Sensitivity of Volume-regulated Anion Current to Cholesterol Structural Analogues

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ABSTRACT Depletion of membrane cholesterol and substitution of endogenous cholesterol with its structural analogues was used to analyze the mechanism by which cholesterol regulates volume-regulated anion current (VRAC) in endothelial cells. Depletion of membrane cholesterol enhanced the development of VRAC activated in a swelling-independent way by dialyzing the cells either with GTPγS or with low ionic strength solution. Using MβCD–sterol complexes, 50–80% of endogenous cholesterol was substituted with a specific analogue, as verified by gas-liquid chromatography. The effects of cholesterol depletion were reversed by the substitution of endogenous cholesterol with its chiral analogue, epicholesterol, or with a plant sterol, β-sitosterol, two analogues that mimic the effect of cholesterol on the physical properties of the membrane bilayer. Alternatively, when cholesterol was substituted with coprostanol that has only minimal effect on the membrane physical properties it resulted in VRAC enhancement, similar to cholesterol depletion. In summary, our data show that these channels do not discriminate between the two chiral analogues of cholesterol, as well as between the two cholesterols and β-sitosterol, but discriminate between cholesterol and coprostanol. These observations suggest that endothelial VRAC is regulated by the physical properties of the membrane.

KEY WORDS: Cl− channels • cholesterol analogues • physical properties of the membrane • cell swelling • vascular endothelial cells

INTRODUCTION

Changes in the level of cellular cholesterol are known to affect numerous membrane proteins, including several types of ion channels (e.g., Bolotina et al., 1989; Barrantes, 1993; Chang et al., 1995; Lundbaek et al., 1996; Romanenko et al., 2002b). Two general mechanisms have been proposed for the regulation of membrane proteins by cholesterol: (a) changing the physical properties of the membrane or (b) direct binding between cholesterol and the proteins (for reviews see Barrantes, 1993; Bastiaanse et al., 1997; Burger et al., 2000). The basis for the first mechanism is a well-known observation that an increase in membrane cholesterol restricts the motion of phospholipids, resulting in lipid ordering (e.g., Demel et al., 1972; Stockton and Smith, 1976), decrease in membrane fluidity (e.g., Brunet and McConnell, 1976; Cooper, 1978; Xu and London, 2000), and increase in membrane stiffness (Evans and Needham, 1987; Needham and Nunn, 1990). The latter was proposed to increase membrane deformation energy altering the energetic cost of the transitions between the conformational states of the ion channels (Lundbaek et al., 1996). This hypothesis is supported by the studies showing that in model membranes, increase in spontaneous monolayer curvature and thickness of the membrane bilayer, factors that are among the determinants of the membrane deformation energy (Huang, 1986; Nielsen et al., 1998), affect open probability of antibiotic ion channels, gramicidin (Lundbaek et al., 1997), and alamethicin (Keller et al., 1993), and of bacterial mechanosensitive MscL channels (Perozo et al., 2002). There is little evidence, however, for the regulation of mammalian anion channels by cholesterol-induced changes in the physical properties of the membrane bilayer in living cells.

We focus on endothelial volume-regulated Cl− current, a key process in cell volume homeostasis (for review see Nilius et al., 1996) that is also implicated in the regulation of cell cycle (Shen et al., 2000), cell proliferation (Manolopoulos et al., 1997; Voets et al., 1997), and angiogenesis (Manolopoulos et al., 2000). In addition, more recent studies show that VRAC is sensitive to mechanical fluid shear stress (Barakat et al., 1999; Romanenko et al., 2002a), suggesting that it may contrib-
ute to shear stress-induced regulation of the endothelium. Our earlier study has shown that VRAC is inversely dependent on the level of cellular cholesterol (Levitan et al., 2000). The first goal of this study is to determine whether the effect of cholesterol on VRAC may be accounted for by altering the mechanical constraint to cell swelling that is due to cytoskeleton. This possibility has to be considered because cholesterol depletion has been shown to release several cytoskeletal proteins, such as actin, α-actinin, and ezrin, from the cellular membrane fractions (Harder et al., 1997), suggesting that it may uncouple the membrane from the underlying cytoskeleton and, consequently, decrease cell stiffness and allow cells to swell more. In this study, however, we show that cholesterol depletion enhances VRAC in a swelling-independent way.

