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Modulation of Endothelial Inward-Rectifier K⁺ Current by Optical Isomers of Cholesterol

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ABSTRACT Membrane potential of aortic endothelial cells under resting conditions is dominated by inward-rectifier K⁺ channels belonging to the Kir 2 family. Regulation of endothelial Kir by membrane cholesterol was studied in bovine aortic endothelial cells by altering the sterol composition of the cell membrane. Our results show that enriching the cells with cholesterol decreases the Kir current density, whereas depleting the cells of cholesterol increases the density of the current. The dependence of the Kir current density on the level of cellular cholesterol fits a sigmoid curve with the highest sensitivity of the Kir current at normal physiological levels of cholesterol. To investigate the mechanism of Kir regulation by cholesterol, endogenous cholesterol was substituted by its optical isomer, epicholesterol. Substitution of ~50% of cholesterol by epicholesterol results in an early and significant increase in the Kir current density. Furthermore, substitution of cholesterol by epicholesterol has a stronger facilitative effect on the current than cholesterol depletion. Neither single channel properties nor membrane capacitance were significantly affected by the changes in the membrane sterol composition. These results suggest that 1) cholesterol modulates cellular K⁺ conductance by changing the number of the active channels and 2) that specific cholesterol-protein interactions are critical for the regulation of endothelial Kir.

INTRODUCTION

Cholesterol is one of the major lipid components of the plasma membrane of mammalian cells. A normal physiological level of cholesterol in the plasma membrane is essential for cell function and growth, but its excess is cytotoxic (Yeagle, 1985, 1991; Kellner-Weibel et al., 1999; Simons and Ikonen, 2000). The basis for the cholesterol requirement and for its cytotoxicity is believed to lie in its ability to alter the function of integral membrane proteins. Several studies have demonstrated that changes in the cholesterol/phospholipid molar ratio of cellular membranes modulate the activity of a number of ion channels. Elevation of membrane cholesterol decreases the open probability of the antibiotic ion channel gramicidin (Lundbaek et al., 1996) and of large-conductance Ca²⁺-dependent K⁺ channels (Bolotina et al., 1989; Chang et al., 1995), inhibits L-type Ca²⁺ current (Jennings et al., 1999), and suppresses the development of volume-regulated anion current (Levitan et al., 2000). Although in most cases the activity of the ion channels is decreased by the elevation of membrane cholesterol, elevated cholesterol may also increase the activity of the channels, as has been shown for the nicotinic acetylcholine receptor (reviewed by Barrantes, 1993). Changes in membrane cholesterol have also been shown to shift the voltage sensitivity of inactivation of N-type Ca²⁺ channels (Lundbaek et al., 1996) and to shift the voltage sensitivity of both activation and inactivation of delayed rectifier K⁺ channels, K_v 1.5 (Martens et al., 2000, 2001).

Here we examine the role of cholesterol in the regulation of an inwardly rectifying K⁺ current in bovine aortic endothelial cells (BAECs). These channels dominate the ionic conductance of endothelial cells under resting conditions and are responsible for maintaining negative membrane potential (Voets et al., 1996; Kamouchi et al., 1997). Endogenous endothelial inwardly rectifying K⁺ channels belong to a family of strong rectifiers, Kir 2.0, and are homologous to Kir 2.1 (Forsyth et al., 1997; Kamouchi et al., 1997). A powerful tool to study the mechanism by which cholesterol modifies the function of ion channels is the substitution of endogenous cholesterol with its optical isomer (stereoisomer), epicholesterol. Stereoisomers (isomers that differ only in the spatial orientation of their component atoms) are widely used to distinguish between specific and nonspecific effects of different biological molecules because, typically, stereoisomers have similar physical properties but are strikingly different in their specific biological interactions. A known synthetic stereoisomer of cholesterol (3 β -hydroxy-5-cholestene) is epicholesterol (3 α -hydroxy-5-cholestene) that differs from cholesterol in the rotational angle of the hydroxyl group at position 3. The effects of epicholesterol on membrane fluidity and on formation of the lipid domains have been shown to be indistinguishable from those of cholesterol (Gimpl et al., 1997; Xu and London, 2000). Our results demonstrate that changes in the cholesterol level strongly influence Kir current density and that the chirality of the sterol is important. In contrast, neither enriching the cells with cholesterol nor the substitution of cholesterol by epicholesterol affect the unitary conductance and the open probability of the channels, suggesting that the level of cellular cholesterol modulates the number of the channels in the membrane rather than the biophysical properties of the channels. We also show that cholesterol-depen-

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dent regulation of Kir current density is observed less than 2 h after the sterol treatment and is not accompanied by any change in the cell capacitance. These observations suggest that neither gene expression nor membrane insertion/retrieval mechanisms can be responsible for the observed effects. We suggest, therefore, that endothelial Kir channels may exist in the membrane in two modes, an active and a silent mode, and that the level of cholesterol alters the equilibrium between the active and the silent populations of the channels.

MATERIALS AND METHODS

Cell culture

BAECs between passages 10 and 30 were grown in Dulbecco's modified Eagle's medium (DMEM; Cell Grow, Washington, DC) supplemented with 10% bovine serum (Gibco BRL, Grand Island, NY). Cell cultures were maintained in a humidified incubator at 37°C with 5% CO₂. The cells were fed and split every 3–4 days.

Modulation of cellular cholesterol level and substitution of cholesterol with epicholesterol

BAECs were enriched with or depleted of cholesterol by incubating them with methyl- β -cyclodextrin (M β CD) saturated with cholesterol or with empty M β CD (not complexed with cholesterol), as described previously (Levitani et al., 2000). Briefly, a small volume of cholesterol stock solution in chloroform:methanol (1:1, v:v) was added to a glass tube, and the solvent was evaporated. Then, 2.5 mM or 5 mM M β CD solution in DMEM without serum was added to the dried cholesterol. The tube was vortexed, sonicated, and incubated overnight in a shaking bath at 37°C. M β CD was saturated with cholesterol at a M β CD:cholesterol molar ratio of 8:1, the saturation limit of M β CD (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 M β CD-saturated solution or with M β CD solution containing no cholesterol (empty M β CD) 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 M β CD. After exposure to M β CD, cells were washed three times with serum-free medium and returned to the incubator. Cells that were incubated in serum-free medium maintained the elevated or the decreased level of cholesterol for at least 48 h, providing the time window for the electrophysiological recordings. M β CD and cholesterol were purchased from Sigma Chemical Co. (St. Louis, MO).

