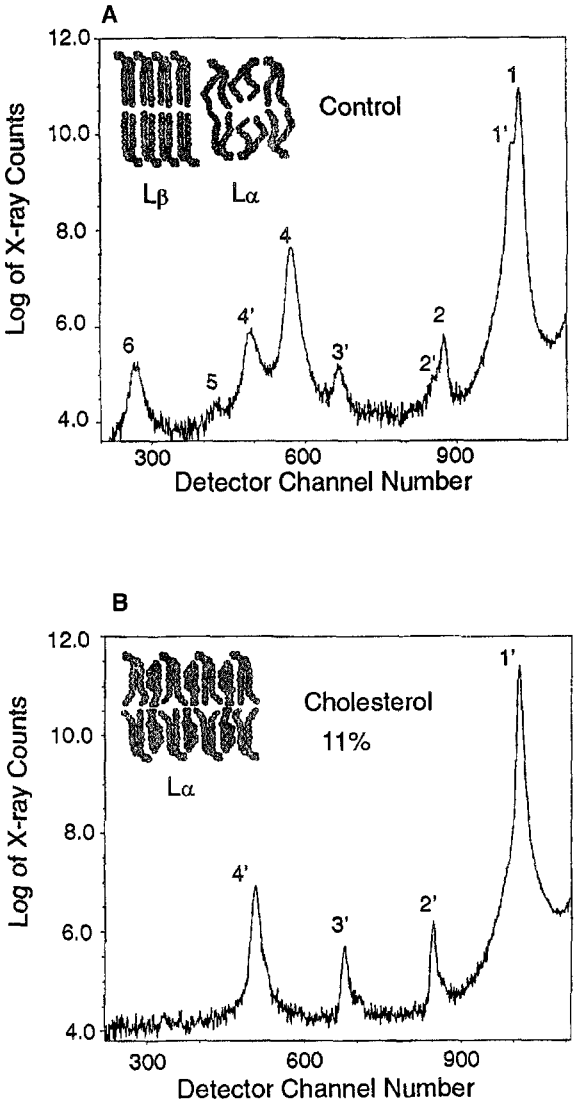

Fig. 6. X-ray scattering from oriented POPE/POPS samples in the absence (*A*) and presence (*B*) of 11% cholesterol at 25°C. The ordinate represents the intensity of the scattered x-ray and the abscissa represents the channels for the one-dimensional position-sensitive detector. In the absence of cholesterol, the x-ray diffraction pattern is consistent with two separated lamellar lipid bilayer phases. Diffraction orders *1–6* correspond to a lipid bilayer in the gel state with a *d*-space (distance from the center of one bilayer to the next, including water) of 62 Å while diffraction orders *1′–4′* correspond to a lipid bilayer in the liquid crystalline state with a *d*-space of 54 Å. In *B*, x-ray scattering from samples containing 11% by weight cholesterol indicated a single liquid-crystalline lipid bilayer phase with a *d*-space of 55 Å. The panel insets are schematic illustrations of the hypothetical structure of the samples under these experimental conditions, where L_α represents the liquid crystalline state and L_β represents the gel state.

on channel properties, we tested the effects of temperature on channel behavior at an intermediate concentration of cholesterol (11% by weight). Figure 7 shows typical channel activity in the absence (left column) and the presence of cholesterol (right column) at three different temperatures (32°C in row *A;* 22.5°C in row *B;* and 10°C in row *C*). In general, channel amplitudes decreased as the temperature was lowered. For example, under control conditions (left column) channel amplitude dropped from 15.4 ± 0.02 pA (1,600 events; 32°C) to 8.9 ± 0.02 pA (167 events; 10°C). By comparison (right column), channel amplitude dropped from 14.7 ± 0.7 pA (395 events; 32°C) to 8.0 ± 0.3 pA (40 events; 10°C) in bilayers containing 11% cholesterol. Figure 7 also shows that channel open times increased as the temperature was lowered, with the longest open times being found in control bilayers at 10°C. The effects of temperature on the mean open time, in the absence and presence of cholesterol are summarized in Fig. 8. As the temperature was decreased, t_o increased in a linear fashion, in both conditions. However, in the presence of cholesterol, the slope of the relationship was attenuated significantly (the correlation coefficient for the control curve was $r = -0.96$ and that for the cholesterol relationship was $r = -0.99$). The respective slopes were –0.6 msec/°C for control and –0.27 msec/°C for cholesterol-containing bilayers. ANCOVA (analysis of covariance) testing showed these values to be significantly different

