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# Specificity of cholesterol and analogs to modulate BK channels points to direct sterol–channel protein interactions

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The activity (Po) of large-conductance voltage/Ca<sup>2+</sup>-gated K<sup>+</sup> (BK) channels is blunted by cholesterol levels within the range found in natural membranes. We probed BK channel-forming  $\alpha$  (cbv1) subunits in phospholipid bilayers with cholesterol and related monohydroxysterols and performed computational dynamics to pinpoint the structural requirements for monohydroxysterols to reduce BK Po and obtain insights into cholesterol's mechanism of action. Cholesterol, cholestanol, and coprostanol reduced Po by shortening mean open and lengthening mean closed times, whereas epicholesterol, epicholestanol, epicoprostanol, and cholesterol trisnorcholelenic acid were ineffective. Thus, channel inhibition by monohydroxysterols requires the  $\beta$  configuration of the C3 hydroxyl and is favored by the hydrophobic nature of the side chain, while having lax requirements on the sterol A/B ring fusion. Destabilization of BK channel open state(s) has been previously interpreted as reflecting increased bilayer lateral stress by cholesterol. Lateral stress is controlled by the sterol molecular area and lipid monolayer lateral tension, the latter being related to the sterol ability to adopt a planar conformation in lipid media. However, we found that the differential efficacies of monohydroxysterols to reduce Po (cholesterol  $\geq$  coprostanol  $\geq$  cholestanol  $\gg \gg$  epicholesterol) did not follow molecular area rank (coprostanol  $\gg$  epicholesterol  $\geq$  cholesterol  $\geq$  cholestanol). In addition, computationally predicted energies for cholesterol (effective BK inhibitor) and epicholesterol (ineffective) to adopt a planar conformation were similar. Finally, cholesterol and coprostanol reduced Po, yet these sterols have opposite effects on tight lipid packing and, likely, on lateral stress. Collectively, these findings suggest that an increase in bilayer lateral stress is unlikely to underlie the differential ability of cholesterol and related sterols to inhibit BK channels. Remarkably, *ent*-cholesterol (cholesterol mirror image) failed to reduce Po, indicating that cholesterol efficacy requires sterol stereospecific recognition by a protein surface. The BK channel phenotype resembled that of  $\alpha$  homotetramers. Thus, we hypothesize that a cholesterol-recognizing protein surface resides at the BK  $\alpha$  subunit itself.

## INTRODUCTION

Cholesterol is an essential component of cell membranes in eukaryotes and plays a critical role in regulating the activity of membrane-spanning proteins, including ion channels (Fielding and Fielding, 2003; Epand, 2006; Levitan et al., 2010). Membrane cholesterol is absolutely required for ligand-mediated opening of nicotinic acetylcholine receptors (nAChRs) (Fong and McNamee, 1986; Barrantes, 2004), and an excess in membrane cholesterol increases calcium uptake, supposedly by activating calcium channels in the cell membrane (Bialecki and Tulenko, 1989). On the other hand, increased membrane cholesterol blunts the activity of volume-regulated anion channels (VRACs) and inwardly rectifying K<sup>+</sup> (Kir2) channels (Levitan et al., 2000; Epshtein et al., 2009).

Cholesterol modulation of ion channel function has been attributed to two types of interactions between the

sterol and its biological target(s). The first involves the direct recognition of cholesterol by specific protein regions that are provided by the ion channel itself and/or closely associated proteins. Indeed, cholesterol-sensing protein regions have been identified in nAChRs (Addona et al., 2003) and Kir2 channels (Epshtein et al., 2009). The second type of interaction occurs between cholesterol and membrane lipids and leads to changes in the physical properties of bulk bilayer lipids. These changes are sensed by the channel protein, eventually resulting in modification of channel protein function. For example, cholesterol modulation of VRAC is considered to occur in response to cholesterol-induced increase in the deformation energy of the membrane that is associated with channel opening (Levitan et al., 2000; Romanenko et al., 2004).

Large-conductance Ca<sup>2+</sup>- and voltage-gated K<sup>+</sup> (BK) channels are ubiquitously expressed (Salkoff et al., 2006).

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Abbreviations used in this paper: BK, large-conductance Ca<sup>2+</sup>- and voltage-gated K<sup>+</sup>; nAChR, nicotinic acetylcholine receptor; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine sodium salt; SAR, structure–activity relationship; VRAC, volume-regulated anion channel.

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Inhibition of BK channel function by cholesterol has been reported both in native cell membranes (Bolotina et al., 1989; Lin et al., 2006) and after channel protein reconstitution into artificial lipid bilayers (Crowley et al., 2003; Bukiya et al., 2008b). However, inhibition of BK currents in response to cholesterol exposure is not universal (King et al., 2006). Lack of cholesterol-induced inhibition of BK current may reflect the contribution of a variety of factors, including changes in channel expression and/or cell membrane sorting to the overall cholesterol effect on macroscopic current, as well as inappropriate access of cholesterol from a bath-perfusing solution to the vicinity of the BK channel. The vast majority of studies, however, concur that an elevation in membrane/lipid bilayer cholesterol content leads to a significant reduction in BK channel steady-state activity (Po) and, thus, current (Bolotina et al., 1989; Chang et al., 1995; Crowley et al., 2003; Lam et al., 2004; Lin et al., 2006). Cholesterol-induced reduction in BK current may have drastic consequences for neuronal excitability, neurotransmitter release, smooth muscle tone regulation, and other processes that are heavily dependent on BK channel activity.

In most tissues, BK channels are heterooligomers that result from the association of channel-forming  $\alpha$  (encoded by *Slo1* or *KCNMA1*) subunits with tissue-specific accessory  $\beta$  subunits (encoded by *KCNMB1-4*) (Orio et al., 2002; Lu et al., 2006). Many physiologically relevant steroids, including estrogens, androgens, glucocorticoids, mineralocorticosteroids, and bile acids increase BK channel activity. Notably, this effect depends on the presence of, or at least is heavily regulated by, distinct accessory BK  $\beta$  subunits (Valverde et al., 1999; Dick and Sanders, 2001; King et al., 2006; Bukiya et al., 2007). Moreover, a structure–activity relationship (SAR) study unveiled the specific structural requirements in the bile acid molecule for steroid activation of  $\beta 1$  subunit-containing BK channels, and an actual docking site in the BK  $\beta 1$  subunit for bile acids has been proposed (Bukiya et al., 2008a). In contrast, cholesterol inhibition of BK channels does not depend on the presence of BK accessory  $\beta 1$  subunits (Bukiya et al., 2008b), and the structural determinants in the cholesterol molecule that lead to BK channel inhibition remain unknown. Furthermore, it has been consistently interpreted that cholesterol reduction of BK channel activity is primarily determined by changes in the physical properties of the bulk lipid bilayer upon cholesterol insertion in the bilayer (Bolotina et al., 1989; Chang et al., 1995; Crowley et al., 2003; Morris and Juranka, 2007).

Here, we tested the ability of cholesterol and related monohydroxysterols to inhibit BK channels and performed computational dynamics to pinpoint the structural requirements for monohydroxysterols to reduce BK Po. This approach also led us to obtain critical insight into the type of interaction (cholesterol–ion

channel protein vs. cholesterol–bulk lipid bilayer) primarily determining cholesterol regulation of BK channel activity. Thus, we reconstituted BK  $\alpha$  subunits (cbv1; AY330293) (Jaggar et al., 2005; Liu, J., P. Liu, J. Crowley, M. Asuncion-Chin, and A. Dopico. 2005. Cloning and Functional Characterization of BKCa Channel-forming (rslo cbv) Subunit. Society for Neuroscience. Abstr. 960.13.) into a two-species (i.e., binary) phospholipid bilayer: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE)/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine sodium salt (POPS) 3:1 (wt/wt), dissolved in decane as described elsewhere (Bukiya et al., 2008b). This simple system minimized any possible contribution of cytosolic signaling, cholesterol cell metabolism, complex membrane cytoarchitecture, and heterogeneous lipid domains to steroid action, thereby drastically reducing the number of possible elements involved in cholesterol recognition. On the other hand, the binary bilayer chosen reproduces faithfully the cholesterol regulation of native BK channel function observed in native cell membranes (Chang et al., 1995; Bukiya et al., 2008b).

The cholesterol molecule contains three clearly distinct regions: (1) a rather rigid, steroid tetra-ring system with a double bond between C5 and C6; (2) a small polar hydroxyl group at C3, which provides the molecule with a bit of amphiphilic character; and (3) a hydrophobic iso-octyl lateral chain attached to C17 (Fig. 1 A and Table I). This side chain is a significant contributor to the overall hydrophobicity of the molecule and plays a crucial role in cholesterol insertion and partitioning into both natural membranes and artificial phospholipid bilayers, with the cholesterol hydrophobic regions (steroid ring system and lateral chain) reaching the hydrophobic core of the bilayer (Worcester and Franks, 1976; Bittman, 1997). Our data demonstrate that BK channel inhibition by cholesterol and related analogues strictly depends on the  $\beta$  configuration of the sterol C3 hydroxyl and is facilitated by the hydrophobic nature of the side chain, while having rather lax structural requirements on the A/B ring fusion within the hydrophobic steroid ring system. These requirements are consistent with a cholesterol target site within a hydrophobic environment.

Cholesterol and analogues have been widely used to discriminate between direct sterol–ion channel protein interaction versus perturbation of bulk bilayer lipid properties in cholesterol modification of ion channel function (Gimpl et al., 1997; Addona et al., 2003; Romanenko et al., 2002, 2004). Moreover, predictions from SAR studies have often been verified by structural data (Byfield et al., 2006; Epshtein et al., 2009). Our SAR data from a wide variety of cholesterol-related monohydroxysterols do not support an exclusive or primary role of perturbation of bulk bilayer lipid in cholesterol inhibition of BK channels. In addition, our study

documents that cholesterol inhibition of BK channels strictly depends on the optical isomery of the sterol: in contrast to natural cholesterol, its enantiomer (*ent*-cholesterol) is totally ineffective in blunting BK channel activity. Because cholesterol and *ent*-cholesterol affect membrane physical properties similarly, their differential effects on protein activity are widely recognized as an indicator of a direct cholesterol–protein interaction mediating cholesterol action (Crowder et al., 2001; Alakoskela et al., 2008). Thus, our study strongly suggests that cholesterol inhibition of BK channels is mediated primarily by selective recognition of this steroid by a protein surface. Given the simple composition of our channel reconstitution system and the  $\alpha$ -subunit homomeric phenotype of the BK current under study, we hypothesize that the cholesterol–protein recognition interface is provided by the BK channel–forming  $\alpha$  subunit itself.