M β CD-epicholesterol solution was prepared as described for M β CD-cholesterol. The substitution was performed either in two-step or in one-step procedures. In the first case, cells were first depleted of cholesterol by exposing them to empty M β CD and then exposed to M β CD saturated with epicholesterol. In the second case, the cells were exposed to M β CD-epicholesterol without previous depletion. The two procedures were similarly effective in substituting cholesterol with epicholesterol. The amount of epicholesterol incorporated into the membranes after 1 h of incubation was 12 ± 3 μ g/mg protein constituting ~50% of the total sterol content of the membrane. Prolonging the incubation from 1 h to 12 h did not significantly increase the amount of epicholesterol incorporated into the membrane, indicating that epicholesterol reaches its equilibrium level within 1 h. A one-step, 1-h incubation with M β CD-epicholesterol was, therefore, used for all electrophysiological experiments. Epicholesterol was purchased from Steraloids (Newport, RI).

Measurement of cellular sterols

Lipids were extracted from the washed cell monolayer using isopropanol as previously described (McClosky et al., 1987). Total cholesterol mass analysis was done by gas-liquid chromatography (GLC) as previously described (Ishikawa et al., 1974; Klansek et al., 1995). Briefly, before lipid extraction, the medium was removed and the cells were dried in air. Dried cells were extracted with 4 ml/well isopropanol containing 2 μ g of cholesteryl methyl ether (CME) as an internal standard. After 4 h, the extracts were dried in the flow of N₂ at 35°C, re-extracted, dissolved in CS₂, and analyzed by GLC. 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.

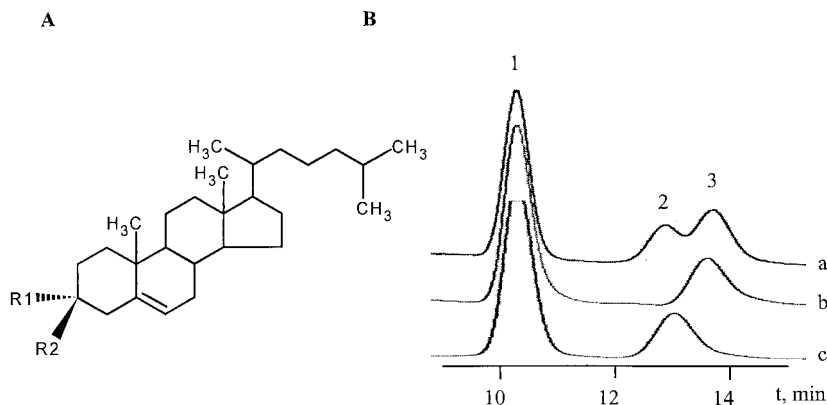
Electrophysiological recording

Ionic currents were measured using the whole-cell and cell-attached configurations of the standard patch clamp technique (Hamill et al., 1981). Pipettes were pulled (SG10 glass; Richland Glass, Richland, NJ) to give a final resistance of 2–6 M Ω when the above recording solutions were used. Pipettes were coated with Sylgard (Dow Corning, Midland, MI) to decrease their electrical capacitance. These pipettes generated high-resistance seals without fire polishing. A saturated salt agar bridge was used as reference electrode. Currents were recorded using an EPC9 amplifier (HEKA Elektronik, Lambrecht, Germany) and accompanying acquisition and analysis software (Pulse and PulseFit, HEKA Elektronik) running on a PowerCenter 150 (Mac OS) computer. Pipette and whole-cell capacitance was automatically compensated. Whole-cell capacitance and series resistance were compensated and monitored throughout the recording. Current was monitored by 500-ms linear voltage ramps or series of voltage steps from –160 mV to +60 mV at an interpulse interval of 5 s. The holding potential between the ramps was –60 mV. Normal cellular current convention was used when referring to the direction of current; e.g., inward current refers to inward K⁺ ion flow. Single-channel recordings were done in 1.6-s sweeps with 0.1-ms sampling interval and filtered at 500 Hz. All experiments were performed at room temperature (22–25°C). The external recording solution contained (in mM) 150 NaCl, 6 KCl, 10 HEPES, 1.5 CaCl₂, 1 MgCl₂, and 1 EGTA, pH 7.3, or 156 KCl, 10 HEPES, 1.5 CaCl₂, 1 MgCl₂, and 1 EGTA, pH 7.3. The pipette solutions for the whole-cell configuration contained (in mM) 140 KCl, 10 HEPES, 1 MgCl₂, 4 ATP, and 1 EGTA, pH 7.3 (KOH) and for the cell-attached configuration contained 156 KCl, 10 HEPES, 1.5 CaCl₂, 1 MgCl₂, and 1 EGTA, pH 7.3, identical to the extracellular solution. For recording nonselective cation current the pipette solution (in mM) was designed to suppress activation of volume-regulated anion current and block Kir channels: 140 CsGlu, 10 HEPES, 0.1 CaCl₂, 5 EGTA, and 4 ATP, pH 7.3 (with osmolarity ~5% lower than of external recording solution). The chemicals for the recording solutions were obtained from Fisher Scientific (Fairlawn, NJ) or Sigma. The osmolarities of all solutions were determined immediately before recording with a vapor pressure osmometer (Wescor, Logan, UT) and were adjusted by the addition of sucrose, as required.

Analysis

Statistical analysis of the data was performed using a standard two-sample Student's *t*-test assuming unequal variances of the two data sets. Statistical significance was determined using a two-tailed distribution assumption and was set at the 5% level ($p < 0.05$). The time constants of voltage-dependent inactivation were measured by fitting a single-exponential function $V(t) = Ae^{-t/\tau}$ where A is current amplitude and τ is the time constant. The fits were obtained with Levenberg-Marquardt algorithm using PulseFit software (HEKA Elektronik). Analysis of the single-channel properties was performed using TAC software (Bruyton, Seattle, WA). The unitary con-

FIGURE 1 Structure and chromatographic profiles of cholesterol and epicholesterol. (A) Cholesterol and epicholesterol differ in the orientation of the 3-hydroxyl group. For cholesterol, R1 = H and R2 = OH; for epicholesterol, R1 = OH and R2 = H. (B) The two isomers can be separated and quantified using GLC (see Materials and Methods): peak 1 is CME (internal standard), peak 2 is epicholesterol, and peak 3 is cholesterol. The three chromatographic profiles lipid extracts of the cells treated with M β CD-epicholesterol (a), a mixture of CME and cholesterol (b), and a mixture of CME and epicholesterol (c).



ductance of the channels was calculated from the amplitude histograms of the current, and the kinetic parameters of the channels (mean open time and mean closed time) were calculated from the dwell-time histograms of the current. The number of channels per cell (N) was calculated as $N = I/(iP_o)$, where I is the whole-cell K^+ current, i is the Kir unitary current, and P_o is the open probability of the channels.