The second question addressed in this study is whether the sensitivity of VRAC to cholesterol is due to cholesterol-induced changes in the physical properties of the membrane bilayer or due to specific sterol–protein interactions defined as direct binding between cholesterol and the channel protein or a modulatory protein. Development of β-cyclodextrin–cholesterol donor/acceptor system that allows precise adjustment of the level of cellular cholesterol to different levels (Christian et al., 1997) and the substitution of cholesterol with its chiral isomer epicholesterol in intact vascular endothelial cells without apparent cell damage (Romanenko et al., 2002b). Here, we extend this approach to demonstrate a strong correlation between the effects of cholesterol structural analogues on VRAC and their known effects on the physical properties of the membrane lipid bilayer providing the first evidence that VRAC is regulated by membrane physical properties.

MATERIALS AND METHODS

Modulation of Cellular Cholesterol Level and Substitution of Cholesterol with Sterol Analogues

Bovine aortic endothelial cells (BAECs) between passages 10 and 30 were grown in DMEM (Cell Grow) supplemented with 10% FBS (GIBCO BRL). BAECs were enriched with or depleted of cholesterol by incubating them with methyl-β-cyclodextrin (MβCD) saturated with cholesterol or with empty MβCD (not complexed with cholesterol), as described previously (Levitan et al., 2000). Briefly, a small volume of cholesterol stock solution in chloroform:methanol (1:1, vol:vol) was added to a glass tube and the solvent was evaporated. Then, 2.5 or 5 mM MβCD solution in DMEM medium without serum was added to the dried cholesterol. The tube was vortexed, sonicated, and incubated overnight in a shaking bath at 37°C. MβCD was saturated with cholesterol at an MβCD:cholesterol molar ratio of 8:1, the saturation limit of MβCD (Christian et al., 1997). In preparation for an experiment, cells were washed three times with serum-free DMEM to remove the serum from the growth medium. Cells were then incubated with MβCD saturated solution or with MβCD solution containing no cholesterol (empty MβCD) for 60 or 120 min. During the incubation, cells were maintained in a humidified CO₂ incubator at 37°C. Control cells were treated similarly and incubated with serum-free DMEM solution without any MβCD. After exposure to MβCD, cells were washed three times with serum-free medium and returned to the incubator. After treatment, cells were kept in serum-free medium and were found to maintain the elevated or the decreased level of cholesterol for at least 48 h, providing the time window for the electrophysiological recordings (Table I). Analogous procedure was used for substitution of membrane cholesterol with epicholesterol, β-sitosterol, and coprostanol using respective analogue instead of cholesterol for preparation of MβCD–sterol complex. MβCD and sterols were purchased from Sigma-Aldrich or Steraloids.

Measurement of Cellular Sterols

Quantitative analysis of membrane cholesterol and epicholesterol was done as described previously (Romanenko et al., 2002b). Briefly, total lipid was extracted from the cell monolayers using isopropanol with addition of known amount of cholesteryl methyl ether (CME) as an internal standard. The extracts were dried in the flow of N₂ at 35°C, reextracted with chloroform:methanol:water system (Bligh and Dyer, 1959). dissolved in CS₂ and analyzed by gas-liquid chromatography (GLC) as described previously (Ishikawa et al., 1974; Klansek et al., 1995). Cell protein was determined on the lipid-extracted monolayer using a modification (Markwell et al., 1978) of the method of Lowry (Lowry et al., 1951). All mass values were normalized on the basis of cell protein. Quantifications of membrane β-sitosterol and coprostanol were performed using the same procedure.

Electrophysiological Recording

Ionic currents were measured using the whole-cell and outside-out excised patch configurations (Hamill et al., 1981). Pipettes were pulled (SG10 glass; Richland Glass) to give a final resistance of 2–6 MΩ. A saturated salt agar bridge was used as the reference electrode. Currents were recorded using an EPC9 amplifier (HEKA Electonik) and accompanying acquisition and analysis software (Pulse & PulseFit; HEKA Electronics). Whole-cell capacitance and series resistance were compensated and monitored throughout the recording. Whole-cell currents were elicited by 500-ms linear voltage ramps from −60 to +60 mV with an interpulse interval of 5 s. The holding potential between the ramps was −60 mV. Excised patches were pulled from the cells after full VRAC development and single channel current was recorded using a voltage-step pro-

| Conditions          | Cholesterol, µg/mg protein |
|---------------------|----------------------------|
| 5 mM MβCD, 2 h      | 7.45 ± 0.45                |
| 2.5 mM MβCD, 1 h    | 14.91 ± 0.96               |
| Control             | 22.62 ± 2.02               |
| 2.5 mM MβCD-cholesterol, 1 h | 31.92 ± 2.16 |
| 5 mM MβCD-cholesterol, 2 h | 46.44 ± 1.46 |