Table 2. Effect of temperature on the d-space

	d-space (l.c.:gel)		
Temperature	5°C	25°C	35°C
Control	0:62	54:62	52:0
11% cholesterol	54:61	55:0	n.a.

l.c. = liquid crystalline phase; gel = gel phase; n.a. = not available, 0 = not observed. d-space units are given in Å.

($F < 0.05$). The open probability P_o was also reduced in the control bilayers, from 58% (32°C) to 11% (10°C). In cholesterol-containing bilayers, P_o was reduced from 2% (32°C) to 1% (10°C).

An analysis of the effects of temperature on channel conductance (g) is summarized in Fig. 9. In both cases, g decreased as the temperature was lowered and these relationships are both linear ($r = 0.8$ for the control and $r = 0.98$ for the cholesterol-containing bilayers) but the slopes of the lines (7.9 pS/°C for control and 7.8 pS/°C in the presence of cholesterol) are not significantly different ($F > 0.05$). Nevertheless, conductance values in the presence of cholesterol were consistently lower.

Because channel properties are temperature dependent, we sought to obtain a quantitative estimate of the activation energies for channel transitions from the open to closed state under control and cholesterol-containing conditions. The multiple-state transition of the BK channel can be approximated by a two-state (open to closed) model if the transition between open states is much faster than the one between the open and closed state (Moczydlowski & Latorre, 1983). The transition rate from open to closed states is approximated to be $1/t_o$. Figure 10 shows Arrhenius plots whose slopes reflect the activation energy required for channel transitions from the open to the closed state. At 22°C, under control conditions, this value was 10.1 ± 1.2 kcal/mol; in the cholesterol-containing (11%) bilayers the value was 4.5 ± 0.4 kcal/mol. Similarly, the activation energy of ionic conductance was calculated from the slope of ln(g) *vs.* the reciprocal of the absolute temperature. The activation energies for conductance were found not to be different when comparing the control and cholesterol paradigms. Using the formulation shown in Materials and Methods, the thermodynamic properties of the BK channel transition, from the open to the closed state, and channel conductance were calculated at 22°C (Table 3). These values are within the same range as previously reported for Na^+ (Correa, Bezanilla & Latorre, 1992) and K^+ channels (McLarnon, Hamman & Tibbits, 1993). For the open-to-closed transition, the change in enthalpy (ΔH) was reduced in bilayers containing 11% cholesterol when compared with controls. The change in entropy (ΔS) increased and the change in the free energy (ΔG) was unaffected. The corresponding values of ΔH, ΔS and ΔG for conductance, however, showed no difference between cholesterol-containing and control bilayers.

Discussion

Cholesterol is found in most cell membranes (Hauser & Poupert, 1992) and when present, it is often the second most abundant lipid (Gennis, 1989; Hauser & Poupert, 1992). The presence of cholesterol and its structural effects on lipids suggest that it plays an essential role in modifying membrane structure and consequently modulating the activity of membrane integral proteins. Assessing the effects of cholesterol is facilitated by real time measurements of transitions of a single protein—an ion channel—as it goes through its activity cycle. While providing access to a membrane-integral protein function, elucidating the effects of cholesterol on the channel is still fraught with difficulties because of the complex interactions of cholesterol with other membrane lipids even in simple, binary systems (Marbrey, Mateo & Sturtevant, 1978; Dufourc et al., 1984; Leonard & Dufourc, 1991). For example, cholesterol has been shown to increase the order parameter of such systems above the phase transition temperature but to reduce the lipid order below the transition temperature (Cortijo et al., 1982; Dufourc et al., 1984; Yeagle 1985, 1991). The addition of cholesterol to lipid bilayers has also been shown to induce structural stress by increasing the modulus of compressibility K, which is defined as:

$$\Delta T = K(\Delta\alpha/\alpha), \text{ therefore } K = \Delta T(\alpha/\Delta\alpha) \quad (5)$$

where ΔT is the change in tension, accompanied by an induced change in the total surface area of a bilayer (α) equal to $\Delta\alpha$ (Helfrich, 1973; Evans & Needham, 1987). This increase in K, in turn, increases the lateral elastic stress energy (E_1) for the lipid molecules which builds up in the bilayer, according to the relationship:

$$E_1 = K\,(\Delta A)^2/(2A) \quad (6)$$

where ΔA is the change in the cross-sectional area (A) of each lipid molecule (Helfrich, 1973; Israelachvili, 1991). In addition, cholesterol can promote the formation of nonlamellar hexagonal (H_{II}) lipid structures (Tilcock et al., 1984) which tend to produce bending in the bilayer and hence the induction of a curvature-related physical stress that also increases the structural stress in planar bilayers (Gruner, 1985). Thus, as the concentration of cholesterol increases, physical stress builds up and this force is available to act on membrane integral proteins such as ion channels.

The functional hallmark of ion channel activity is the transition from the closed to the open state. This transition is thought to be associated with a conforma-

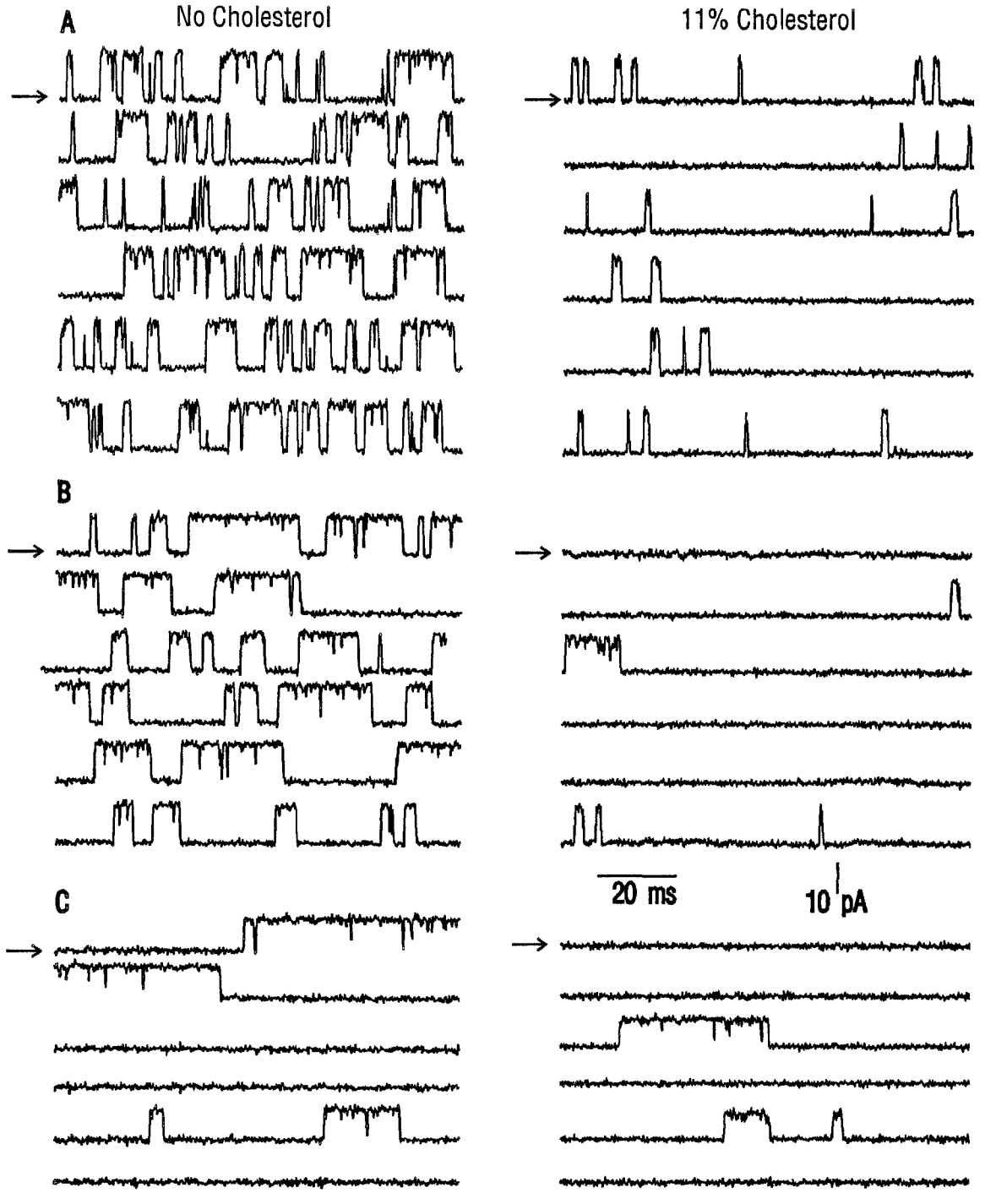

Fig. 7. Effects of temperature on single channel events from BK channels reconstituted into POPE/POPS (55/45 by weight; left column) and into bilayers containing, in addition, 11% cholesterol (right column). In all cases, $E_h = +10$ mV. Other details are as in Fig. 1. The temperature for the experiments shown in row (*A*) was 32°C; for row (*B*) the temperature was 22.5°C; and for row (*C*) the temperature was 10°C. Arrows indicate the closed state of the channel.