RESULTS

Modulation of cellular cholesterol and substitution of endogenous cholesterol by epicholesterol using M β CD

M β CD, a cyclic oligosaccharide, provides a precise and reproducible method for modulating cholesterol content in cellular membranes (Klein et al., 1995; Christian et al., 1997; Levitan et al., 2000). When M β CD is saturated with cholesterol, it acts as a cholesterol donor, increasing the cellular cholesterol level, whereas in its empty form, when not complexed with cholesterol, it acts as a cholesterol acceptor and depletes cholesterol from cells. M β CD can also be used to substitute endogenous cellular cholesterol with epicholesterol. Despite the structural similarity between the two analogs, they can be separated by GLC (Fig. 1). The figure shows the GLC peaks obtained from the cellular extract of cells treated with M β CD-epicholesterol, compared with the peaks obtained from the solutions of pure cholesterol and of epicholesterol relative to an internal standard (cholesteryl methyl ether). Although the peaks of cholesterol and epicholesterol overlap, they are clearly separable by the integrator. The separation coefficient, R_s , between the two peaks is defined as $R_s = 2t/(w_1 + w_2)$, where t is the distance between the two peaks and w_1 and w_2 are the width of the first and the second peaks, respectively. R_s varied in different experiments between 0.9 and 1.1. The amount of each sterol was determined as the area under the appropriate peak normalized to the area-to-mass ratio of the internal standard, corrected for the sensitivity difference of the detector for the internal standard and for the sterols.

Exposure of BAECs to M β CD saturated with epicholesterol for 60 min resulted in the substitution of ~40% of the endogenous cholesterol with epicholesterol (Fig. 2). Consistent with our earlier study (Levitan et al., 2000), a similar amount of cholesterol was removed by exposing the cells to empty M β CD. Because in endothelial cells cellular cholesterol exists predominantly in the form of free cholesterol (Levitan et al., 2000), only the changes in free cholesterol levels are shown. Prolonging the exposure to M β CD-epicholesterol to 6 or 12 h did not result in an additional increase in the cellular epicholesterol content, suggesting that the steady state between the M β CD-sterol complexes and the membrane is achieved when the levels of the two isomers in the membrane are similar. One hour of incubation, therefore, was chosen for testing the effect of epicholesterol on Kir. Because no significant recovery was observed after 24 and 48 h (not shown), the currents were recorded within the 48-h window after the exposure to M β CD.

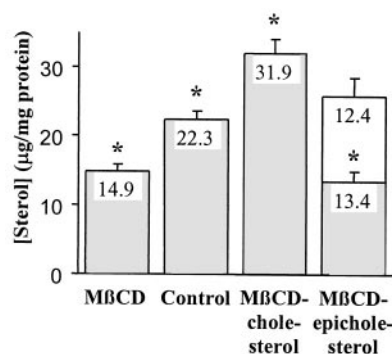
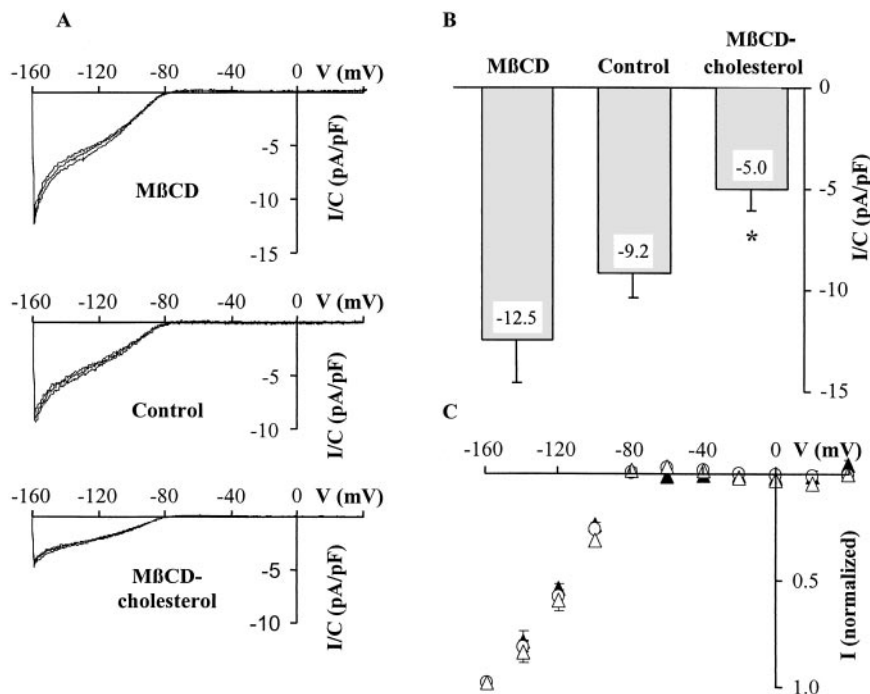


FIGURE 2 Modulation of membrane cholesterol and substitution of endogenous cholesterol by epicholesterol using M β CD. Cells were treated with 2.5 mM M β CD, M β CD-cholesterol, or M β CD-epicholesterol in DMEM with no serum. Control cells were treated with serum-free DMEM. Dark bars represent the concentration of cholesterol; the lighter portion of the bar is the epicholesterol level. All values are means \pm SE ($n = 3-15$). Cholesterol levels after all treatments are different from the control cell level ($p < 0.05$).

FIGURE 3 Kir currents in cells depleted of or enriched with cholesterol. (A) Typical current traces recorded from three individual cells: a cell exposed to 2.5 mM empty M β CD (*top*), a control cell that was not exposed to M β CD (*middle*), and a cell that was exposed to 2.5 mM M β CD saturated with cholesterol (*bottom*). Three superimposed traces are shown for each cell. The currents were elicited by 500-ms linear voltage ramps from -160 mV to $+60$ mV, applied with intervals of 5 s for the duration of the experiment. The holding potential between the ramps was -60 mV. The currents were normalized to cell capacitance recorded before each voltage ramp. (B) Peak current densities (-160 mV) of the three cell populations. The data are pooled from 15, 18, and 10 cells for M β CD-treated, control, and M β CD-cholesterol-treated cells, respectively. All values are means \pm SE; * $p < 0.05$ vs. control. (C) Normalized current-voltage relationships at the three experimental conditions: \blacktriangle , cells treated with 2.5 mM M β CD; \circ , control cells; \triangle , cells treated with 2.5 mM M β CD-cholesterol. Each point is the mean \pm SE ($n = 4-7$).