Cholesterol levels in BAECs exposed to MβCD and MβCD-cholesterol complexes for indicated periods and in control cells measured by GLC (n = 8–12).
tocel, as described previously by (Jackson and Strange, 1995). Single-channel recordings were done with a 50-μm sampling interval and filtered at 500 Hz. The external recording solution contained (in mM): 150 NaCl, 1 EGTA, 2 CaCl₂, 10 HEPES, pH 7.3. The basic internal solution contained (in mM): 120 or 140 Cs-Glutamate, 10 HEPES, 4 ATP, pH 7.3 (CsOH), with free [Ca²⁺] ≈ 10 nM (0.1 CaCl₂, 1.1 EGTA). VRAC was activated by either supplementing the internal solution with 100 μM GTPγS or by lowering the concentration of Cs-Glutamate to 90 mM. The osmolarities of all solutions were determined immediately before recording with a vapor pressure osmometer (Wescor, Inc.) and were adjusted by the addition of sucrose, as required. All chemicals for the recording solutions were obtained from Fisher Scientific or Sigma-Aldrich. Only cells that retained normal morphology after the MβCD treatments were taken for the analysis.

**Analysis**

Ionic strength (Iₛ) was calculated as: $Iₛ = 1/2 \sum m_i x_i^2$, where the sum of the molalities of each ion in the solution (mᵢ) multiplied by the square of its charge (zᵢ) (Moore, 1972). It was approximated that zwitterions of HEPES and glutamate contribute to ionic strength of the solutions as monoanionic acids with pKa 7.6 and 4.3, respectively. 120 Cs-Glutamate, 10 HEPES, 0.1 CaCl₂, 1.1 EGTA, 4 ATP, pH 7.3 (CsOH) has ionic strength 0.133 (μ = 1.039 ± 0.002 kg/l). Analogous solutions with 90 or 140 mM Cs-Glutamate have ionic strengths of 0.104 and 0.153, respectively (μ were 1.041 ± 0.004 kg/l and 1.056 ± 0.002 kg/l, respectively). The calculations were performed using WINMAX v2.40 software (Bers et al., 1994).

Activation rates of VRAC were calculated as maximal slopes of the linear regression of the time-courses of the current increase normalized by the cell capacitance. Analysis of the single-channel properties was performed using TAC software (Bruxton). Statistical analysis of the data was performed using a standard two-sample Student's t test and was considered significant if two-tailed P values were <0.05. All values are presented as means ± SE.

**RESULTS**

**Cholesterol Depletion Enhances VRAC in the Absence of Osmotic Stress**

Two strategies were employed in this study to activate VRAC in the absence of osmotic stress: (a) dialyzing the cells with guanosine 5′-O-(3-thiotriphosphate) (GTPγS) (Doroshenko et al., 1991; Nilius et al., 1999; Estevez et al., 2001), and (b) a decrease in the intracellular ionic strength (Iₛ) (Nilius et al., 1998; Voets et al., 1999; Sabirov et al., 2000). To test the sensitivity of GTPγS-induced and Iₛ-induced VRAC to the level of cellular cholesterol, the cells were exposed to “empty” MβCD for 2 h resulting in removal of ~70% of cellular cholesterol (Fig. 1 A). Intracellular dialysis of the cells with GTPγS (100 μM) induced transient development of VRAC (Fig. 1 B), consistent with the earlier studies (Doroshenko et al., 1991; Nilius et al., 1999; Estevez et al., 2001). Depletion of cellular cholesterol resulted in a significant increase in current density (Fig. 1, B and C), indicating that the effect of cholesterol can occur in the absence of cell swelling and it cannot be accounted for by an increase in cell swelling. The rate of current activation was not affected by cholesterol depletion.