tional change (Miller & White, 1984; Catterall, 1986; Guy & Conti, 1990) which may be accompanied by a change in volume (Heinemann, Stühmer & Conti, 1987). A conformational change from the closed to the open state, coupled to an increase in protein volume, is likely to generate a lateral stress force in the lipid structure surrounding the channel protein. The surrounding lipids would then generate a counter force which would be

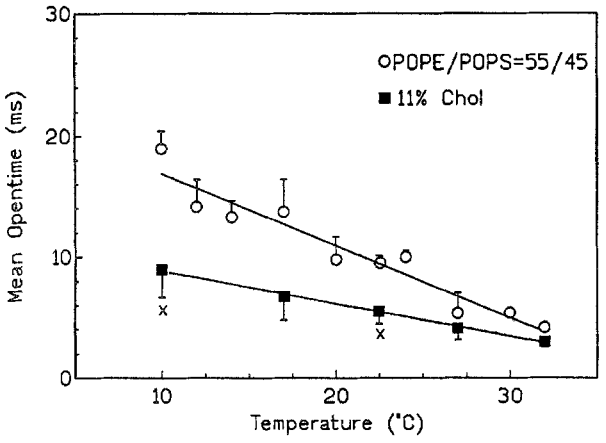

Fig. 8. Effects of temperature on the BK channel mean open time in the absence (○) and the presence (■) of cholesterol (11% by weight). The linear coefficient $r = -0.96$, the slope of the line is −0.6 msec/°C for the control data. In the presence of cholesterol, $r = -0.99$, slope = −0.27 msec/°C. Covariance analysis (ANCOVA) of these two lines shows the difference in the slope to be significant ($F < 0.05$). Each point represent the mean ± SEM of 2 to 14 experiments. Mean open times which are significantly different from their corresponding control values are marked with an (×).

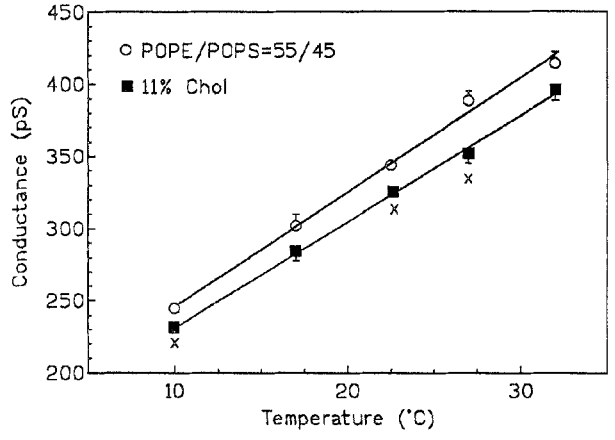

Fig. 9. Effects of temperature on the BK channel conductance in the absence (○) and presence (■) of cholesterol (11% by weight). In control bilayers (POPE/POPS = 55/45), $r > 0.99$, the slope of the line is 7.93 pS/°C. In bilayers with cholesterol, $r > 0.99$, slope = 7.76 pS/°C. The difference between the slopes is not significant ($F > 0.05$). Other details are as in Fig. 7.