## MATERIALS AND METHODS

### Bilayer experiments

BK channel–forming (cbv1) subunit cDNA was cloned from rat cerebral artery myocytes (AY330293; Jaggar et al., 2005; Liu, J., P. Liu, J. Crowley, M. Asuncion-Chin, and A. Dopico. 2005. Cloning and Functional Characterization of BKCa Channel-forming (rslo cbv) Subunit. Society for Neuroscience. Abstr. 960.13.). HEK293 cells transiently transfected with cbv1 using Lipofectamin 2000 (Invitrogen) were grown to confluence, pelleted, and resuspended on ice in 10 ml of buffer solution (in mM): 30 KCl, 2 MgCl<sub>2</sub>, 10 HEPES, and 5 EGTA, pH 7.2. A membrane preparation was obtained using a sucrose gradient, as described previously (Crowley et al., 2003), and aliquots were stored at  $-80^{\circ}\text{C}$ .

Cbv1 channels were incorporated by adding 10–15  $\mu\text{l}$  of membrane preparation onto bilayers cast of POPE and POPS, 3:1 (wt/wt). Cholesterol or any of its monohydroxysterol analogues (Fig. 1 A and Table I) were dissolved in chloroform and then introduced into the lipid mixture at a final concentration of 20% (wt/wt), which corresponds to  $\approx 33$  mol% monohydroxysterol. The lipid mixture was dried under N<sub>2</sub> gas and resuspended at 25 mg per ml of decane.

Vertical 80–120-pF bilayers were formed by painting the lipid mix across a 200- $\mu\text{m}$  diameter hole in a deldrin cup (Warner Instruments). Vesicle fusion was promoted by osmosis, with the cis chamber (to which the membrane preparation was added) being hyperosmotic to the trans chamber solution. Recording solutions consisted of (in mM): 300 KCl, 10 HEPES, 1.47 HEDTA, and 1.05 CaCl<sub>2</sub> (free Ca<sup>2+</sup>  $\approx 10$   $\mu\text{M}$ ), pH 7.2 (cis chamber); 30 KCl, 10 HEPES, and 0.1 HEDTA, pH 7.2 (trans chamber). Nominal free Ca<sup>2+</sup> in solution was calculated using the MaxChelator Sliders program (C. Patton, Stanford University, Stanford, CA) and validated experimentally, as described elsewhere (Dopico, 2003). The trans chamber was held at ground while the cis chamber was held at potentials relative to ground. Only channels with their intracellular Ca<sup>2+</sup> sensors oriented toward the cis chamber were considered for experimentation.

Ion currents were obtained during 3 min of continuous recording at 0 mV by using an amplifier (BC-525D; Warner Instruments), low-pass filtered at 1 kHz with the four-pole Bessel filter built in the amplifier, and sampled at 5 kHz with Digidata 1322A/pCLAMP 8 (MDS Analytical Technologies). However, for dwell-time analysis, gap-free recordings were acquired for up to 30 min, low-pass filtered at 10 kHz, and sampled at 50 kHz.

For a proper comparison with data previously obtained by us (Crowley et al., 2003, 2005; Bukiya et al., 2008b; Vaithianathan et al., 2009) and others (Bolotina et al., 1989; Chang et al., 1995; Yuan et al., 2007), all studies were conducted at room temperature (20–25°C).

### Chemicals

Cholesterol, epicholesterol, coprostanol, epicoprostanol, cholestanol, epicholestanol, and cholesterol trisnorcholenic acid were purchased from Steraloids. IUPAC nomenclature for these steroids is presented in Table I. POPE and POPS were purchased from Avanti Polar Lipids, Inc. *Ent*-cholesterol was synthesized as described elsewhere (Belani and Rychnovsky, 2008). All other chemicals were purchased from Sigma-Aldrich.

### Computational modeling

Three-dimensional structures of cholesterol and epicholesterol were modeled using MOE software (Chemical Computing Group). Models were optimized with the MMFF94 force field (Halgren, 1996) to a root mean square gradient of 0.1 kcal  $\cdot$  mol<sup>-1</sup>  $\cdot$   $\text{\AA}^{-1}$ . The list of stable conformations and their corresponding energies was obtained using the stochastic conformational search routine in MOE, with the dielectric constant set at 3. This value is characteristic of the lipid bilayer hydrophobic core (Shibata et al., 2003).

### Data analysis

As an index of channel steady-state activity, we used the product of the channel open probability (Po) and the maximum number of functional channels present in the bilayer (N). NPo was obtained from all-points amplitude histograms (Dopico et al., 1996; Liu et al., 2008). N was determined by exposing the intracellular side of the BK channel to a solution containing 100  $\mu\text{M}$  of free Ca<sup>2+</sup> at the end of the recording period. This treatment raised Po to  $\approx 0.9$ , namely, close to its theoretical maximum (Fig. S1 A).

Dwell-time histograms were constructed from idealized records by using the half-threshold criterion for event detection. Original records were obtained from 30 min of gap-free data acquisition in bilayers where N = 1. Single-channel currents were low-pass filtered at 10 kHz and sampled at 50 kHz. Open- and closed-time distributions were plotted as log-linear histograms with 13 bins/decade. A Levenberg-Marquardt search algorithm with the sum of squared error minimization routine built in Clampfit 9.2 (MDS Analytical Technologies) was used to fit log variant of exponential probability function to distributions of open and closed intervals. An *F* table ( $P < 0.05$ ) was used to determine the minimum number of exponential components to appropriately fit dwell-time histogram data. The number of components in the exponential fit to the open- (closed-) time distribution provided a minimum estimate of the number of open (closed) states in which the channel population sojourned (Colquhoun and Hawkes, 1983).

Data plotting and statistical analysis were conducted using Origin 7.0 (OriginLab) and InStat 3.0 (GraphPad Software, Inc.). Data were analyzed with one-way ANOVA followed by Dunnett's test, and statistical significance was set at  $P < 0.05$ . Data are expressed as mean  $\pm$  SEM; *n* = number of bilayers.

### Online supplemental material

Fig. S1 shows basic functional characteristics of cbv1 channels reconstituted into control versus cholesterol-containing bilayers. Fig. S2 documents the lack of significant variations in BK Po from two control bilayers within 30 min of continuous recording. Fig. S3 depicts direct comparisons of channel inhibition evoked by respective monohydroxysterol isomers. Fig. S4 displays dwell-time distributions of cbv1 channels incorporated into control and epicoprostanol-containing bilayers. Table S1 shows dwell-time constants

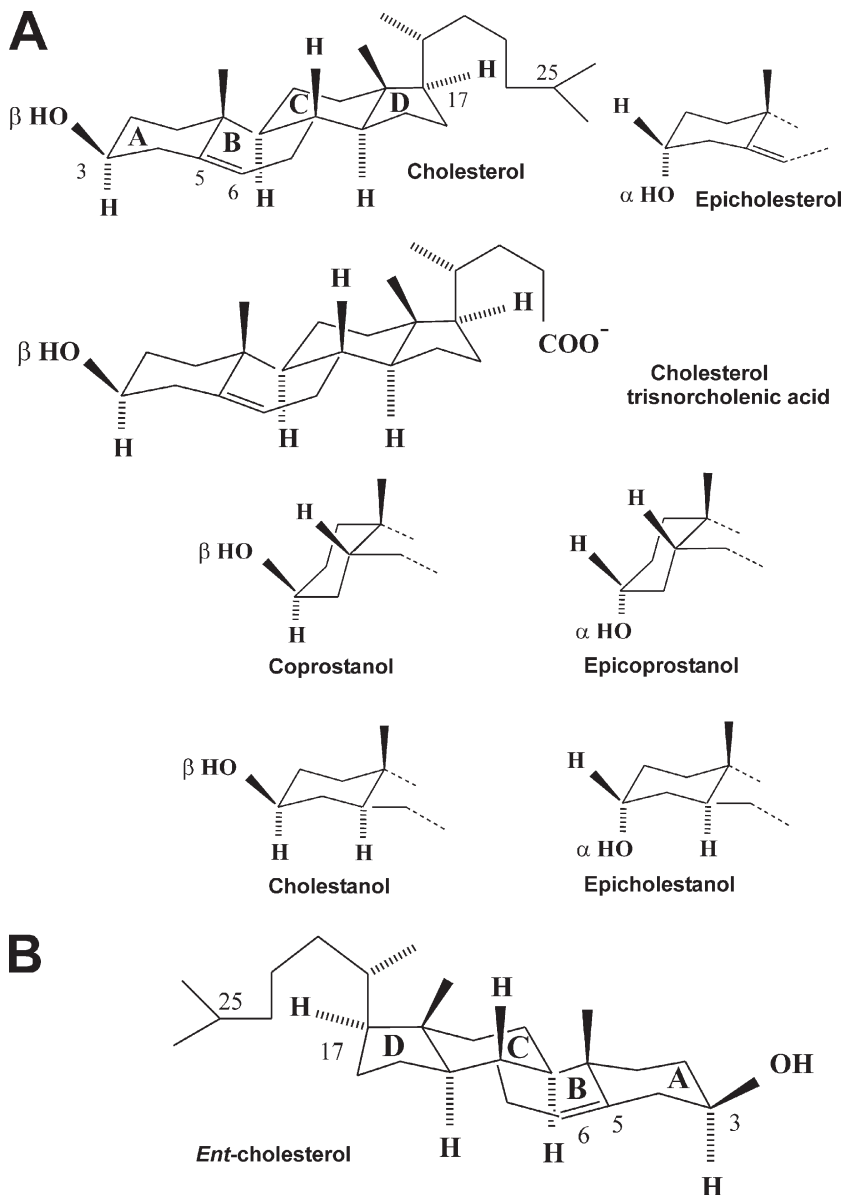
(mean  $\pm$  SEM) from control and inhibitory sterol-containing bilayers. The online supplemental material is available at <http://www.jgp.org/cgi/content/full/jgp.201010519/DC1>.

## RESULTS

We first set out to determine whether a hydrophobic side chain attached to the steroid ring at C17 (Fig. 1 A and Table I) is necessary for cholesterol to inhibit BK channels. Thus, we reconstituted cholesterol-sensitive (Bukiya et al., 2008b) BK channel-forming (*cbv1*) subunits into binary phospholipid bilayers containing cholesterol trisnorcholelenic acid and compared channel activity to those in cholesterol-containing and control (sterol-free) bilayers. Cholesterol trisnorcholelenic acid contains a carboxyl group at the end of its side chain (C24), which significantly diminishes the hydrophobic-

ity of the lateral chain (Fig. 1 A and Table I). As positive control, BK channel-forming *cbv1* subunits were incorporated into POPE/POPS (3:1 wt/wt, dissolved in decane) bilayers containing 20% (wt/wt) cholesterol (molar fraction = 0.33). This molar fraction is within the cholesterol content range found in the plasma membranes of most tissues (30–50 mol%; Gennis, 1989) and also corresponds to the  $IC_{90}$  for cholesterol inhibition of *cbv1* (Bukiya et al., 2008b) and human brain BK channel-forming (*hsl01*) subunits (Crowley et al., 2003) reconstituted into the same bilayer type.