Modulation of Kir by the level of cellular cholesterol

Enriching the cells with cholesterol resulted in a significant decrease in the Kir current density (Fig. 3). The concentrations of K^+ in the recording solutions were maintained in the physiological range (6 mM and 156 mM in the extracellular and intracellular solutions, respectively), and the currents were elicited by linear voltage ramps from -160 mV to $+60$ mV. Fig. 3 A shows typical K^+ currents recorded from three individual cells: a cell that was exposed to 2.5 mM M β CD (upper family of traces), a control cell (middle family), and a cell exposed to 2.5 mM M β CD saturated with cholesterol (lower family). Mean current densities measured at -160 mV are shown in Fig. 3 B. No differences were observed between the currents recorded within 5-, 24-, or 48-h window after the M β CD exposure.

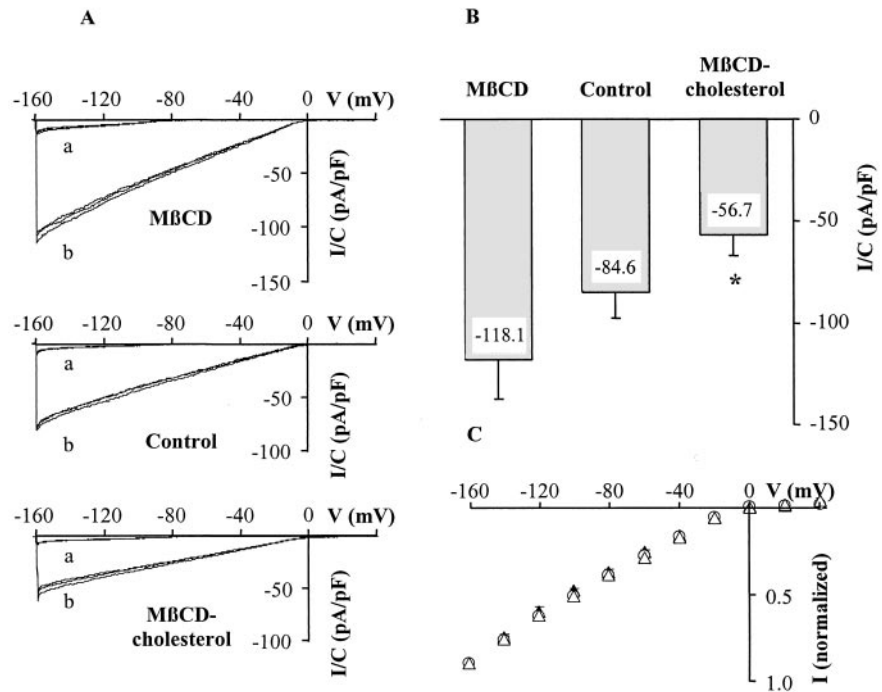
Reversal potentials of the currents were -81 ± 2 mV, -77 ± 1 mV, and -78 ± 2 mV in cholesterol-enriched cells, cholesterol-depleted cells, and control cells, respectively, indicating that the current is carried mainly by K^+ ions (the theoretical reversal potential for K^+ at these ionic conditions, calculated from the Nernst equation, is -80 mV). The inward rectification observed in the current traces is typical for the strong inward rectifiers Kir 2 that underlie the resting K^+ conductance in aortic endothelial cells (Kamouchi et al., 1997). Changes in cellular cholesterol had no effect on the rectification properties of the current (Fig. 3 C).

A typical feature of Kir 2.0 subfamily channels is the dependence of the current amplitude on the extracellular K^+

concentration (Sakmann and Trube, 1984; Ishihara and Hiraoka, 1994). As expected, increasing the extracellular K^+ concentration (K_o^+) from 6 mM (physiological level of K_o^+) to 156 mM strongly increased the amplitude of the current and shifted the reversal potential of the current from -79 ± 1 mV to $+5 \pm 3$ mV. This is in excellent agreement with the shift predicted from the Nernst equation for our recording solutions, from -80 mV to $+2$ mV, thus confirming that the membrane conductance is dominated by K^+ channels (Fig. 4 A). Although the currents recorded in high K_o^+ were significantly higher than those recorded in low K_o^+ , the dependence of the current density on cellular cholesterol in high and low K_o^+ was very similar (Fig. 4, A and B). Rectification properties of the current in symmetrical K^+ solutions were also unaffected by the changes in membrane cholesterol (Fig. 4 C).

To test further the quantitative relationship between membrane cholesterol and Kir current density, the cells were exposed to either 2.5 mM or 5 mM M β CD or M β CD-cholesterol complex. As expected, the efficiency of depletion/enrichment was higher when the cells were exposed to the higher concentration of M β CD/M β CD-cholesterol complex. Fig. 5 shows the Kir peak current density plotted as a function of membrane cholesterol. The peak current density and the cholesterol level at every experimental condition were normalized to those in control cells recorded in the same experiment. The figure shows that Kir current density has a strong sigmoidal dependence on membrane cholesterol (the curve was fit by a sigmoid curve with a correlation coefficient of 0.98). The current density of control cells

FIGURE 4 Comparison of the cholesterol effect on Kir current in low K_o^+ and in high K_o^+ conditions. (A) Typical current traces recorded before (a) and after (b) the extracellular medium was exchanged from 6 mM extracellular K^+ to 156 mM K^+ . The experimental protocols for the exposure to 2.5 mM M β CD and to 2.5 mM M β CD-cholesterol and the protocol for current recordings were identical to those in Fig. 2. (B) Peak current densities (-160 mV) at 156 mM extracellular K^+ . The data are pooled from 6, 5, and 6 cells for M β CD-treated, control, and M β CD-cholesterol treated cells, respectively. All values are means \pm SE; * p < 0.05 vs. control. (C) Normalized current-voltage relationships at the three experimental conditions recorded under high K_o^+ conditions: \blacktriangle , cells treated with 2.5 mM M β CD; \circ , control cells; \triangle , cells treated with 2.5 mM M β CD-cholesterol. Each point is the mean \pm SE (n = 4-7).



appears to be at the midpoint of the linear portion of the sigmoid (inflection region of the curve), suggesting that the sensitivity of the Kir current to cholesterol is the highest at normal physiological levels of cholesterol. The effects of cholesterol depletion/enrichment on Kir recorded at physiological and at high K_o^+ were identical.