deflected back onto the channel. The magnitude of this force would depend on the modulus of compressibility of the membrane, and would thus facilitate the return of the channel to the closed state at different rates. The sensitivity of ion channels to mechanical perturbations has been reported for mechanosensitive channels. Increasing the transmembrane pressure gradient, for example, results in activation of such channels (Morris, 1990; Sachs & Lecar, 1991; Corey & Howard, 1994).

When, as in our experiments, a specific mechanical

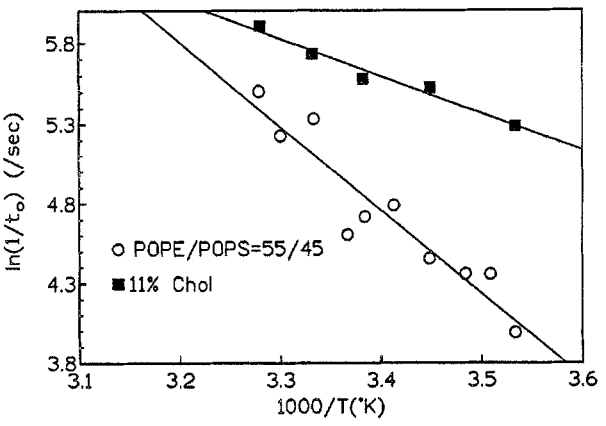

Fig. 10. Arrhenius plots of the channel closing rate ($1/t_o$). The slope is −5,200 ± 600 (SE) °K/sec and the activation energy (*see text* for details) is 10.1 ± 1.2 kcal/mol, as calculated from the slope for the channel in the control bilayers (○). In the presence of cholesterol (■), the slope is −2,300 ± 200 °K/sec and the activation energy is 4.5 ± 0.6 kcal/mol. ANCOVA shows the difference in the slope between the two lines is significant ($F < 0.05$).

Table 3. Thermodynamic variables of BK channel properties

Open-to-Closed Transition	% Cholesterol	11% Cholesterol
E_a (kcal/mol)	10.1 ± 1.2	4.5 ± 0.4
ΔH (kcal/mol; 22°C)	9.5 ± 1.2	3.9 ± 0.4
ΔG (kcal/mol; 22°C)	14.3 ± 0.9	13.8 ± 2.1
ΔS (cal/mol °K; 22°C)	−15.6 ± 6.8	−34.6 ± 8.1
Conductance Parameters		
E_a (kcal/mol)	4.2 ± 0.2	4.2 ± 0.2
ΔH (kcal/mol; 22°C)	3.6 ± 0.2	3.6 ± 0.2
ΔG (kcal/mol; 22°C)	13.7 ± 0.1	13.8 ± 0.1
ΔS (cal/mol °K; 22°C)	−34.2 ± 1.0	−34.6 ± 1.0

E_a = activation energy, ΔH = change in enthalpy, ΔG = change in Gibb's energy, ΔS = change in entropy. Values are calculated based on linear regression and propagation of error.

property of lipid membranes (the lateral stress force), is increased by the insertion of cholesterol, several predictions can be made regarding channel transitions. Thus, we propose that as the lateral stress of the bilayer increases, the ability of the channel to remain in the open state will decrease resulting in shorter open times. When the lateral stress increases further, channel opening events may be compromised altogether resulting in a reduced probability of finding the channel in the open state. Finally, an increase in cholesterol concentration might also affect channel conformation more severely to the extent of causing a "squeezing" or "bending" action such that channel conductance proper is expected to decrease. As discussed below, our data show a significant reduction in channel kinetics and conductance as the concentration of cholesterol increases. Similar findings, with respect to altered kinetics, have been reported by

Bolotina et al. (1989) who showed a reduction in the P_o of a BK channel from rabbit vascular smooth muscle after exposing excised membrane patches to a cholesterol-containing solution. Bolotina's study also demonstrates that the effects of cholesterol, similar to those which we report here, can take place even at different ranges of cholesterol and in complex (native) lipid membranes. Thus, while not quantitatively comparable because of the profoundly different lipid compositions, the effects of cholesterol are expressed qualitatively in a similar fashion in lipid membranes of vastly different compositions.