In both the presence and absence of cholesterol, *cbv1* channel incorporation into the POPE/POPS bilayer rendered unitary current events that displayed all basic features of BK channel openings: (a) a large unitary conductance (see ion current records in Figs. 2 A, S1 A, and S2, and unitary current–voltage plots in Fig. S1 C);



**Figure 1.** Molecular structures of cholesterol and analogues. (A) Structures of cholesterol and cholesterol trisnorcholelenic acid are shown in full. Only the steroid nucleus around the A/B fusion is shown for the remaining compounds to emphasize differences in the planar orientation of the C3 hydroxyls and geometry of the A/B ring fusion. The free carboxyl group (pKa = 5.5) at the end of the side chain in cholesterol trisnorcholelenic acid is shown in its ionized form, which likely predominates under our experimental conditions (pH 7.2). (B) The structure of cholesterol enantiomer (*ent*-cholesterol) is shown in full to emphasize that this molecule is a mirror image of cholesterol. For all compounds, trivial names are provided. For IUPAC nomenclature, please refer to Table I.

(b) at constant transbilayer voltage, an increase in Po as Ca<sup>2+</sup> was increased (Fig. S1 A); and (c) at constant free Ca<sup>2+</sup> in the cis chamber solution, an increase in Po when the voltage at the cis side of the bilayer (i.e., intracellular side of the cbv1 channel) was made more positive (Fig. S1 B). In addition, the low levels of channel activity (Po) observed at negative voltages and 10 μM Ca<sup>2+</sup> in the cis solution (Fig. S1 B) were characteristic of channels made by homomeric cbv1 subunits (Bukiya et al., 2008b).

Cbv1 Po in the presence of 33 mol% cholesterol was significantly lower (≈28%) than that in the control sterol-free bilayer (Fig. 2, A and B). This result is consistent with previous findings from recombinant hslol (Crowley et al., 2005) and native rat brain BK channels reconstituted into phospholipid bilayers (Chang et al., 1995), as well as native BK channels in aortic smooth muscle membranes (Bolotina et al., 1989). Thus, our data buttress the idea that BK channel-forming α subunits and a bare lipid environment (e.g., a binary phospholipid bilayer) are sufficient to support cholesterol-induced reduction of BK channel activity.

Cholesterol-induced reduction of cbv1 steady-state activity was not accompanied by any noticeable change in unitary current amplitude when recorded in 300/30 mM [K<sup>+</sup>]<sub>i</sub>/[K<sup>+</sup>]<sub>o</sub> within a wide voltage range (−80 to 70 mV). Indeed, in cholesterol-containing bilayers, cbv1 conductances from unitary current–voltage plots averaged 330 ± 26 pS (*n* = 7), a value indistinguishable from that in the sterol-free bilayer (340 ± 22 pS; *n* = 5). This result is in agreement with studies of cholesterol actions on recombinant hslol reconstituted into bilayers (Crowley et al., 2003) and BK channels in rabbit aorta (Bolotina et al., 1989), while differing from those on rat brain BK channels reconstituted into bilayers, in which a 7% decrease in unitary current amplitude in the pres-

ence of 11% (wt/wt) cholesterol has been reported (Chang et al., 1995). As discussed previously (Crowley et al., 2003), differences in the cis/trans K<sup>+</sup> gradient used likely contribute to explain the discrepancies on cholesterol modulation of BK channel unitary conductance between the different studies.

In contrast to cholesterol, the incorporation of 33 mol% cholesterol trisnorcholenic acid into the phospholipid–decane mix consistently (six out of six bilayers) resulted in bilayers where cbv1 Po was identical to that observed in sterol-free bilayers (Fig. 2, C–E). These data indicate that the combination of a small polar hydroxyl group at C3 and a rigid steroid tetra ring system with a double bond between C5 and C6 is not sufficient to support a cholesterol-like inhibition of BK channels. Rather, it appears that a hydrophobic (e.g., iso-octyl) side chain attached to the steroid nucleus is critical for this cholesterol action. The iso-octyl side chain plays a crucial role in cholesterol insertion and partitioning into phospholipid bilayers, with the steroid ring system and side chain reaching the bilayer hydrophobic core (Worcester and Franks, 1976; Bittman, 1997). Because the carboxyl pK<sub>a</sub> ≈ 5.5, the side chain in cholesterol trisnorcholenic acid likely remains significantly ionized in our media (pH 7.2), making it difficult for this sterol to partition into the hydrophobic bilayer region as cholesterol does (Ohvo-Rekilä et al., 2002). Conceivably, the failure of cholesterol trisnorcholenic acid to inhibit BK channels reflects the inability of this acidic sterol to properly insert and partition in the bilayer as cholesterol does (see Discussion).

Cholesterol insertion into phospholipid bilayers at a sterol molar fraction similar to that used here (≥30 mol%) favors tight lipid packing (also referred to as “cholesterol condensation effect”) (Demel et al., 1972;

TABLE I  
Distinctive chemical features of cholesterol and analogs

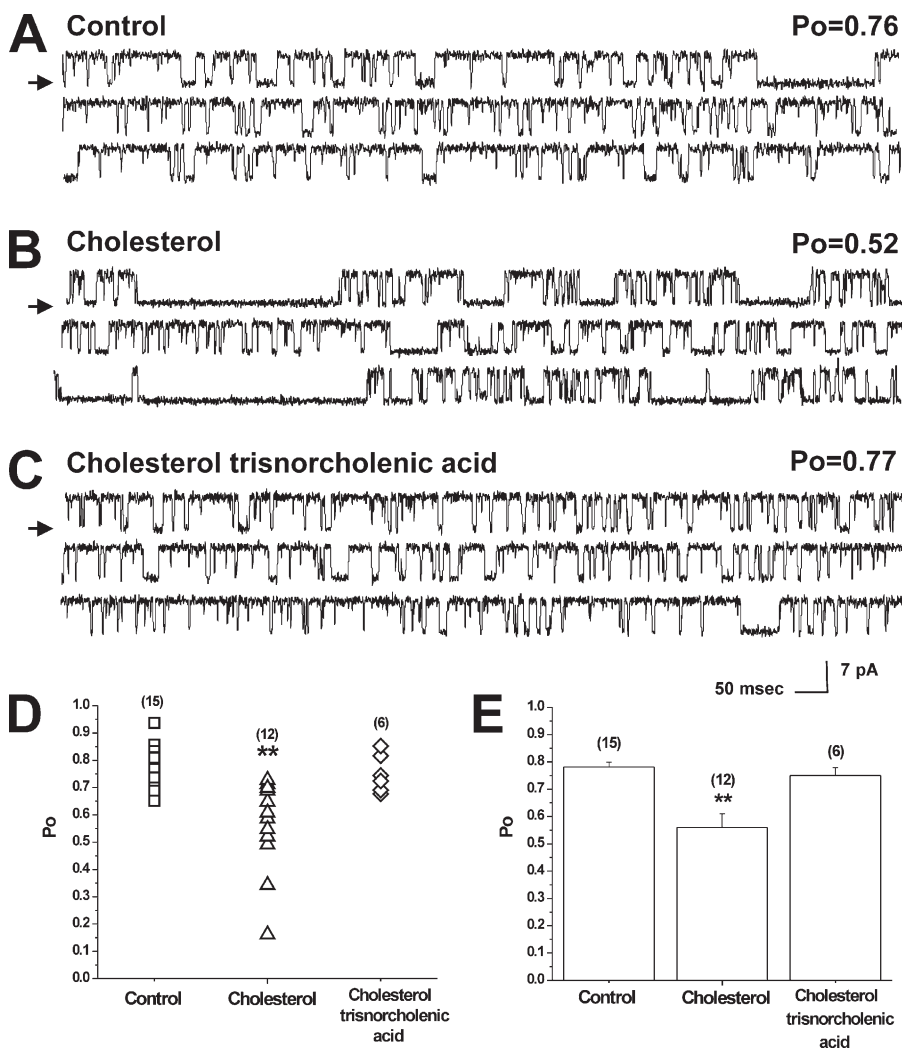
Trivial name	IUPAC nomenclature	3-OH orientation	A/B junction
Cholesterol	(3 <i>S</i> , 10 <i>R</i> , 17 <i>R</i> )-10,13-dimethyl-17-(( <i>R</i> )-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1 <i>H</i> -cyclopenta[ <i>a</i> ]phenanthren-3-ol	β	<i>n/a</i> <sup>a</sup>
Epicholesterol	(3 <i>R</i> , 10 <i>R</i> , 13 <i>R</i> , 17 <i>R</i> )-10, 13-dimethyl-17-(( <i>R</i> )-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1 <i>H</i> -cyclopenta[ <i>a</i> ]phenanthren-3-ol	α	<i>n/a</i> <sup>a</sup>
Coprostanol	(3 <i>S</i> , 5 <i>R</i> , 9 <i>S</i> , 10 <i>S</i> , 13 <i>R</i> , 14 <i>S</i> , 17 <i>R</i> )-10,13-dimethyl-17-(( <i>R</i> )-6-methylheptan-2-yl)hexadecahydro-1 <i>H</i> -cyclopenta[ <i>a</i> ]phenanthren-3-ol	β	<i>cis</i>
Epicoprostanol	(3 <i>R</i> , 5 <i>R</i> , 9 <i>S</i> , 10 <i>S</i> , 13 <i>R</i> , 14 <i>S</i> , 17 <i>R</i> )-10,13-dimethyl-17-(( <i>R</i> )-6-methylheptan-2-yl)hexadecahydro-1 <i>H</i> -cyclopenta[ <i>a</i> ]phenanthren-3-ol	α	<i>cis</i>
Cholestanol	(3 <i>S</i> , 5 <i>S</i> , 8 <i>R</i> , 9 <i>S</i> , 10 <i>S</i> , 13 <i>R</i> , 14 <i>S</i> , 17 <i>R</i> )-10,13-dimethyl-17-(( <i>R</i> )-6-methylheptan-2-yl)hexadecahydro-1 <i>H</i> -cyclopenta[ <i>a</i> ]phenanthren-3-ol	β	<i>trans</i>
Epicholestanol	(3 <i>R</i> , 5 <i>S</i> , 8 <i>R</i> , 9 <i>S</i> , 10 <i>S</i> , 13 <i>R</i> , 14 <i>S</i> , 17 <i>R</i> )-10,13-dimethyl-17-(( <i>R</i> )-6-methylheptan-2-yl)hexadecahydro-1 <i>H</i> -cyclopenta[ <i>a</i> ]phenanthren-3-ol	α	<i>trans</i>
<i>Ent</i> -cholesterol	(3 <i>R</i> ,8 <i>R</i> ,9 <i>R</i> ,10 <i>S</i> ,13 <i>S</i> ,14 <i>R</i> ,17 <i>S</i> )-10,13-dimethyl-17-(( <i>S</i> )-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1 <i>H</i> -cyclopenta[ <i>a</i> ]phenanthren-3-ol	β	<i>n/a</i> <sup>a</sup>

<sup>a</sup>Because of the presence of the double bond at C5–C6 (Fig.1), the A/B junction presents neither *cis* nor *trans* geometry.