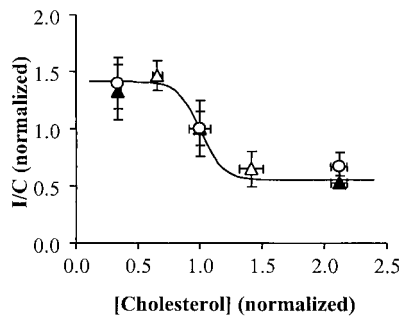


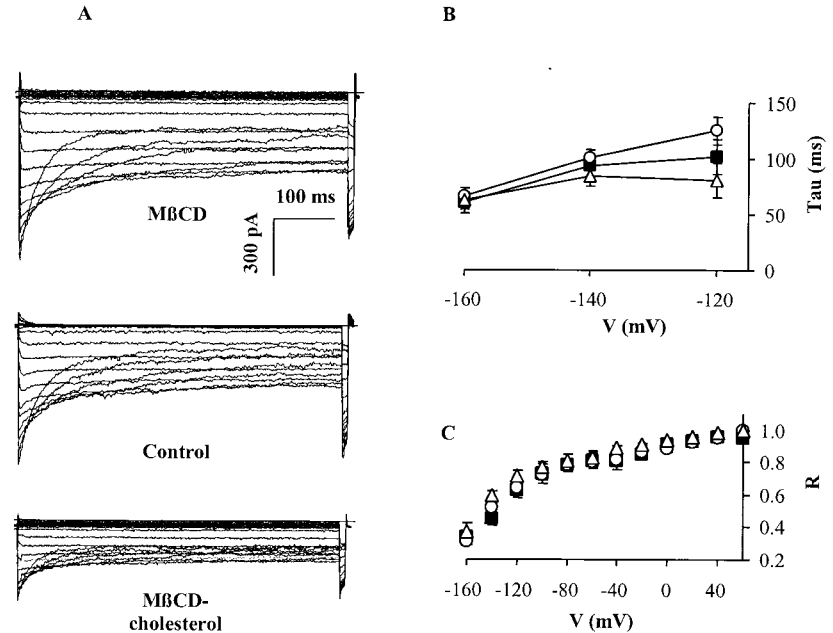
FIGURE 5 The functional dependence of normalized Kir current on the level of membrane cholesterol. The points represent normalized peak currents (-160 mV) plotted as a function of normalized level of free cholesterol in cells exposed to various experimental conditions: cells treated with 5 mM M β CD or M β CD-cholesterol and cells treated with 2.5 mM M β CD or M β CD-cholesterol. The currents were recorded under physiological (6 mM) or symmetrical (156 mM) extracellular K^+ concentrations. Both peak current densities and cholesterol concentrations in the cell extracts were normalized to those of control cells measured in the same series of experiments: \circ , cells treated with 5 mM M β CD and M β CD-cholesterol and the currents recorded in 156 mM K_o^+ ; \blacktriangle , cells treated with 5 mM M β CD and M β CD-cholesterol and the currents recorded in 6 mM K_o^+ ; \triangle , cells treated with 2.5 mM M β CD and M β CD-cholesterol and the currents recorded in 6 mM K_o^+ . Each point is the mean \pm SE (n = 5-35). The data were fit to a sigmoid with a correlation coefficient of 0.98.

Pronounced voltage-dependent inactivation was observed in control cells, in cell enriched with cholesterol, and in cells depleted of cholesterol (Fig. 6 A). The time course of the current decay could be adequately fit by a single exponent in all three conditions. The time constant of the inactivation becomes faster as the membrane potential becomes more negative, as described earlier (Sakmann and Trube, 1984; Shieh, 2000). The time constants of the inactivation were similar in all three conditions (Fig. 6 B). The inactivation ratio (the ratio between the current amplitude of a test pulse delivered after a preconditioning pulse to the current amplitude of a control test pulse) was also unaffected, indicating that the voltage dependence of the inactivation is not altered by changes in membrane cholesterol (Fig. 6 C).

Substitution of cholesterol with epicholesterol increases Kir current density

Fig. 7 shows that partial substitution of the endogenous cholesterol with its optical isomer epicholesterol resulted in more than a twofold increase in the Kir current density. The increase in current density was not accompanied by changes in the reversal potentials of the current (-78 \pm 2 mV and -79 \pm 2 mV in control and in epicholesterol-treated cells, respectively) or by changes in the current rectification properties (Fig. 7 C). In this experiment, the cells were exposed to 2.5 mM M β CD-epicholesterol. Note that the decrease in cholesterol level after exposure to 2.5 mM M β CD-epicholesterol (13.4 μ g/mg protein) was not as strong as that in cholesterol-depleted cells exposed to 5 mM M β CD (7.5 μ g/mg protein). Nevertheless, the increase in current den-

FIGURE 6 Effect of cholesterol on Kir inactivation. (A) Typical families of current traces elicited by two-pulse voltage protocol in cells depleted of cholesterol, enriched with cholesterol, and control. The two-pulse voltage protocol is used to compare the steady-state inactivation properties of the current. The 500-ms voltage pulses were applied from -160 to $+60$ mV with increments of 10 or 20 mV and immediately followed by 10-ms test pulses to -100 mV. The experiments were performed at 6 mM K_o^+ . (B) Time constant of inactivation (τ) plotted against voltage (single-exponential fit): ■, cells treated with 2.5 mM M β CD; ○, control cells; △, cells treated with 2.5 mM M β CD-cholesterol. Each point is the mean \pm SE ($n = 3-6$). (C) Inactivation ratio (R) was determined as the ratio of the current amplitude in response to a test voltage pulse to that of a $+60$ -mV test voltage pulse: ■, cells treated with 2.5 mM M β CD; ○, control cells; △, cells treated with 2.5 mM M β CD-cholesterol. Each point is the mean \pm SE ($n = 4-24$).



sity induced by cholesterol/epicholesterol substitution was much more pronounced than that induced by cholesterol depletion even in cells exposed to the higher concentration of M β CD. Specifically, cholesterol depletion resulted only in an ~ 1.4 -fold increase in the current density, whereas partial substitution of cholesterol by epicholesterol resulted in up to a 2.4-fold increase (compare Figs. 3 and 7). The increase in current density induced by the cholesterol/epicholesterol substitution was retained when the extracellular K_o^+ was increased from the physiological level to 156 mM (Fig. 8). Again, cholesterol/epicholesterol substitution resulted in a 3-fold increase in current density, whereas cho-

lesterol depletion resulted only in a 1.6-fold increase (compare Figs. 4 and 8). These findings indicate that the effect of the substitution of endogenous cholesterol by epicholesterol cannot be explained solely by the depletion of endogenous cholesterol.