To test whether the observed effect may be due to the effect of cholesterol depletion on the unitary conductance of VRAC channels, single-channel events were recorded in excised outside-out configurations after the current has developed (Fig. 1 D). After the excision, single-channel events can be observed under strong depolarization that induces channel closing. In most cases, when the channels close they stay closed for a prolonged period of time (Fig. 1 D, a), but in some cases the channels may flicker between the open and closed states for a short period of time before they close completely (Fig. 1 D, b). The amplitudes of the single-channels events were indistinguishable in cells depleted of cholesterol and in control cells (Fig. 1 E).

In our previous study we have shown that swelling-activated VRAC becomes insensitive to changes in the membrane cholesterol level when activated by strong osmotic gradient, indicating the maximal number of swelling-activated channels is not altered by changes in the membrane cholesterol (Levitan et al., 2000). Here we also tested whether the effect of cholesterol depletion on GTPγS-induced VRAC is different at low and at saturating levels of GTPγS. VRAC currents were recorded in BAECs dialyzed with increasing concentrations of GTPγS (Fig. 2 A). The figure shows that at 50–100 μM GTPγS VRAC reached an apparent maximum. To confirm that at 100 μM GTPγS the current indeed reaches the maximal value, average maximal current densities were compared at 100 μM and 2 mM GTPγS concentrations when recorded the same day and were found virtually indistinguishable (P = 0.96, n = 13–15). Fig. 2 B shows that the effect of cholesterol depletion on VRAC activated at low GTPγS concentration is similar to that at saturating GTPγS concentration.

Similarly to GTPγS-activated VRAC, cholesterol depletion significantly enhanced VRAC development when the current was activated by decreasing the intracellular ionic strength (Fig. 3 A and B). The intracellular ionic strength was reduced by dialyzing the cells with low salt solution (Iₛ = 104 mM) where the solution was adjusted with sucrose to maintain normal osmolality (310 mOsm). Furthermore, cholesterol depletion alone was sufficient to induce spontaneous VRAC activity (Fig. 3, C and D). When dialyzed with isosmotic intracellular solution with Iₛ = 153 mM, control cells develop only a low-amplitude, nonselective cation current and virtually no VRAC (Levitan and Garber, 1998). In contrast, cells depleted of cholesterol show clear VRAC development (Fig. 3 C) with the maximal current density in cholesterol depleted cells being ~4 times higher than the baseline current density (Fig. 3 D).

To analyze further the quantitative relationship between GTPγS-induced and low Iₛ-activated VRAC and...
cellular cholesterol, the level of cellular cholesterol was titrated by exposing the cells to either 2.5 or 5 mM MβCD and in control cells measured by GLC (n = 8–12; *P < 0.01). The typical families of current traces recorded from individual cells dialyzed with 100 μM GTPγS elicited by application of linear voltage ramps MATERIALS AND METHODS. The time-courses of VRAC current densities that developed in the same cells calculated by normalizing the maximal current amplitudes (+60 mV) of each individual ramp by the cell capacitance. (C) The activation rates and mean peak current densities of VRAC in cells treated with MβCD or control cells (n = 11–19). (D) Single-channel currents recorded in the outside-out excised patch configuration after a depolarization step from −60 to +140 mV. Examples of recordings when the channels were closing for a prolonged period of time (a) and the channels that flickered between the open and closed states (b). (E) Unitary conductances of the channels recorded in the membranes of the cells depleted of cholesterol and control cells.

As described in our previous study (Romanenko et al., 2002b), exposing the cells to 2.5 mM MβCD saturated with epicholesterol resulted in removal of ~50% of endogenous cholesterol, which was substituted with the similar amount of epicholesterol during the same exposure (Fig. 5 A). The effect of cholesterol/epicholesterol substitution on VRAC development was tested for both GTPγS-induced and low Γ3-induced currents (Fig. 5, B and D, respectively). Our observations show that substitution of 50% of endogenous cholesterol with epicholesterol had no significant effect on either GTPγS-induced or low Γ3-induced VRAC, indicating that the channels are insensitive to the chirality of the cholesterol isomer in the membrane (Fig. 5, C and D).
deed, when VRAC activity in MβCD-epicholesterol-treated cells was plotted as a function of total cellular sterol level it fell exactly on the same exponential curve that was obtained by titrating the levels of cellular cholesterol (Fig. 9). The equivalent result was observed when cells, depleted of cholesterol by exposing them to empty MβCD, were reloaded with epicholesterol by exposing them to MβCD complexed with epicholesterol. After the reloading, VRAC current density returned to the control level. Since the sensitivities of GTPγS- and Γi-induced VRAC to both cholesterol depletion and to the substitution of cholesterol with epicholesterol appear to be very similar, the rest of the substitution experiments were performed only for GTPγS-induced VRAC.