Xu and London, 2000; McConnell and Radhakrishnan, 2003). Cholesterol and coprostanol share an identical hydrophobic tail and polar group. However, coprostanol's A/B ring junction is in cis configuration (Fig. 1 A and Table I). Notably, this structural difference leads to coprostanol having an inhibitory effect on tight lipid packing ("anticonsolidation effect") (Xu and London, 2000). Remarkably, the presence of 33 mol% coprostanol in our bilayer system resulted in cbv1 Po values that were significantly lower ( $\approx 25\%$  reduction) than those in the sterol-free bilayer (Fig. 3). This result demonstrates that at equimolar lipid fraction, coprostanol is approximately as effective as cholesterol in inhibiting BK channels. Because coprostanol and cholesterol have opposite effects on lipid condensation, lipid condensation secondary to sterol insertion in the bilayer is unlikely to underlie the ability of these steroids to inhibit BK channels.

The fact that both coprostanol and cholesterol inhibit BK channel activity also appears to indicate that the particular configuration of the junction between steroid rings A and B and/or the degree of saturation between C5 and C6 are not essential for cholesterol-like

sterols to inhibit BK channels. To further test the structural requirements around rings A and B for cholesterol analogues to inhibit BK channels, we next investigated cbv1 function in bilayers containing cholesterol. This monohydroxysterol contains a C17 side chain and C3 hydroxyl group identical to those of cholesterol and coprostanol but has a trans A/B fusion. Indeed, cholesterol and cholesterol both lack the cis configuration of the A/B ring fusion, a typical feature of coprostanol. However, cholesterol shares with coprostanol the full hydrogen saturation of the steroid ring system (Fig. 1 A and Table I). As found with cholesterol and coprostanol, cholesterol's presence in the POPE/POPS bilayer usually resulted in cbv1 Po values significantly smaller than those in sterol-free bilayers (Fig. 3, A, C, and D). However, in spite of having a ring structure different from those of cholesterol and coprostanol, the average Po values in the presence of cholesterol were not significantly different from those of coprostanol (Fig. 3, E and F). These data indicate that the A/B ring fusion geometry and the C5–C6 degree of saturation are not critical for monohydroxysterols to inhibit BK channels. Thus, any possible



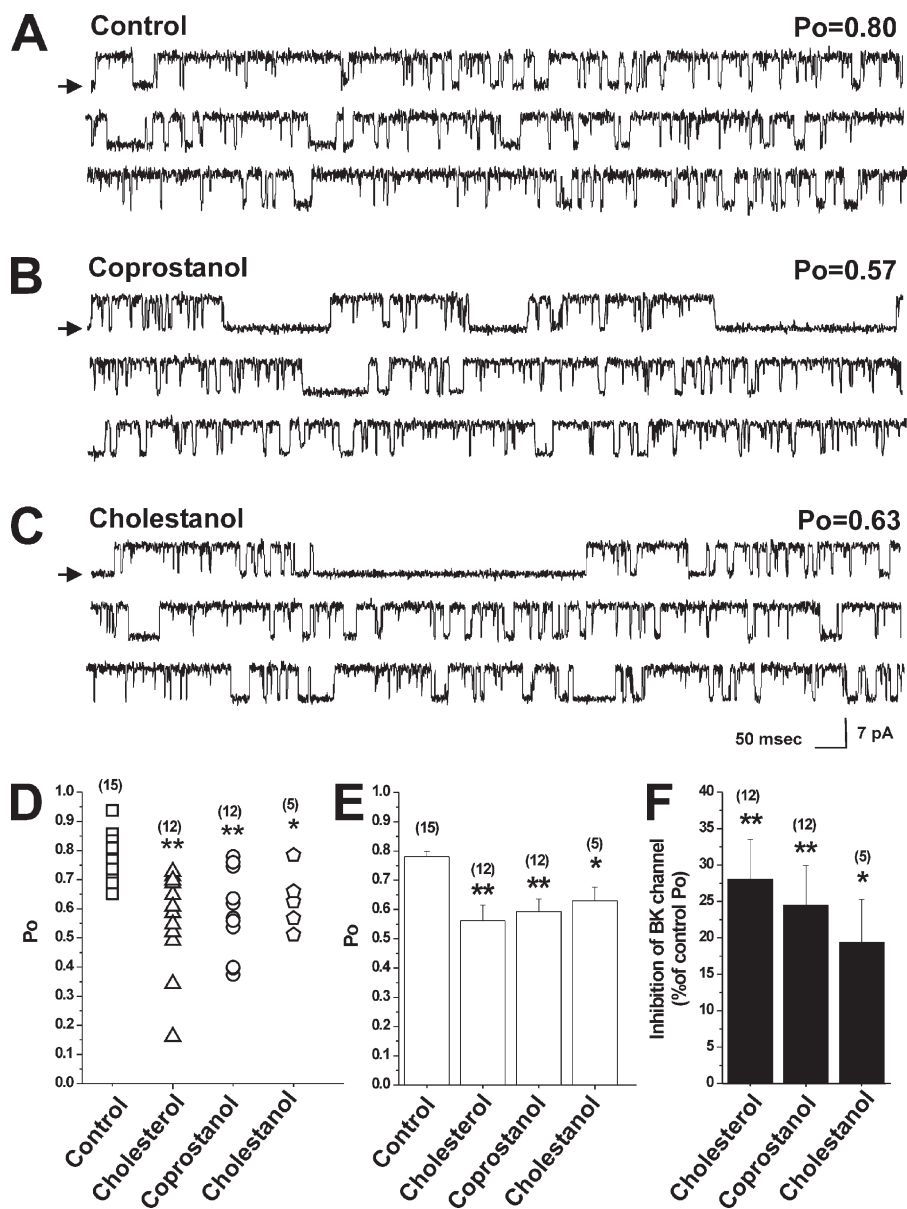
**Figure 2.** Substitution of cholesterol hydrophobic side chain with a chain including a highly polar (carboxyl) group abolishes sterol inhibition of BK channels. Representative single-channel recordings obtained after incorporation of cbv1 into control (A), cholesterol-containing (B), or cholesterol trisnorcholeonic acid-containing (C) POPE/POPS (3:1 wt/wt) bilayers. Steroid levels corresponded to 33 mol% in the lipid mix. For A–C, channel openings are shown as upward deflections; arrows on the top trace of each panel indicate the baseline level. Po values were obtained from all-point amplitude histograms (Dopico et al., 1996; Liu et al., 2008) constructed from 3 min of continuous recording in each bilayer type. The membrane potential was set to 0 mV and free  $[Ca^{2+}]_i \approx 10 \mu M$ . (D) Scatter graph showing individual cbv1 Po values in control (sterol-free) and in the presence of cholesterol or cholesterol trisnorcholeonic acid in the lipid mix. Each data point corresponds to one independent bilayer preparation. (E) Average Po values show that cholesterol but not cholesterol trisnorcholeonic acid significantly reduced activity from controls. \*\*, significantly different from control ( $P < 0.01$ ); n, number of bilayers. In this and all studies shown in Figs. 3, 4, 6–8, S1, S3, and S4, control refers to sterol-free POPE/POPS (3:1 wt/wt). All lipid mixtures, whether sterol-containing or not, were dissolved in decane as described in Materials and methods.

sterol site of action that leads to BK channel inhibition has lax structural requirements that accommodate variant A/B ring junction structures.

Cholesterol, coprostanol, and cholestanol all have a single C3 hydroxyl group in  $\beta$  configuration. In contrast, epicholesterol contains its single C3 hydroxyl in  $\alpha$  configuration. Furthermore, this is the only structural difference between epicholesterol and cholesterol (Fig. 1 A and Table I). Thus, we next evaluated cbv1 channel activity in bilayers containing 33 mol% epicholesterol to determine whether the  $\beta$  configuration of the C3 hydroxyl was required for cholesterol to inhibit BK channels. Remarkably, epicholesterol in the phospholipid mix was totally ineffective at reducing  $P_o$  from values found in sterol-free bilayers (Fig. 4, A, B, and E–G). This result indicates that the site(s) involved in cholesterol inhibition of BK channels is able to specifically recog-

nize the  $\beta$  configuration of the single polar group in the cholesterol molecule.

Moreover, we set out to determine whether the differential efficacy on BK channel activity driven by the specific configuration of the C3 hydroxyl was limited to cholesterol–epicholesterol or, rather, could be extended to all active monohydroxysterols. Thus, we next studied cbv1 channels reconstituted into bilayers including either epicoprostanol or epicholestanol, as these monohydroxysterols contain their single C3 hydroxyl in  $\alpha$  configuration (Fig. 1 A and Table I). As found with epicholesterol-containing bilayers, epicoprostanol or epicholestanol presence in the phospholipid bilayer consistently evoked cbv1  $P_o$  values similar to those found in sterol-free control bilayers (Figs. 4, A and C–G, and S3). Collectively, the fact that cholesterol, coprostanol, or cholestanol in the bilayer reduced cbv1  $P_o$ , while their