The onset of the change in Kir current after the substitution of cellular cholesterol with epicholesterol on Kir current was rapid. Kir current density was measured in BAECs after incubation with M β CD-epicholesterol for 1 h, followed by a 1-h recovery period (Fig. 7 B). Immediately after the treatment, Kir current density increased significantly to 17 ± 3 pA/pF ($n = 8$). Although we observed a slight

FIGURE 7 Substitution of cholesterol by epicholesterol increases Kir current density. (A) Typical current traces recorded from a cell exposed to M β CD-epicholesterol and from a control cell. Both cells were maintained in 6 mM extracellular K^+ . (B) Peak current densities in control cells ($n = 31$) and in the cells 0–1 h ($n = 8$), 1–2 h ($n = 13$), 2–3 h ($n = 9$), and 5–48 h ($n = 32$) after treatment with M β CD-epicholesterol. The currents were recorded in 6 mM extracellular K^+ . All values are means \pm SE; $*p < 0.05$ vs. control. (C) Normalized current-voltage relationships: ○, control cells; ▲, cells treated with 2.5 mM M β CD-epicholesterol. Each point is the mean \pm SE ($n = 3-5$).

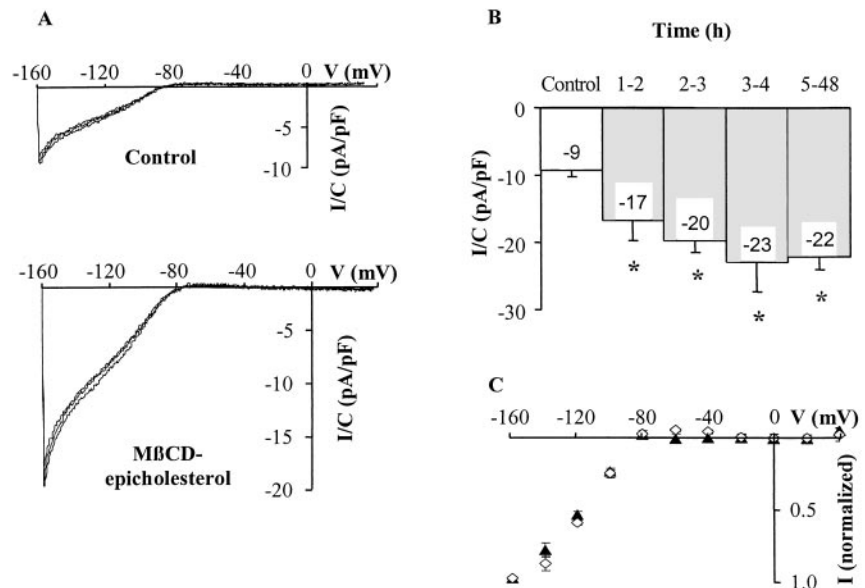
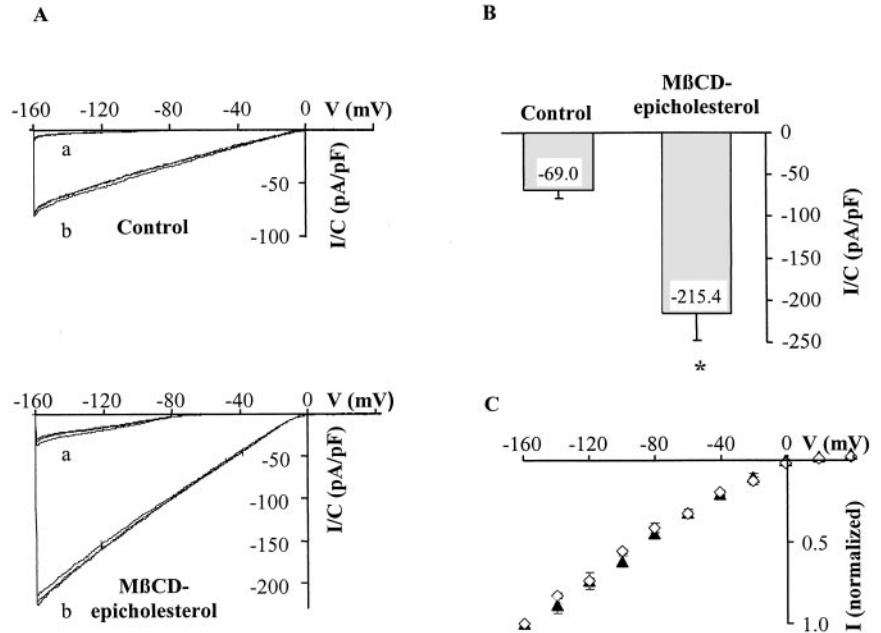


FIGURE 8 Comparison of the epicholesterol effect on Kir current in low K_o^+ and in high K_o^+ conditions. (A) Current traces recorded from a cell exposed to M β CD-epicholesterol and from a control cell in symmetrical extracellular K^+ . (B) Peak current densities at 156 mM extracellular K^+ ($n = 12$ and $n = 9$ for M β CD-epicholesterol treated cells and for control cells, respectively). All values are means \pm SE; * $p < 0.05$ vs. control. (C) Normalized current-voltage relationships under high K_o^+ conditions: \circ , control cells; \blacktriangle , cells treated with 2.5 mM M β CD-epicholesterol. Each point is the mean \pm SE ($n = 4-5$).