Substitution of Endogenous Cholesterol with β-sitosterol has a Small Effect on VRAC Activity

To verify further that VRAC activity is regulated by the physical properties of the membrane bilayer but not by specific cholesterol–protein interactions, we have substituted endogenous cholesterol with a plant sterol, β-sitosterol that, similarly to the two chiral forms of cholesterol, decreases membrane fluidity (Gimpl et al., 1997) and induces formation of the structural lipid domains (Xu and London, 2000). As with cholesterol/epicholesterol substitution, we have optimized the conditions of the exposure so that ~50% of endogenous cholesterol was substituted with the appropriate amount of β-sitos-

**Figure 2.** Effect of cholesterol depletion is conserved when VRAC is induced by various concentrations of GTPγS. (A) Peak current densities of VRAC were measured in control cells (n = 5–13). (B) The mean peak current densities of VRAC in cells treated with MβCD or control cells (n = 5–13).

**Figure 3.** Cholesterol depletion up-regulates low Γi-induced VRAC and induces VRAC activation at high Γi. (A and C) Typical families of current traces and time courses recorded in cholesterol-depleted and control cells dialyzed with solution with either Γi = 104 mM (A) Γi = 153 mM (C). (B and D) The activation rates and the mean peak current densities in cells dialyzed with either low or high Γi solutions, respectively (n = 3–10).
Regulation of VRAC by Cholesterol Analogues

We have found, however, that cholesterol/sitosterol substitution invariably resulted in some decrease in the total sterol level. Typically the level of total sterol was decreased by \( \sim 20\% \) (Fig. 6 A). The reason for this partial depletion effect is probably based on the relative affinities of MβCD and of the membrane to the particular sterol. The level of VRAC current density in sitosterol–treated cells was slightly but not significantly higher than in control cells (Fig. 6, B and C). This increase, however, is accounted for by the decrease in the total sterol level in these cells, which is apparent from plotting VRAC current density as a function of the total sterol level for different experimental conditions (Fig. 9).

**Effect of Cholesterol/coprostanol Substitution Is Similar to that of Cholesterol Depletion**

Finally, we have substituted endogenous cholesterol with coprostanol that, in contrast to cholesterol, has only minimal effect on fluidity of the membrane (Gimpl et al., 1997; Xu and London, 2000). Coprostanol partitioned easily into the membrane resulting in 70% cholesterol/coprostanol substitution with no change in total sterol level (Fig. 7 A). Under these experimental conditions, VRAC activity was strongly facilitated (Fig. 7, B and C) and was almost identical to the current in cholesterol-depleted cells (Fig. 9).

**Substitution of Endogenous Cholesterol with its Analogues has No Effect on VRAC Unitary Conductance**

Similarly to cholesterol depletion, we tested whether partial substitution of cellular cholesterol with epi-cholesterol, β-sitosterol, or coprostanol affects the unitary conductance of VRAC channels (Fig. 8). We have found that the amplitudes of the single-channel events were indistinguishable in cells at all four conditions.

**Dependence of VRAC on Total Sterol Level Versus Cholesterol Alone**

Fig. 9 summarizes the findings of this study by showing maximal VRAC amplitudes in different substitution experiments as a function of total cellular sterol (Fig. 9 A) or as a function of cholesterol alone (Fig. 9 B). The figure shows that when cholesterol is substituted with epi-cholesterol or with β-sitosterol, VRAC current density is identical to the value predicted from the VRAC/total sterol curve but when cholesterol is substituted with coprostanol the level of VRAC current density is significantly higher than that predicted by the VRAC/total sterol curve. In contrast, if VRAC is plotted as a function of cholesterol alone, the picture reverses. When cholesterol is substituted with coprostanol, VRAC current density is identical to the value predicted by the VRAC/cholesterol curve, whereas when cholesterol is substituted with epi-cholesterol or β-sitosterol, it falls significantly lower than the predicted value. These observations lead to the conclusion that VRAC is regulated by the physical properties of the membrane bilayer and not by specific sterol–protein interactions.