We studied the effects of cholesterol on ion channel activity in an artificial bilayer in order to better control the lipid composition around the BK channel. Although this system still contains native lipids, extracted with the reconstituted channel, this contamination can be shown to be very small compared to the composition of the bilayer itself. Vesicles in the P3 preparation from rat brain have been estimated to have a diameter of *ca.* 0.5 μm (Worley, 1985) with a surface area of $\sim 8 \times 10^{-13}$ m^2. Our experiments were carried out with only one functional BK channel in each bilayer. Therefore, the expected ratio of the surface area of the membrane vesicle to that of the bilayer is very small and hence contamination of bilayer lipids with native lipids is likely to be less than 10^{-4}. The probability of the presence of other, silent, channels is very low since (i) BK channels are abundant and if only one is observed the likelihood is that other channels did not simultaneously incorporate; (ii) we could monitor Cl^- channel activity (through its reversal potential) and eliminated such bilayers from our study. These observations suggest that the fusion rate of membrane vesicles into the bilayer is low and therefore the amount of native lipid expected to contaminate the bilayer is likely to be inconsequential. In addition, the exchange rate between the bulk lipid of the bilayer and the lipid surrounding the channel protein (initially the native lipids) is in the range of 10^{-7} sec (Ryba et al., 1987). Thus, the original native lipid surrounding the channel protein will be replaced and diluted by bulk lipids very rapidly. Furthermore, although the bilayers we used contain decane as a constant contaminant in all experiments, we believe that its contribution to the results is insignificant. This is based on previous observations which show that decane does not significantly affect the conductance of the voltage-dependent K^+ channel reconstituted from sarcoplasmic reticulum (Labarca, Coronado & Miller, 1980). Therefore, although we do not know the actual composition of the annular lipids, our results are most likely to reflect the modulation of channel properties by changes induced in the physical properties of the bilayer and, to a much lesser extent, by direct interaction between specific lipids with the channel protein.

The kinetic and conductance responses of the ion channel to the increased cholesterol concentration (Figs. 3–5) reveal a nonlinear relationship which was fitted well by a sigmoidal function ($R^2 > 0.96$; on the other hand, a linear fit resulted in much lower correlation coefficients in the range 0.6 to 0.8), with a sharp break at about 11 weight % cholesterol. This abrupt transition may be correlated with the phase alteration observed with low angle x-ray diffraction (Fig. 6). Under control conditions, two phases—a gel phase and a liquid-crystalline phase—coexist in the bilayer. After the addition of cholesterol (11% by weight), only one phase—the liquid-crystalline phase—was detected. The details of the bilayer structure, under control conditions, are unclear. The two phases may represent the segregation between POPE and POPS similar to those reported by Onishi and Ito (1974). We speculate here that the BK channel may be partitioned into the liquid crystalline domain at low (or zero) concentrations of cholesterol; then, as the cholesterol concentration is increased, the channel may be forced into a phase which has an increased K value due to the presence of cholesterol. As indicated by the x-ray diffraction data, this phase transition may occur in the range 11% cholesterol. It should be noted, however, that the x-ray diffraction study was performed on a stack of bilayers separated by at least 10& (Mason & Chester, 1989) with free water in between stacks (Lundberg, Svens & Ekman, 1978) and the reconstitution buffer for the x-ray studies contained no calcium. Therefore, extrapolations from these data must be done carefully as these differences in conditions, from the ones used in the ion channel reconstitution experiments, may result in bilayer structural changes that are not entirely comparable. Another possible explanation for the sigmoidal relationship between channel properties and the concentration of cholesterol is that the increase of lipid structural stress, induced by cholesterol, is sigmoidal by itself as shown for SOPC vesicles (Needham & Nunn, 1990).