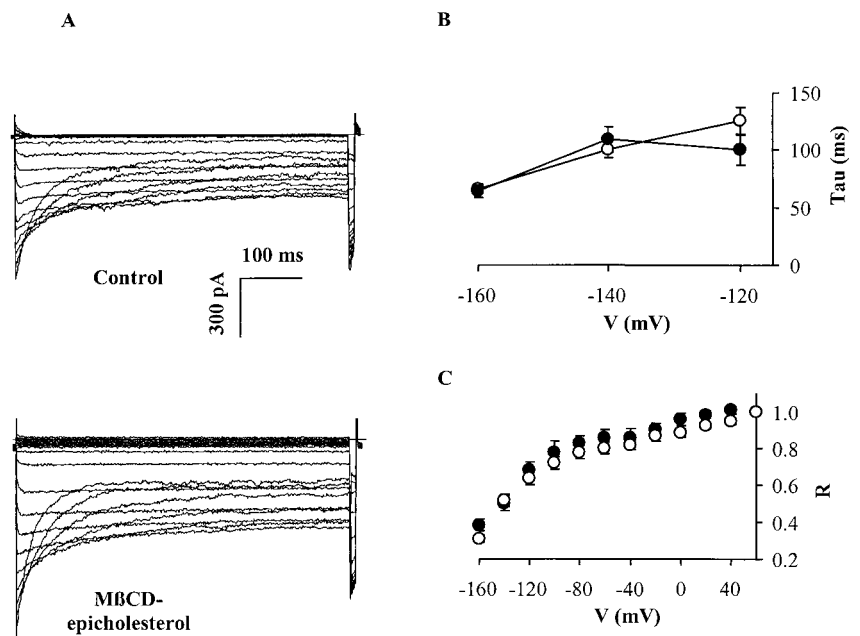
further increase in the current density during the next 2 h, the average current density measured within the 1–2-h window was not significantly different from the level of the plateau. These observations indicate that the observed effects are not caused by de novo protein synthesis.

In contrast to current density, the inactivation properties of the current were not affected by the substitution of cholesterol with epicholesterol (Fig. 9). The figure shows that both the time constant (Fig. 9 B) and the steady-state inactivation ratio (Fig. 9 C) were similar in control and in epicholesterol-treated cells.

Sterols have no effect on single-channel properties of the Kir channels

Fig. 10 A shows typical single-channel recording of a K^+ channel in BAECs under control conditions. The unitary conductance of the channel, calculated as the slope of the unitary current-voltage relationship, is 42 ± 6 pS, similar to the unitary conductance of the Kir channel in bovine vascular endothelial cells reported in the earlier studies, which was identified as Kir 2.1 by the PCR analysis (Kamouchi et al., 1997). Fig. 10, B and C, show that neither enriching the

FIGURE 9 Effect of epicholesterol on Kir inactivation. (A) Current traces elicited by two-pulse voltage protocol in epicholesterol-treated and control cells. (B and C) Time constants of inactivation (B) and inactivation ratios (C): \circ , control cells; \bullet , cells treated with 2.5 mM M β CD-epicholesterol. Each point is the mean \pm SE ($n = 3-6$).



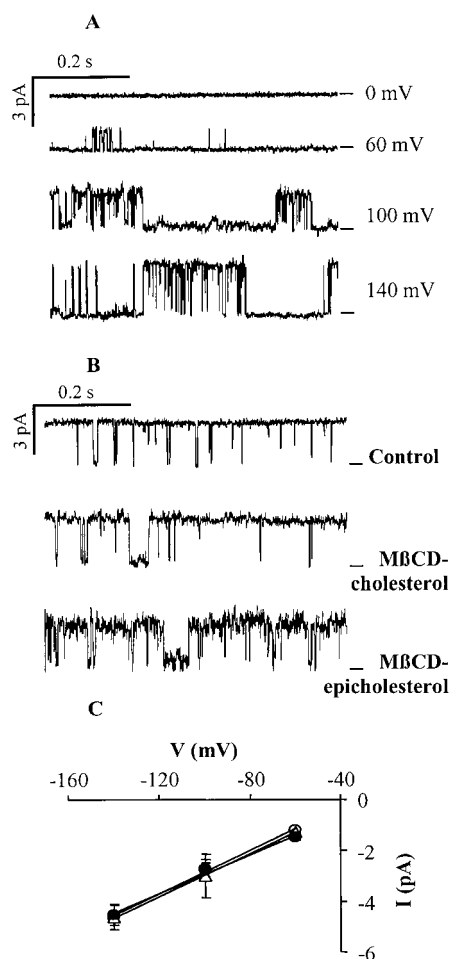


FIGURE 10 Single-channel conductance of Kir is unaffected by the membrane sterols. (A) Typical single-channel currents at different membrane potentials recorded in the cell-attached configuration in a control cell. Bath and pipette solution contained 156 mM K^+ . The currents were recorded at 0.1-ms sample intervals and filtered at 500 Hz. The closed level is indicated by the bar to the right of each trace. (B) Typical single-channel currents recorded in a control cell and in cells treated with 2.5 mM M β CD-cholesterol and M β CD-epicholesterol. (C) Current-voltage relationship for the unitary currents determined from the amplitude histograms: \circ , control cells; \triangle , cells treated with 2.5 mM M β CD-cholesterol; \bullet , cells treated with 2.5 mM M β CD-epicholesterol. Each point is the mean \pm SE ($n = 3$ –11).

cells with cholesterol nor substituting cholesterol with epicholesterol has any effect on the unitary conductance of the channels. Furthermore, modulation of the sterol composition of the membrane has no effect on the open probabilities of the channels (Table 1). In all three conditions, the open probabilities of the channels ranged between 0.8 and 0.9 with similar mean open and mean closed times. The similarity between the single-channel properties of the Kir channels in the three experimental conditions indicates that the change in Kir current density is caused by a change in the number of active channels. The number of the active channels in the membrane can be estimated by comparing the

TABLE 1 Single-channel properties of Kir are unaffected by membrane sterol composition

Treatment	Open probability	Mean open time (s)	Mean closed time (s)
Control	0.8 ± 0.1	0.3 ± 0.1	0.04 ± 0.02
M β CD-cholesterol	0.8 ± 0.2	0.5 ± 0.2	0.02 ± 0.01
M β CD-epicholesterol	0.9 ± 0.04	0.3 ± 0.1	0.02 ± 0.005

Values are means \pm SE ($n = 4$ –7).

whole-cell current density with the unitary conductance of the channels, as described in Materials and Methods. We estimate that enriching the cells with cholesterol decreases the number of active channels from ~ 320 channels per cell in control cells to ~ 210 channels per cell. In contrast, the substitution of cholesterol by epicholesterol increased the number of Kir channels to ~ 710 channels per cell.