**DISCUSSION**

The role of cholesterol in regulation of VRAC development was studied by analyzing the sensitivity of VRAC to changes in the cellular level of cholesterol and to substitution of endogenous cholesterol with its structural analogues. The main findings of this study are: (a) cholesterol depletion enhances VRAC activity in a swelling-independent way, (b) VRAC unitary conductance is not affected by changes in the membrane sterol composition, and (c) VRAC is insensitive to the substitution of cholesterol with the sterols whose effects on membrane fluidity were shown to be similar to that of cholesterol, but is strongly affected by the substitution of cholesterol with a sterol whose effect on membrane fluidity is similar to that of cholesterol depletion.

The fundamental question for understanding the mechanisms by which cholesterol affects the function of membrane proteins is whether cholesterol regulates protein function by specific sterol–protein interactions (direct binding to the protein of interest or to a modulatory protein) or by changing the physical properties of the lipid environment. Three experimental approaches are
commonly used to test the effect of cholesterol on membrane proteins in living cells. The first approach is depleting the cells of or enriching them with cholesterol by exposing the cells to cyclodextrins, water-soluble cyclic carbohydrates with high specificity for cholesterol (e.g., Klein et al., 1995; Christian et al., 1997; Gimpl et al., 1997). As expected, cholesterol removal increases membrane fluidity (Gimpl et al., 1997) and decreases lipid ordering (Gidwani et al., 2001) but altering the level of cholesterol alone cannot discriminate between the two mechanisms because both the amount of cholesterol and the physical properties of the membrane are altered at the same time. Another approach is to sequester cholesterol with a polyene antibiotic filipin (e.g., (Gimpl et al., 1997; Scanlon et al., 2001; Mitter et al., 2003)). This treatment, however, decreases the local concentration of cholesterol and, therefore, bears the same limitations as cholesterol depletion. Finally, membrane cholesterol can be modified by its enzymatic degradation with cholesterol oxidase (e.g., Gimpl et al., 1997; Pang et al., 1999; Scanlon et al., 2001). This method has the advantage of preserving membrane fluidity (Gimpl et al., 1997); however, it tests only the impact of one specific modification of cholesterol structure. In this study, we use an alternative approach of quantitative substitution of endogenous cholesterol by its structural analogues. This approach is based on application of cyclodextrin that was first used to titrate cellular cholesterol to different levels (Klein et al., 1995; Christian et al., 1997) and then developed further to introduce various sterols into membrane vesicles (Gimpl et al., 1997).

In this study, we substitute endogenous cellular cholesterol with three of its structural analogues: the cholesterol synthetic chiral analogue epicholesterol, the plant sterol β-sitosterol, and coprostanol. Comparing effects of cholesterol and epicholesterol is the most commonly used method to discriminate between specific and nonspecific effects of cholesterol on protein function (Mickus et al., 1992; Gimpl et al., 1997; Sook-saware and Simmonds, 2001). Indeed, cholesterol and epicholesterol are known to reside at significantly different angles in the membrane (Murari et al., 1986).
Figure 6. Substitution of cholesterol with β-sitosterol has no significant effect on GTPγS-induced VRAC. (A) Structure of β-sitosterol (note the additional ethyl group at position 24), GLC profile of the lipid extract of β-sitosterol-treated cells [(a) CME, (b) cholesterol, and (c) β-sitosterol], and the concentrations of cholesterol (lighter portion of the bar) and of β-sitosterol (darker portion) measured in cells treated with 5 mM MβCD saturated with β-sitosterol and in control cells (n = 6). (B) Typical families of current traces and the time-courses of GTPγS-induced VRAC recorded in a β-sitosterol–treated cell and in a control cell. (C) The peak current densities in treated cells were not significantly different than in control cells (n = 15–21).