We propose here that the reduction of channel kinetics (mean open time and probability of the channel being in the open state) can be explained by the lateral elastic stress hypothesis. To test this, we calculated the thermodynamic properties of the BK channel open-to-closed transition in the presence and absence of cholesterol from Arrhenius plots which correlate the effects of cholesterol as a function of temperature. The calculated reduction in the activation energy (Table 3), which is required to move the channel from the open to the closed state, is consistent with the hypothesis that cholesterol destabilizes the open state of the channel causing it to close sooner than in the absence of cholesterol. To provide futher evidence for this point, we have estimated the lateral elastic stress energy produced by cholesterol, calculated from the equation for E_1 (Eq. 6) to be about 30–50 kcal/mol (*see* Appendix for details). This energy is considerably higher than the activation energy (from

Table 4. Qualitative effects of cholesterol on bilayer and BK channel properties

	LES (K)[a]	CS[b]	S[c]	t_o	g
Cholesterol ↑ ($T > T_c$)	↑	↑	↑	↓	↓
Cholesterol ↑ ($T < T_c$)	↑	n.a.	↓	↓	↓

LES = lateral elastic stress; K = modulus of compressibility; CS = curvature stress; S = order parameter; t_o = mean opening; g = conductance; n.a. = not available; ↑ = increase in parameter; ↓ = decrease in parameter.

[a] Lis et al. (1982); Evans and Needham (1987); Rand et al. (1988); Needham and Nunn (1990).

[b] Tilcock et al. (1984).

[c] Cortijo et al. (1982); Dufourc et al. (1984).

the Arrhenius plot) required to move the channel from the open to the closed state (*ca.* 5–10 kcal/mol; Table 3). Thus, the energies produced within the lipid bilayer, through the addition of cholesterol, and those required for channel transitions are consistent with our interpretation of the action of cholesterol through the development of lateral elastic stress. Although the considerations of lateral stress being responsible for the earlier closure of the channel are consistent with our findings and those of others (e.g., Hui & Sen, 1989; Helfrich & Jakobsson, 1990), it is possible that other factors, such as an interference of calcium binding to the channel through a change in the conformation of the channel protein by cholesterol, may also contribute to our results. Although this possibility cannot be eliminated, there are presently no data which bear directly on this point.

The calculated entropy change [ΔS (Table 4)], for a BK channel going from the open to closed state in the absence of cholesterol, is *ca.* −16 cal/(mol °K). The negative sign indicates that the entropy for the open state is larger than the entropy for the transitional state. The difference in entropy between the open and the transitional state may reflect conformational changes of the protein related to a volume change of the channel (Heinemann et al., 1987). (To a first approximation, we ignore here possible influences of lipids and water). In support of this notion are the reported changes in entropy of globular proteins in solution and of rhodopsin in bilayer membranes which have been correlated with a partial unfolding of the protein (Rothchild, Gillespie & DeGrip, 1987; Gibson & Brown, 1993). The addition of cholesterol results in a reduction in entropy; the calculated ΔS is −34 cal/(mol °K). Although this extra decrease in entropy for the open to closed transition is expected to favor the open state entropically, it is accompanied by a reduction in enthalpy (Table 3) which would appear to compensate for the gain in entropy. The destabilization of the open state is reflected by the large reduction in the activation energy for the open-to-closed transition. These thermodynamic variables reflect the state of the entire system (consisting of bilayer lipids, the ion channel protein and the water phase). Changes in these components will therefore contribute to the thermodynamic variables, but their contribution is likely to be smaller than that of cholesterol and to be relatively constant under the various conditions we examined here. Since cholesterol has been shown to change protein structures (e.g., AChR channels; Fong & McNamee, 1987; Butler & McNamee, 1993), it is possible that the extra reduction in entropy, for the open-to-closed transition of the channel is also related to a structural change induced by cholesterol.