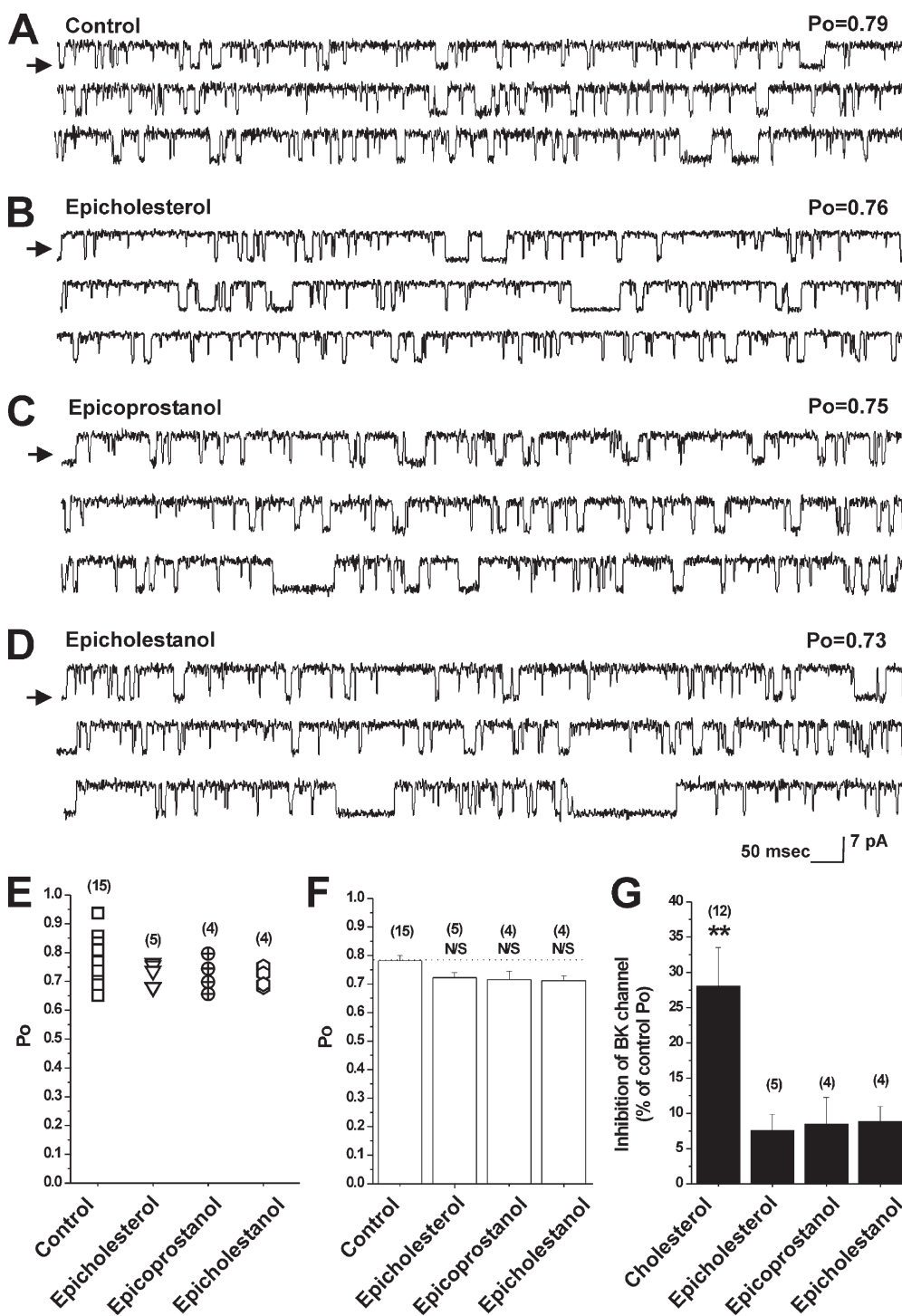


**Figure 3.** Variant degree of saturation and geometry of the A/B ring fusion enables monohydroxysterol inhibition of BK channels. Representative single-channel recordings obtained after cbv1 incorporation into control (A), coprostanol- (B), or cholestanol-containing (C) POPE/POPS (3:1 wt/wt) bilayers. Sterols were introduced at 33 mol% in the lipid mix. For A–C, channel openings are shown as upward deflections; arrows on the top trace of each panel indicate the baseline. Channel activity ( $P_o$ ) was calculated as described in the legend to Fig. 2. (D) Scatter graph showing individual  $P_o$  values in control and in the presence of cholesterol, coprostanol, or cholestanol in the lipid mixture. Each data point corresponds to one independent bilayer preparation. (E) Averaged  $P_o$  data show that cholesterol, coprostanol, and cholestanol are all able to significantly reduce BK channel activity from control values. (F) Averaged degree of sterol-induced modification of cbv1 channel  $P_o$  given as a percentage of inhibition from control bilayers. For D–F: \*, significantly different from control ( $P < 0.05$ ); \*\*, significantly different from control ( $P < 0.01$ ).



$\alpha$  epimers all failed to do so, indicates that the C3 hydroxyl in cholesterol and analogues must be specifically constrained to the  $\beta$  configuration for these monohydroxysteroids to inhibit BK channels. This strict stereochemical requirement favors the idea that specific chemical interactions between the steroid C3  $\beta$  hydroxyl and a protein recognition site(s) may exist for cholesterol and related monohydroxysteroids to inhibit BK channels (see Discussion).

Cholesterol inhibition of BK channels, however, has been previously hypothesized to result mainly from the increase in bilayer lateral stress caused by steroid insertion into the bilayer (Chang et al., 1995). In addition, lipid disordering in protein-free media by monohydroxy anesthetic steroids is affected by the  $\alpha$  versus  $\beta$  configuration of the single hydroxyl (Lawrence and Gill, 1975). Thus, the differential efficacy of  $\beta$ - versus  $\alpha$ -hydroxyl-containing monohydroxysteroids to reduce BK channel



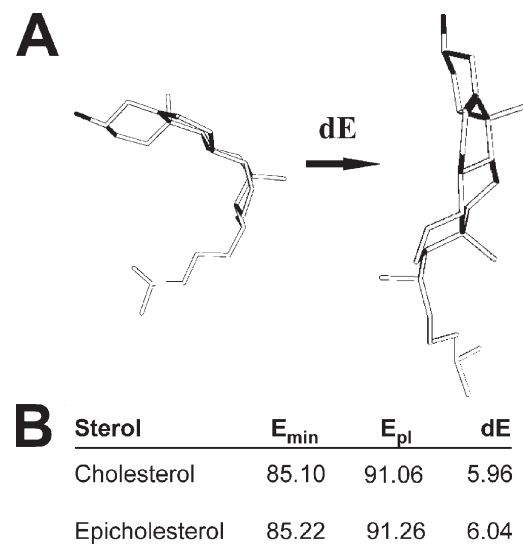
**Figure 4.** The  $\beta$  configuration of the sterol C3 hydroxyl is crucial for cholesterol and related monohydroxysteroids to inhibit BK channels. Representative single-channel recordings obtained after cbv1 incorporation into control (A), epicholesterol- (B), epicoprostanol- (C), or epicholestanol-containing (D) POPE/POPS (3:1 wt/wt) bilayers. Sterols were introduced at 33 mol% in the lipid mixture. For A–D, channel openings are shown as upward deflections; arrows on the top trace of each panel indicate the baseline.  $P_o$  was calculated as described in the legend to Fig. 2. (E) Scatter graph showing individual  $P_o$  values in control and in the presence of epicholesterol, epicoprostanol, or epicholestanol in the lipid mixture. Each data point corresponds to one independent bilayer preparation. (F) Averaged  $P_o$  values show that epicholesterol, epicoprostanol, and epicholestanol failed to reduce BK channel activity from control values. (G) Averaged degree of sterol-induced modification of cbv1 channel displayed as a percentage of inhibition from control bilayers. Data from cholesterol-containing bilayers are shown as positive control. \*\*, significantly different from controls ( $P < 0.01$ ).

activity could also be attributed to differences in the bilayer lateral stress that is generated by sterol incorporation into the phospholipid bilayers: the different spatial characteristic of the C3 hydroxyl imposed by the  $\beta$  versus  $\alpha$  configuration modify sterol overall molecular volume (area per molecule) (Demel et al., 1972), a likely contributor to the bilayer lateral stress that results from sterol insertion. To analyze a possible contribution of lateral stress to cholesterol inhibition of BK channels, we considered that the lateral stress exerted on transmembrane ion channel proteins is a direct function of two factors: the sterol condensation effect, in turn determined by the steroid molecular area (Demel et al., 1972), and the lipid monolayer lateral tension (Lundbaek and Andersen, 1994; Cantor, 1999; Bezrukov, 2000). However, the differential efficacies of monohydroxysterols to inhibit cbv1 channels (cholesterol  $\gg$  coprostanol  $\gg$  cholesterol  $\gg$  epicholesterol; Figs. 3 and 4) did not follow the molecular area rank (coprostanol  $\gg$  epicholesterol  $\gg$  cholesterol  $\gg$  cholestanol) (Demel et al., 1972). In particular, because of their different A/B ring fusion configuration (Fig. 1 A), coprostanol and cholesterol have a significantly different molecular area: 43.8 versus 39 Å, at 12 dynes/cm, 22°C (Demel et al., 1972). However, these steroids did not differ significantly in their ability to cause channel inhibition (Fig. 3). Therefore, steroid molecular area per se or its possible contribution to bilayer stress is unlikely to underlie the differential degree of BK channel inhibition caused by cholesterol and related monohydroxysterols.

Cholesterol-induced increase in lipid monolayer lateral tension is a function of the sterol facility to insert normally to the bilayer plane. In turn, this insertion depends on the ability of cholesterol to adopt a planar conformation in a lipid environment (Kessel et al., 2001). Thus, we next determined whether sterol efficacy to inhibit BK channels was a function of the molecule's ability to adopt a planar conformation. We performed a computational stochastic search for energetically favorable conformations of active (cholesterol) and inactive (epicholesterol) monohydroxysterols. From the list of computationally predicted stable conformations and their corresponding energies, we chose the lowest energy and the energy nearest to the planar conformation and computed their difference (dE) for each compound. As shown in Fig. 5, both the energy nearest the planar conformation and dE are practically identical for cholesterol and epicholesterol, indicating that both active and inactive isomers are similarly posed to adopt a planar conformation. This result strongly suggests that possible changes in lipid monolayer tension by these monohydroxysterols cannot be different enough to explain their drastic differential efficacies on BK Po.

Finally, we used dwell-time distribution analysis to obtain mean dwell times in the absence and presence of the three monohydroxysterols that inhibit cbv1 channels

(cholesterol, coprostanol, and cholestanol) and, thus, determined the relative contribution of mean open time shortening to the overall reduction in Po evoked by these sterols. Under fixed voltage ( $V_m = 0$  mV) and activating calcium concentration ( $[Ca^{2+}]_{free} = 10$   $\mu$ M), the open- and closed-time distributions from channels in control and sterol-containing bilayers could be well fitted with three and four exponentials, respectively, indicating the existence of at least three open and four closed states (Fig. 6). Representative dwell-time distributions from cbv1 channels in cholesterol-containing versus control bilayers (Fig. 6, first vs. second row of panels) show that cholesterol presence drastically modifies both open- and closed-time distributions, as reported with other BK channels (Chang et al., 1995; Crowley et al., 2003). In particular, cholesterol presence resulted in the shortening of long open-time constants:  $\tau_3 = 15.2$  versus 7.7 ms in control versus cholesterol-containing bilayers. On the other hand, cholesterol increased the contribution of long closed events to the total time spent in closed states, indicated by a significant redistribution of closed times from events with a time constant that averaged less than 1 ms ( $\tau_1$ ) to those that averaged  $\sim 7$ –8 ms ( $\tau_3$ ) and  $\sim 50$  ms ( $\tau_4$ ). These changes in dwell-time distributions resulted in cholesterol-induced major modifications in both mean open and mean closed times (Fig. 7, A and B). Indeed, the