Figure 7. Substitution of cholesterol with coprostanol significantly enhances GTPγS-induced VRAC. (A) Structure of coprostanol (note the reduced Δ5 double bond), GLC profile of the lipid extract of 1 mM MβCD-coprostanol treated cells [(a) CME, (b) coprostanol, and (c) cholesterol], and the concentrations of the sterols cholesterol (lighter portion of the bar) and coprostanol (darker portion) (n = 3). (B) Typical families of current traces and VRAC time-courses of GTPγS-induced VRAC in a coprostanol-treated cell and in a control cell. (C) The peak current densities (n = 21–23; *P < 0.01).
and therefore are expected to differ in direct binding to membrane proteins. In addition, cholesterol was substituted with \( \text{Hepatitis} \), which incorporates a structural modification at the opposite end of the cholesterol molecule than epicholesterol but, similarly to epicholesterol, is known to have an effect on membrane fluidity indistinguishable from that of cholesterol. Co-prostanol, on the other hand, was chosen because its effect is similar to that of cholesterol depletion. Effects of these sterols on the physical properties of the membrane were measured both in simple reconstituted lipid systems (Xu and London, 2000) and in membrane vesicles isolated from epithelial cells HEK293 (Gimpl et al., 1997; Xu and London, 2000). The effects of the sterols on membrane fluidity in membrane vesicles are very similar to their effects in reconstituted lipid systems suggesting that these observations reflect general effects of the sterols on membrane properties. Our study shows that the effects of the three analogues on VRAC correlate closely to their effects on the physical properties of the membrane. Therefore, while it is not feasible to completely exclude the possibility that

**Figure 8.** Substitution of cholesterol with its analogues does not affect unitary conductance of GTPyS-induced VRAC. (A) Examples of single-channel currents recorded in the outside-out excised patch configuration after a depolarization step from -60 to +140 mV. (B) Unitary conductances of the channels recorded in the membranes of the cells substituted with cholesterol analogues and control cells (\( n = 3-5 \)).

**Figure 9.** Functional dependence of GTPyS-induced VRAC on the level of total sterol (A) and on the level of endogenous cholesterol (B) in sterol substitution experiments. (A) The mean peak current densities plotted as a function of total sterol level in cells treated with MβCD or MβCD saturated with cholesterol (○), in the cells treated with MβCD saturated with epicholesterol (●), in the cells treated with MβCD saturated with sitosterol (■), and in the cells treated with MβCD saturated with coprostanol (▲). All the values were normalized by their respective values in control cells. (B) The graph represents the same set of data as in A, plotted as a function of cellular cholesterol alone.
the sterols regulate VRAC by directly binding to the channel because the molecular nature of VRAC is not yet identified, our current observations suggest that physical properties of the membrane play an important role in the regulation of VRAC activity.

What are the molecular mechanisms that may be responsible for the regulation of membrane proteins by the physical properties of the membrane? First, since the unitary conductance of VRAC is not affected, we can exclude the possibility that sterols affect VRAC pore properties. We also show that the effect of cholesterol depletion is not abolished at the saturating levels of GTP\textsubscript{S}, indicating that cholesterol does not affect the GTP\textsubscript{S}-dependent transition between the closed and the open states. If the effect is abolished, as we have demonstrated earlier for swelling-activated VRAC (Levitan et al., 2000), then it strongly suggests that it is not due to an increase in the number of the channels. If, however, the effect is not abolished at the saturating level of the stimulus, as is the case for GTP\textsubscript{S}-sensitive VRAC, it suggests that cholesterol affects the transition that is not dependent on GTP\textsubscript{S} and the simplest explanation is that cholesterol alters the distribution between the population of closed unavailable channels and the joint population of closed but available and open channels.

On a mechanistic level, one possibility is that cholesterol affects the distribution between different conformational states of the channels, as was proposed by the membrane deformation model. Specifically, the model suggests that changes in the lipid composition of the membrane that affect the structural stress of the membrane are expected to alter the energetic cost of the transition between the open and the closed states of the channels (Lundbaek and Andersen, 1994; Lundbaek et al., 1996). Indeed, sterols that increase the ordering of phospholipids (cholesterol, epicholesterol, and \( \beta \)-sitosterol) also inhibit the current; whereas cholesterol depletion that has a disordering effect on the membrane (Gidwani et al., 2001) as well as substitution of membrane cholesterol with an analogue, which does not increase ordering (Xu and London, 2000), facilitate channel activity. The correlation of the sterol effects on the transition between the nonavailable and the available channels with the change in bilayer physical properties suggests that this regulation involves a change in the cost of bilayer deformation. Another possible mechanism that may be responsible for the sensitivity of volume-regulated anion channels to cholesterol is segregation of the proteins into cholesterol-rich domains (lipid rafts). Organization of the lipid rafts is driven by lipid–lipid, protein–lipid, and protein–protein interactions (Lai, 2003), and these domains may serve as scaffolds for protein–protein interactions (Brown and London, 1998; Simons and Toomre, 2000). The two mechanisms are distinct but not mutually exclusive because segregation into a raft may affect channel function by altering its lipid environment.

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