We observed a small, but statistically significant, decrease in channel conductance at cholesterol concentrations above 10%. The thermodynamic values for conductance, however, were unchanged. This paradoxical finding may be explained by the fact that ion flux through the channel can be influenced at sites that are different in location from the narrow tunnel region which presumably is located in the midplane of the bilayer. The structure of ion channels is envisioned as a narrow tunnel which contains the rate limiting selectivity filter and is flanked by funnel-shaped vestibules on one or both sides (Latorre & Miller, 1983; Hucho & Hilgenfeld, 1989). We suggest that the lateral stress exerted on the channel protein, which is increased by addition of cholesterol, results in the compression of the vestibule with little or no effect on the narrow tunnel region. The resulting structural change in the vestibule can reduce conductance either by limiting the flux rate of ions on the basis of a reduced vestibule volume (Latorre, 1983; Dani, 1986) or by a change of the local negative charge due to charged residues being moved from the protein/water interface to the interior of the protein molecule. This latter effect could lead to a reduced local concentration of the permeant ion (Dani, 1986; Jordan, 1987). This preferential "squeezing" action on the channel, excluding the narrow tunnel (selectivity portion) region, may result from the structure of the channel tunnel being more dense and more highly packed with electrostatic charges which would therefore oppose physical constriction.

A comparison of the effects of temperature on channel properties, in the presence and absence of cholesterol, shows that cholesterol attenuates the effects of temperature on the mean open time. Cholesterol is known to disorder the structure of fatty acid chains below T_c and to increase the order above T_c (Yeagle, 1985, 1991). The T_c for a POPE/POPS (55/45 by weight) bilayer is between 22–26°C (H. Lamparski, *personal communication*). When we evaluate our data on the basis of the order parameter S (Table 4), the change in order parameter—after addition of cholesterol—cannot fully account for the cholesterol-induced monotomic reduction of channel properties. For example, if the open channel is considered to prefer an ordered lipid structure, then be-

low the phase transition temperature the presence of cholesterol would result in a decrease of t_o. In contrast, above the phase transition temperature, adding cholesterol should result in an increase of t_o. This prediction is not borne out by our observations. However, Table 4 shows that addition of cholesterol correlates well with an increase in the lateral elastic stress both below and above the phase transition temperature. Channel kinetics and conductance are decreased over the same temperature range regardless of the transition temperature. Therefore, we conclude that the concept of the lateral stress energy provides a better explanation of the results reported here.

In conclusion, we show that changing the physicochemical properties of lipid bilayers has profound effects on the function of the BK channel. We propose that these effects may best be explained by considering the increased structural stress, induced by cholesterol, which affects the transition between different conformational states of the channel protein such that an increase in the stress energy favors the closed state of the channel. It is further possible, therefore, that this action of cholesterol may play a role in modulating channel activity in various physiological and pathological states.

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Appendix

Estimation of the Increase in the Lateral Elastic Stress Energy

The increase in the lateral elastic stress energy is calculated from Eq. (6) in the text

$$E_1 = K\,(\Delta A)^2/(2A)$$

where E_1 is the lateral elastic stress energy, K is the modulus of compressibility, and ΔA is the change in the cross-sectional area (A) of each lipid molecule (Helfrich, 1973; Israelachvili, 1991). The K and A values have been reported, at 15°C, for vesicles containing SOPC (1-stearyl-2-oleoyl-phosphatidylcholine) where: T_c = 3–8°C, (Marsh, 1990) and in the presence of cholesterol (Needham & Nunn, 1990). Values for K are unavailable for POPE/POPS bilayers. Therefore, we used the SOPC system to estimate the increase in the lateral elastic stress energy induced by cholesterol. For SOPC vesicles, without cholesterol, K = 193 dyne/cm, and $A = 65\text{Å}^2$. In the presence of cholesterol (14% by weight), the values are: K = 244 dyne/cm, and $A = 53\text{Å}^2$ (Needham & Nunn, 1990). There are no values available for the change in the lipid cross-sectional area and the number of lipid molecules affected when the BK channel opens. However, using an assumption made by Gibson and Brown (1993), we used a value of 10Å^2 change in the cross-sectional area (ΔA) per lipid when the channel opens. We also assume that there are about 60–100 lipid molecules per channel (Gibson & Brown, 1993). Using these values, the calculations show that cholesterol increases E_1 by about 30–50 kcal/mol.