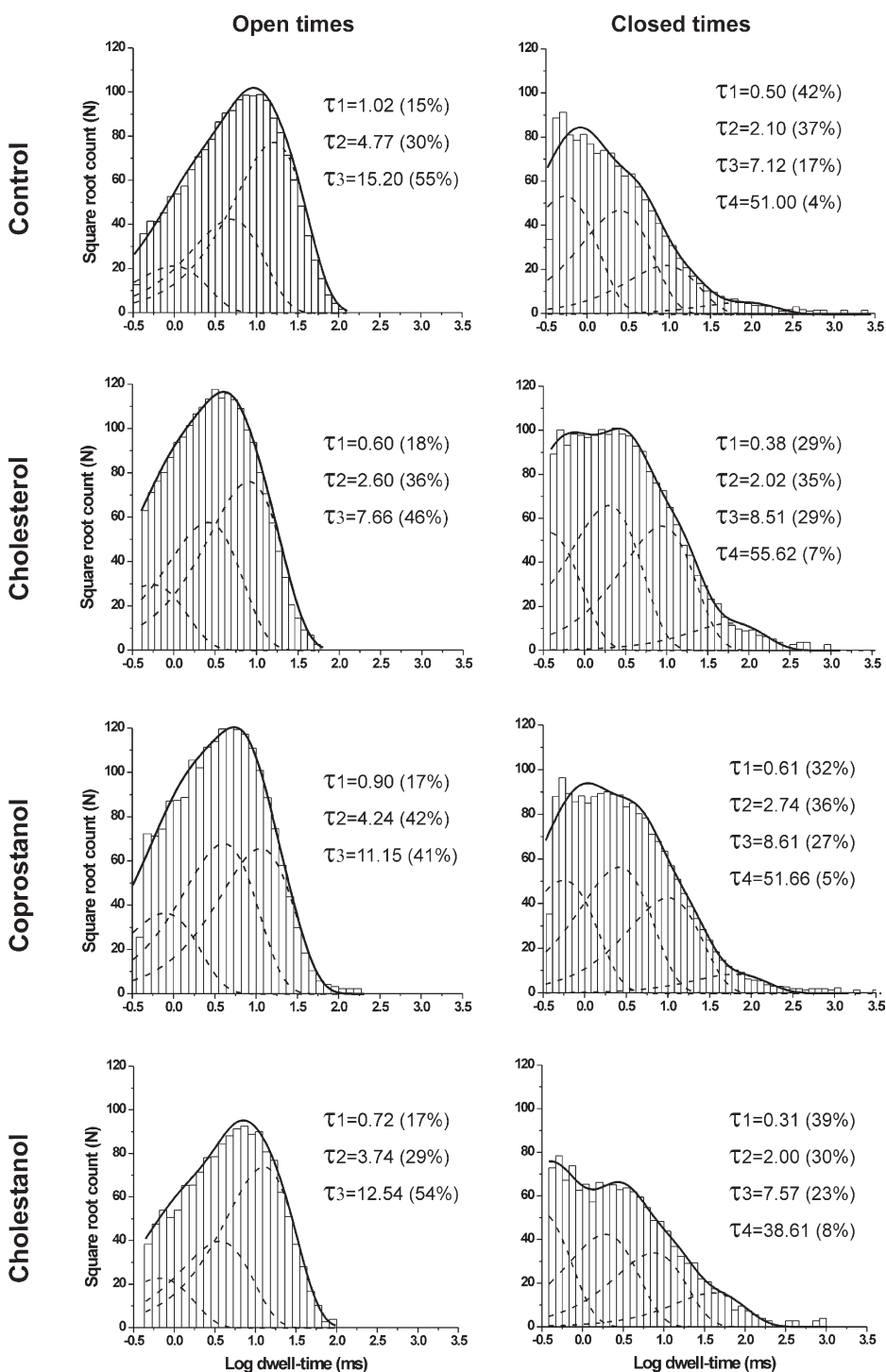


**Figure 5.** Computed energies for an inhibitory sterol (cholesterol) and its inactive diastereomer (epicholesterol) to adopt a planar conformation and, thus, induce membrane deformation during channel opening are similar. (A) Computational model of the cholesterol molecule in its lowest energy (left) and its stable planar conformation (right). The energy associated with the switch from the molecule's lowest energy to its planar conformation (dE) is expected to lead to increased membrane deformation energy barrier during channel opening. (B) Table displaying cholesterol and epicholesterol lowest ( $E_{min}$ ) and planar conformation ( $E_{pl}$ ) energies, and dE for each monohydroxysterol. Energies are given in kilocalorie/mole.

average mean open time decreased from  $8.4 \pm 0.71$  to  $5.46 \pm 0.63$  ms ( $P < 0.01$ ), and the average mean closed time increased from  $3.37 \pm 0.44$  to  $6.01 \pm 0.52$  ms ( $P < 0.01$ ; control [ $n = 6$ ] vs. cholesterol-containing bilayers [ $n = 7$ ]).

Dwell-time analysis of cbv1 channels in coprostanol- and cholestanol-containing bilayers (Fig. 6, third and fourth row of panels) shows that these BK inhibitors also modify both open- and closed-time distributions

(Table S1). Moreover, these modifications resulted in both the shortening of mean open times (Fig. 7 A) and the lengthening of mean closed times (Fig. 7 C), as found with cholesterol. Furthermore, the efficacies of cbv1 channel inhibition by cholesterol, coprostanol, and cholestanol highly correlated with changes in both mean open (Fig. 7 B) and mean closed times (Fig. 7 D). It is noteworthy that cholesterol and cholestanol on one

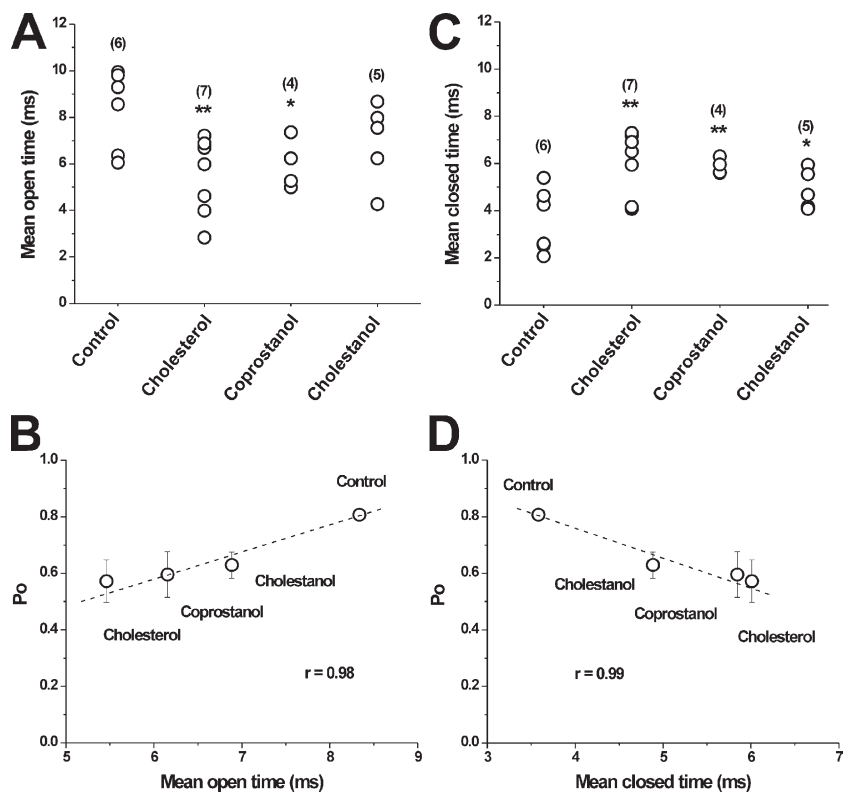


**Figure 6.** Both open- and closed-time distributions are modified by cholesterol and other monohydroxysterols that decrease BK channel activity. Open- (left) and closed- (right panels) time distributions of cbv1 channels incorporated into control (first row of panels), cholesterol- (second row), coprostanol- (third row), or cholestanol-containing (last row) bilayers. Po values are 0.74, 0.34, 0.54, and 0.63 for control, cholesterol-, coprostanol-, and cholestanol-containing bilayers, respectively. Dwell-time distributions were obtained from 30 min of continuous recording of single-channel activity in each bilayer type. Data were fitted to exponential functions, and the minimum number of exponentials used to properly fit the data was determined as described in Materials and methods. Dotted lines indicate the individual component of the fit, with solid lines providing the composite exponential fit. Each panel contains the averaged duration of each time constant (in milliseconds). The relative contribution of each exponential component (defined by  $\tau$ ) to the total time spent in open or closed states is given in parentheses as a percentage of the total fitted area. The summation of products of each  $\tau$  multiplied by its fractional contribution to the total fit was used to obtain the mean open and mean closed times shown in Fig. 7.

hand, and coprostanol on the other, exert opposite effects on tight lipid packing (Gimpl et al., 1997; Xu and London, 2000) and, likely, lateral stress. Thus, even if a lateral stress mechanism could contribute to sterol-induced reduction of BK Po (Chang et al., 1995), additional mechanisms are involved.

Finally, we compared the dwell-time distributions of coprostanol, which decreases BK Po (Fig. 3), and its diastereomer (epicoprostanol), which is ineffective (Fig. 4). It has been claimed that the major determinant of BK mean open-time reduction in response to increased lateral stress is a significant decrease in the contribution of long open events to the total open-time distribution (Chang et al., 1995). We found that epicoprostanol was able to reduce the proportion of long openings (Fig. S4). However, this reduction did not lead to blunted channel activity, suggesting that redistribution of open events from long to shorter openings is not sufficient to ensure Po reduction by cholesterol and related monohydroxysterols. Remarkably, epicoprostanol failed to lengthen closed times (Fig. S4), an alteration that is common to all active compounds (Figs. 6 and 7). Collectively, lack of correlation between molecular area rank and ability to inhibit BK channel, similarity of cholesterol and epicholesterol energies to adopt a planar conformation, and dwell-time analysis and mean lifetime data make it highly unlikely that an increase in the bilayer lateral stress force exerted on the channel by sterol insertion underlies the reduction of BK channel Po evoked by the presence of cholesterol or related monohydroxysterols in the bilayer.

As an alternative to differential modification of physical properties of the bilayer, the differential effectiveness of cholesterol and its related  $\beta$  monohydroxysterols versus epicholesterol and its related  $\alpha$  monohydroxysterols can be attributed to the existence of a protein site(s) embedded in the lipid bilayer that strictly recognizes the  $\beta$  configuration of the C3 hydroxyl while having lax structural requirements on the geometry of the A/B steroid ring junction. Cholesterol enantiomer (*ent*-cholesterol) is a mirror image of natural cholesterol (Fig. 1 B), with both compounds sharing major physicochemical properties. Because the absolute configuration of cholesterol has minor effects, if any, in protein-free phospholipid media (Alakoskela et al., 2008), differential effects of cholesterol versus *ent*-cholesterol have been widely used as a criterion to establish that cholesterol modification of membrane protein function is the result of selective recognition of this sterol by a protein site(s) (Crowder et al., 2001; Westover and Covey, 2004). Thus, we compared cbv1 channel activity in binary phospholipid bilayers containing either cholesterol or *ent*-cholesterol to determine whether a protein site contributes to specific recognition of cholesterol and eventual reduction of BK channel activity. Remarkably, *ent*-cholesterol presence in the binary phospholipid bilayer repeatedly failed to reduce cbv1 Po from values found in sterol-free bilayers, with cholesterol being consistently effective under identical conditions (Fig. 8). Mean closed times in the presence of cholesterol were longer than those in *ent*-cholesterol (6 vs. 4.4 ms), suggesting that



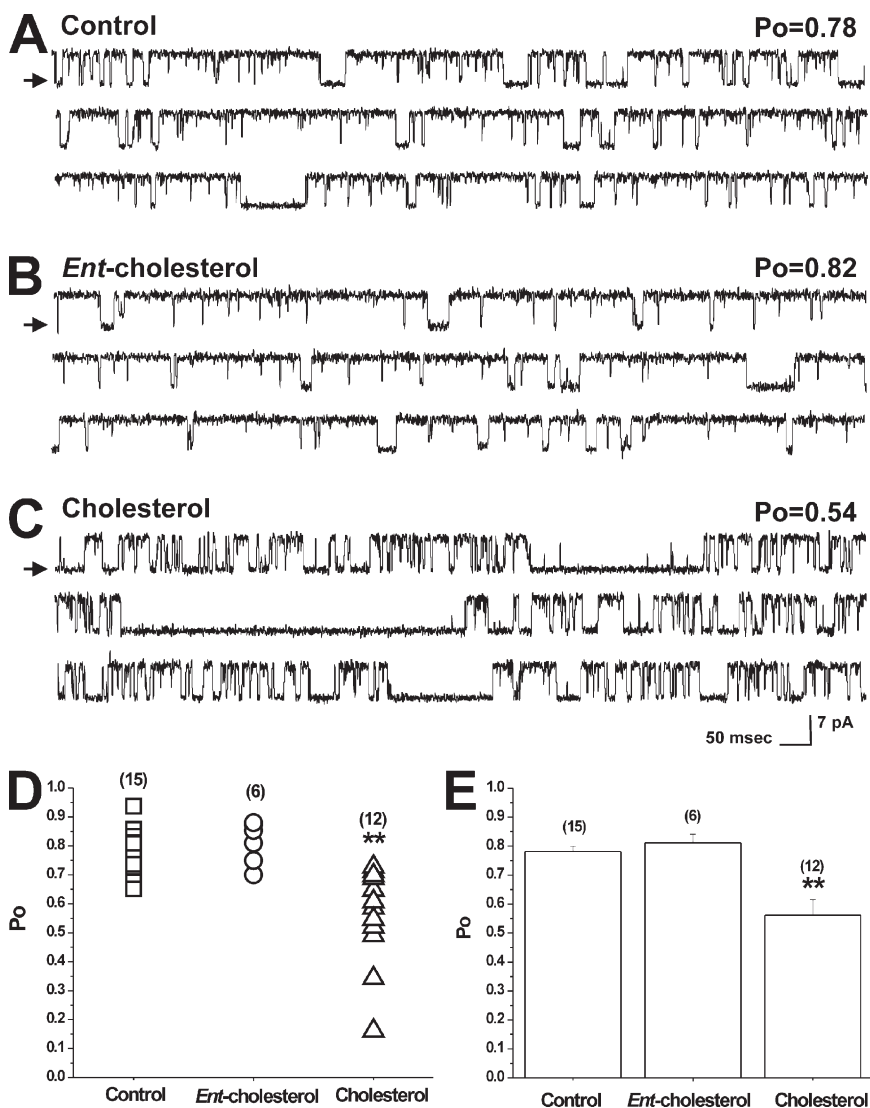
**Figure 7.** Cholesterol and active analogues decrease BK Po both by shortening mean open times and lengthening mean closed times. Scatter graphs showing individual mean open (A) and mean closed (C) times of cbv1 channels reconstituted into control or monohydroxysterol-containing bilayers. For A and C: \*, significantly different from control ( $P < 0.05$ ); \*\*, significantly different from control ( $P < 0.01$ ). Averaged mean open and mean closed times under different conditions are given in B and D. These data show that cholesterol-, coprostanol-, and cholestanol-induced decrease in cbv1 Po is determined by the ability of these steroids to shorten mean open times and lengthen mean closed times. Linear regression was conducted with Origin 7 Software (OriginLab).  $r$ , regression coefficient.

the lengthening of mean closed times and the eventual decrease in  $P_o$  are determined mainly by differential sterol recognition by a protein surface. Mean open times, however, were also different in cholesterol- (5.5 ms) versus *ent*-cholesterol-containing bilayers (8.2 ms), the latter being practically identical to those of controls (8.4 ms). This distinction between cholesterol and its mirror image buttresses the idea that the shortening of mean open times by monohydroxysterols results, at least partially, from enantiospecific interactions between the sterol and a protein site(s). The actual location of such a site remains speculative, yet it must be found at the *cbv1* subunit itself or at another protein that remains tightly associated to *cbv1* after channel reconstitution into the lipid bilayer (see Discussion).

## DISCUSSION

We used cholesterol and a series of structurally related monohydroxysterols, including the enantiomer and a

diastereomer of cholesterol (*ent*-cholesterol and *epi*-cholesterol, respectively), to determine the specific structural features of the sterol molecule that lead to sterol inhibition of BK channels. Data demonstrated that cholesterol at a bilayer molar fraction within the range found in natural membranes reduced the steady-state activity ( $P_o$ ) of recombinant BK channel-forming *cbv1* protein obtained from rat cerebral artery myocytes. This cholesterol action requires a hydrophobic side chain attached to the sterol C17 and the  $\beta$  configuration of the sterol C3 hydroxyl, that is, the single polar group in the cholesterol molecule. However, several chemical manipulations around the configuration of the A/B ring fusion and degree of saturation of the C5–C6 bond failed to drastically alter the efficacy of cholesterol-related monohydroxysterols to inhibit BK channels. Thus, the sterol recognition site(s) has a very strict conformational requirement for the sterol polar group in C3, yet rather lax structural requirements for the non-polar steroid ring structure. SAR studies of steroids and



**Figure 8.** Cholesterol enantiomer (*ent*-cholesterol) consistently fails to inhibit BK channels. Representative single-channel recordings obtained after *cbv1* incorporation into control (A), *ent*-cholesterol- (B), and cholesterol-containing (C) POPE/POPS (3:1 wt/wt) bilayers. *Ent*-cholesterol and cholesterol were introduced at 33 mol% into the lipid mixture. For A–C, channel openings are shown as upward deflections; arrows on the top trace of each panel indicate the baseline. Channel activity ( $P_o$ ) was calculated as described in the legend to Fig. 2. (D) Scatter graph showing individual  $P_o$  values in control and in the presence of *ent*-cholesterol or natural cholesterol in the lipid mix. Each data point corresponds to one independent bilayer preparation. (E) Averaged  $P_o$  values underscoring that *ent*-cholesterol fails to reduce *cbv1*  $P_o$  from control values while cholesterol remains effective under identical recording conditions. C and D: \*\*, significantly different from control ( $P < 0.01$ ).

ionotropic receptors have been widely used to determine the contribution of two major types of molecular interactions in steroid regulation of ion channel function: (1) steroid–bulk lipid interaction leading to changes in the physical properties of the bulk lipid bilayer, and (2) direct steroid–protein interaction (Harrison et al., 1987; Paradiso et al., 2000; Romanenko et al., 2002, 2004; Akk et al., 2007).

#### Change(s) in physical properties of the bulk lipid bilayer is not the main mechanism by which cholesterol and analogues inhibit BK channels

Inhibition of native BK channel activity in aortic myocytes in response to a cholesterol-enrichment medium has been found coincidental with a decrease in the rotational diffusion coefficient of diphenylhexatriene, the latter being interpreted as a decrease in the “fluidity” of the cell membrane (Bolotina et al., 1989). On the other hand, Chang et al. (1995) reconstituted BK channels into binary phospholipid bilayers and determined from Arrhenius plots the activation energy for channel transitions from the open to the closed state(s). Cholesterol 11% (wt/wt) significantly reduced such energy, and thus, cholesterol-induced decrease in  $P_o$  was interpreted to result, at least partially, from destabilization of the BK channel open state(s), likely reflected by a reduction in channel mean open time. The computed lateral elastic stress energy that resulted from cholesterol presence in the lipid mix was significantly higher than the BK activation energy from closed to open state(s), making it possible to postulate that cholesterol-induced increase in lateral stress favors the channel to be deflected back to the closed state. From these early studies, it became accepted that cholesterol reduction of BK channel activity is determined by changes in physical properties of the bulk membrane lipid that occur upon sterol insertion into the bilayer (Crowley et al., 2003; Morris and Juranka, 2007).

Indeed, a wide variety of physical properties of phospholipid bilayers may change upon cholesterol insertion into a phospholipid bilayer in the biologically relevant liquid-order phase, with resulting modification of channel protein conformation and function. At cholesterol molar fractions  $\leq 33$  mol%, such as those used in our study (33 mol%), and which can be found in native membranes (Gennis, 1989; Sackmann, 1995), modification of bilayer properties by cholesterol insertion results mainly from cholesterol–phospholipid interactions, as cholesterol–cholesterol interactions become more significant at higher sterol molar fractions (Demel and De Kruff, 1976; Loura and Prieto, 1997; Hayakawa et al., 1998; Preston Mason et al., 2003). Stronger phospholipid–cholesterol interactions will likely occur with higher sterol solubility in the phospholipid. For example, differential phospholipid solubility between sterols could explain, or at least contribute to, the differential

effectiveness of 33 mol% cholesterol versus epicholesterol in inhibiting BK channels (Fig. 4), as the  $\beta$  monohydroxysterol is more soluble in lecithin than its  $\alpha$  epimer (Demel et al., 1972). However, averaged  $cbv1$   $P_o$  values in cholesterol-containing bilayers are similar to those in cholestanol-containing bilayers (Fig. 3, E and F), yet cholestanol is more soluble in lecithin than cholesterol (Demel et al., 1972). Thus, different phospholipid solubility is unlikely the single mechanism leading to differential inhibition of BK channels by the set of sterols probed in our study. On the other hand, cholesterol and cholestanol share the same hydrophobic side chain (Fig. 1 A), so they likely have a similar capability to form tail-to-tail dimers, as demonstrated for cholesterol in GUV at molar fractions as low as 5 mol% (Loura and Prieto, 1997). Thus, vertical dimer formation is unlikely a primary mechanism involved in sterol inhibition of BK channels. Moreover, both active (cholesterol) and inactive (epicholesterol) inhibitors of  $cbv1$  channels are similarly able to promote sterol-enriched domain formation (Xu and London, 2000). In addition, cholesterol, cholestanol, and coprostanol are all effective in decreasing  $cbv1$   $P_o$  (Figs. 3 and S3), yet only cholesterol and cholestanol produce tight lipid packing/condensation (Demel et al., 1972); this lipid effect is a major determinant of sterol-containing vertical domain formation (Xu and London, 2000). Remarkably, although coprostanol actually inhibits domain formation (“anti-cholesterol effect”) (Xu and London, 2000), it effectively reduces  $cbv1$   $P_o$ . Therefore, it is extremely unlikely that the differential inhibition of  $cbv1$  channels by cholesterol and related monohydroxysterols is primarily determined by differences in phospholipid solubility, sterol–sterol dimer interaction, and/or sterol domain formation among the monohydroxysterol species.

Cholesterol insertion into phospholipid bilayers is also known to modify bilayer elastic properties, introducing negative monolayer curvature and increasing lateral stress in the hydrocarbon chain region (Bezruk, 2000; Lundbaek et al., 2004). These actions translate into decreased channel open times, as demonstrated for gramicidin A (Andersen et al., 2007) and proposed for BK channels themselves (Chang et al., 1995). It has been noted, however, that entropy changes secondary to BK channel open to closed transitions do not tightly follow predictions should lateral stress be the only contributor to cholesterol reduction of BK  $P_o$ . Thus, Chang et al. (1995) suggested that some lateral stress-independent mechanism could contribute to cholesterol inhibition of BK channels.

Several lines of evidence from our study do not support a primary role for lateral stress in cholesterol inhibition of  $cbv1$  channels. Changes in lateral stress secondary to sterol insertion into a phospholipid bilayer are determined by the monolayer curvature and the sterol ability to evoke lipid condensation, in turn dependent

on the sterol molecular volume and shape (Demel et al., 1972; Kessel et al., 2001). The sterols tested in our study represent class II lipids. Thus, they possess relatively large hydrophobic nucleus and tail regions compared with the small polar “head” represented by a single hydroxyl group at C3. Upon insertion into a lipid bilayer, type II lipids increase lateral pressure in the hydrocarbon chain area of the bilayer and, thus, introduce negative monolayer curvature (Bezrukov, 2000). However, some of the sterols tested were effective (those with the C3 hydroxyl in  $\beta$  configuration), whereas some were not (all their  $\alpha$  epimers). Second, it is widely recognized that cholesterol promotes phospholipid condensation in the physiologically relevant liquid crystalline phase, enhancing orientational order (Demel and De Kruyff, 1976; Yeagle, 1985). A major role for increased phospholipid order as mediator of monohydroxysterol inhibition of BK channels is unlikely because (a) coprostanol, an effective *cbv1* channel inhibitor (Fig. 3) has minor effects on bilayer lipid order (Gimpl et al., 1997; Xu and London, 2000); and (b) cholesterol and epicholesterol, although having similar effects on bulk bilayer order as measured by fluorescence polarization anisotropy (Gimpl et al., 1997; Xu and London, 2000), were, respectively, effective and ineffective on *cbv1* activity (Fig. S3). Our data showing (a) similar changes in both mean open and mean closed times and, thus,  $P_o$  by sterols that exert opposite actions on tight lipid packing (e.g., cholesterol vs. coprostanol); (b) similar energies to adopt planar conformations by active and inactive sterols; and (c) lack of correlation between the rank order of the efficacies of monohydroxysterols to inhibit *cbv1* channels and sterol molecular area rank all argue against an exclusive or primary role for lateral stress in mediating decreased BK channel activity by cholesterol and related sterols.

Finally, cholesterol insertion is widely known to alter the thickness of artificial phospholipid bilayers (Ohki, 1969) and natural membranes (Tulenko et al., 1998). Notably, bilayer thickness has been reported to regulate the mean open times of *hslol* BK channel-forming proteins (Yuan et al., 2007). However, it is unlikely that monohydroxysterol inhibition of BK channels is underlain by steroid-induced modification of bilayer thickness: P–P distances in DMPC bilayers that contain epicholesterol are intermediate between those of cholesterol-containing and sterol-free DMPC bilayers (Róg and Pasenkiewicz-Gierula, 2003), yet epicholesterol was totally ineffective in reducing BK channel activity (Figs. 4 and S3).

In summary, modification of bilayer lipid properties is unlikely to underlie cholesterol reduction of BK activity. Rather, this cholesterol action involves sterol-specific recognition by a protein site(s) (see next section). Protein- and lipid-mediated mechanisms could independently contribute to cholesterol-induced reduction of

BK  $P_o$ . Notably, the oxytocin receptor and its structurally related brain cholecystokinin receptor provide precedents where receptor activity is regulated both by changes in membrane fluidity upon steroid incorporation into the membrane and via highly specific steroid–protein receptor interaction (Gimpl et al., 1997). Although we cannot exclude that some part of the action of cholesterol and related sterols on BK channel activity involves modification of bilayer properties, our results neither require nor support such a mechanism.

#### Selective steroid–protein interactions underlie cholesterol inhibition of BK channels

Two major findings from our study point to the involvement of a specific sterol recognition protein site(s) in cholesterol inhibition of BK channel activity: first, the  $\beta$  configuration of the C3 hydroxyl is necessary for channel inhibition, as cholesterol is effective and its C3  $\alpha$  epimer (epicholesterol) is not (Fig. S3). Stereospecificity of cholesterol action on ion channels has been usually interpreted as evidence that a protein surface contributes to the cholesterol-sensing element (Sooksawat and Simmonds, 2001; Romanenko et al., 2004). Second, and more importantly, cholesterol action on *cbv1* channels shows enantiospecificity: *ent*-cholesterol, namely, cholesterol mirror image, was absolutely and consistently ineffective at reducing *cbv1* channel activity (Fig. 8). It is widely accepted that differential cholesterol versus *ent*-cholesterol actions on protein function reflect the existence of a cholesterol-sensing protein site in a proteolipid system like a cell membrane (Crowder et al., 2001; Westover and Covey, 2004).

The chemical requirements of the cholesterol-recognizing protein site involved in BK channel inhibition are lax enough to accommodate variant A/B ring fusions (at the C5–C6 joint), as well as various A/B ring fusion geometries. Lax structural requirements for cholesterol actions on ion channels do not preclude the existence of a distinct protein domain(s) that recognizes steroids; the nAChR provides a well-documented precedent of an ion channel protein that possesses very lax structural requirements for steroid modulation: cholesterol polar conjugates and epicholesterol substitute for cholesterol in supporting agonist-mediated nAChR opening (Addona et al., 2003). Moreover, after identifying two steroid sites in the nAChR TM region by FRET and binding techniques, Barrantes and coworkers demonstrated that both sites are readily accessible to ligands that are structurally unrelated to steroids, such as fatty acids (Antollini and Barrantes, 1998; Barrantes, 2003; Fernández Nievas et al., 2007).

Steroid ion channel SAR data have been used to hypothesize the existence of direct steroid–channel protein interactions. Indeed, a SAR study first identified the structural constraints for steroids to modulate inwardly rectifying  $K^+$  (Kir2) channels, leading to the postulation of a selective cholesterol-sensing region in the Kir2

protein (Romanenko et al., 2002). Later, a combination of protein homology modeling with point mutagenesis identified such a region at the Kir2.1 channel C terminus (Epshtein et al., 2009). Likewise, differential modulation of  $\alpha 4\beta 2$  nAChR (Paradiso et al., 2000) or GABA-A chloride channels (Wittmer et al., 1996) by steroid enantiomers predicted the existence of specific steroid recognition protein sites in each of these ionotropic receptors; these predictions have been confirmed by mutagenesis or computational modeling (Barrantes, 2004; Hosie et al., 2007). In contrast, lack of structural specificity for cholesterol and analogues to modulate VRACs has been interpreted as steroids acting on channel function via changes in the physical properties of the bilayer that result from steroid insertion in the bilayer (Romanenko et al., 2004). This conclusion was confirmed by micropipette aspiration measurements of membrane stiffness, which showed that cholesterol modification of bilayer stiffness determined steroid action on VRACs (Byfield et al., 2006).

The actual location and structural elements of the cholesterol protein site involved in the inhibition of BK channels remain speculative. However, given the differential efficacies of cholesterol versus cholesterol trisnorcholeonic acid (Fig. 2), it is highly likely that such a protein site requires sterol access and/or location into a hydrophobic protein domain or a lipid-protein interface. Conceivably, the highly polar end of the side chain in cholesterol trisnorcholeonic acid makes it difficult for this monohydroxysterol to dive “tail-in” into the bilayer and locate as cholesterol does (Ohvo-Rekilä et al., 2002), which could explain the failure of the acidic steroid to blunt channel activity. Alternatively, trisnorcholeonic acid could dive “head-in” into the bilayer, with its ionized carboxylate located at the lipid-aqueous interface, as proposed for monohydroxy cholanic acids (Bukiya et al., 2008a). In this case, cholesterol trisnorcholeonic acid’s lack of efficacy could be related to (a) limited access to its protein site, as its side chain is shorter than that of cholesterol (Fig. 1 A) and/or the polar group(s) should reside for the most part in the aqueous phase or the phospholipid polar head group region; or (b) improper docking onto the protein site itself. Disregarding the specifics of sterol interactions with the lipid bilayer, the drastic differential modulation of BK channels by cholesterol and cholesterol trisnorcholeonic acid reported here suggests that cholesterol inhibition of BK activity requires sterol interactions via/within a hydrophobic environment.

Our cholesterol data with BK  $\alpha$  (cbv1) subunits in a binary phospholipid bilayer are similar to those observed in more complex systems (Bolotina et al., 1989; Chang et al., 1995) and, after bilayer reconstitution of hslol1 (Crowley et al., 2003), a protein that is >95% identical to cbv1. Conceivably, the cholesterol-recognizing protein surface is provided by a target that is common to all of these systems: the BK channel-forming slo subunit itself

or a closely associated protein. In most tissues, native channels consist of BK channel-forming and accessory ( $\beta$ ) subunits (Orio et al., 2002). In particular, vascular smooth muscle membranes contain auxiliary subunits of the  $\beta 1$  type that are tightly associated to the BK channel-forming tetramer (Orio et al., 2002). We cannot totally rule out the presence in our bilayer of contaminant protein from the membrane preparation, which could serve as, or contribute to, the cholesterol sensor. In our system, however, the single-channel phenotype (Fig. S1) consists of lower levels of  $P_o$  and shorter open times than those introduced by accessory  $\beta 1$  subunits (McManus et al., 1995; Bukiya et al., 2008b). Records (Fig. S1) are basically identical to those reported for BK channels made of recombinant  $\alpha$  subunit homotetramers (Crowley et al., 2003; Bukiya et al., 2007; Liu et al., 2008) and native BK channels thought to result from  $\alpha$  subunit homomers (Bukiya et al., 2009). Thus, it is highly likely that the protein surface that contributes to specifically recognize cholesterol leading to decreased  $P_o$  is provided by the BK  $\alpha$  subunit itself. Any putative contaminant protein that contributed to cholesterol sensing should be selective enough to bind cholesterol and eventually alter cbv1 channel gating without altering cbv1 gating in the absence of the sterol. A simpler and more plausible explanation is that cbv1 itself contains the site(s) for cholesterol sensing/binding that is responsible for cholesterol reduction of BK channel activity. Bilayer lipids, however, could still contribute to the final effect of the sterol on channel activity: cholesterol could insert at a cbv1-lipid interface, with both protein and bilayer lipid surfaces forming part of the cholesterol recognition site.

In conclusion, we have identified the structural determinants in the cholesterol molecule that lead to BK channel inhibition. The strict structural requirements for cholesterol action, including stereo isomery of the single polar group at C3 and, more importantly, optical isomery of the molecule, strongly suggest that a direct cholesterol-protein interaction is required for cholesterol inhibition of BK channels.

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