Cholesterol level of the membrane has no effect on the cell capacitance of BAECs

To test whether cholesterol-induced decrease of the number of active Kir channels in the plasma membrane is caused by plasma membrane retrieval into intracellular compartments, we estimated the total area of the membrane by measuring membrane capacitance. It is typically assumed that the membrane bilayer is homologous to a parallel plate capacitor (Hille, 1984), and therefore, the membrane capacitance is directly proportional to the total surface area of the membrane. Measuring cell capacitance, therefore, is one of the most precise methods to determine whether membrane insertion/retrieval mechanisms are responsible for the regulation of the current density (Penner and Neher, 1989; Fomina et al., 2000; Peters et al., 2001). If the decrease in the number of K^+ channels in cholesterol-enriched cells is because of membrane retrieval, then the capacitance of the membrane is expected to be decreased. Similarly, if the epicholesterol-induced increase in the number of channels is because of membrane insertion, it is expected to be accompanied with an increase in membrane capacitance. However, consistent with our earlier study (Levitan et al., 2000), neither changes in the level of cellular cholesterol nor its substitution with epicholesterol have any effect on membrane capacitance of BAECs (Fig. 11). These observations suggest that membrane insertion/retrieval mechanisms cannot explain the effect of cholesterol on the density of the Kir current in BAECs.

Sterol composition of the membrane has no effect on the nonselective cation current

A low-amplitude, outwardly rectifying nonselective cation current was occasionally observed in a subpopulation of BAECs, as described in our earlier study (Levitan and Garber, 1998). An example of this current in a control cell

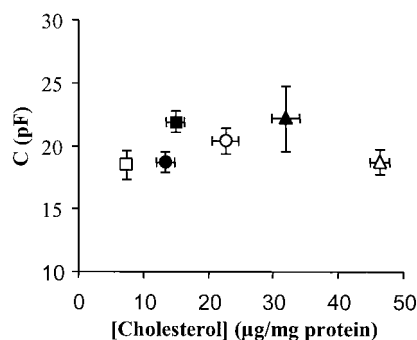


FIGURE 11 Cell capacitance is unaffected by the membrane cholesterol. Plasma membrane capacitance (C) was measured immediately after the whole-cell configuration was established. The capacitance did not change significantly during the recordings in cells treated with 5 mM M β CD (\square) or 2.5 mM M β CD (\blacksquare) (\circ , control cells), and in cells treated with 2.5 mM M β CD-cholesterol (\blacktriangle), 5 mM M β CD-cholesterol (\triangle), or 2.5 mM M β CD-epicholesterol (\bullet). Each point is the mean \pm SE ($n = 8$ –15).

is shown at Fig. 12 *A*. As expected, the reversal potential of the current is 1 ± 5 mV. To test whether the sensitivity to cholesterol is a general phenomenon of endothelial ion channels, we have examined the effects of cholesterol enrichment, cholesterol depletion, and the substitution of cholesterol by epicholesterol on the nonselective cation current. Fig. 12 *B* shows that none of these experimental conditions have any effect on the nonselective cation current in BAECs. These observations indicate that the sensitivity of the endothelial K⁺ channels to cholesterol is channel-type specific.

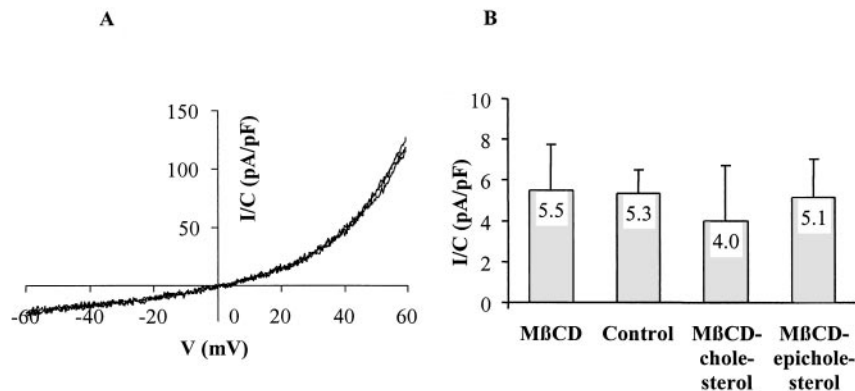
DISCUSSION

This study provides the first evidence that Kir channels are regulated by the cholesterol content of the membrane and is the first to demonstrate the stereospecificity of the cholesterol effect on nonantibiotic ion channels. The main findings of this paper are 1) that elevation of cellular cholesterol in aortic endothelial cells results in a significant decrease in Kir current density, 2) that modulation of Kir by cholesterol

is stereoselective, and 3) that changes in the sterol composition of the membrane have no effect on the single-channel properties of the Kir channels. Taken together, these observations indicate that the level of cellular cholesterol regulates the number of the Kir channels in the membrane. Titrating the level of cellular cholesterol showed that endothelial Kir is most sensitive to changes in membrane cholesterol at the cholesterol level of control cells and that the sensitivity range is limited to an \sim 50% increase or decrease in the cellular cholesterol. The high sensitivity of the endothelial Kir to the cellular cholesterol level suggests that the modulation of K⁺ channels by cholesterol may have important pathophysiological implications. Indeed, a similar (60%) increase in cellular cholesterol is associated with dietary-induced atherosclerosis (Chen et al., 1995).

The mechanism by which membrane cholesterol regulates endothelial Kir channels is different from the mechanisms responsible for cholesterol regulation of Ca²⁺-dependent K⁺ channels (Bolotina et al., 1989; Chang et al., 1995) or volume-regulated anion channels (VRACs) (Levitan et al., 2000). The effect of cholesterol on Ca²⁺-dependent K⁺ channels is due mainly to a decrease in the open probability of the channels (Bolotina et al., 1989; Chang et al., 1995) and to a much smaller extent due to a decrease in the unitary conductance of the channels (Chang et al., 1995). The effect of cholesterol on VRACs was also attributed to a decrease in the channel open probability, as described in our earlier study (Levitan et al., 2000). In contrast, the single-channel properties of the endothelial Kir channels in cells enriched with cholesterol and in cells in which cholesterol was substituted with epicholesterol were identical to those in control cells. It is important to note, however, that the properties of single channels can be analyzed only during the channel activity when the channel flickers between the closed and the open states. If a subpopulation of channels exists in the membrane in an inactive, silent, state, these channels will be invisible for the single-channel recording. The observed effect of cholesterol on the Kir channels must, therefore, be due to the regulation of the number of the active Kir channels in the membrane.

FIGURE 12 Nonselective cation current is unaffected by membrane sterols. (*A*) Typical current traces recorded at nonsymmetric K⁺ and Cl⁻ conditions from a control cell. The currents were elicited by 500-ms linear voltage ramps from -60 mV to $+60$ mV, applied with 5-s intervals for 3–6 min. The holding potential between the ramps was -60 mV. The currents were normalized to cell capacitance recorded before each voltage ramp. (*B*) Peak current densities ($+60$ mV) of the four cell populations: control cells and cells treated with 2.5 mM M β CD, M β CD-cholesterol, and M β CD-epicholesterol. Each point is the mean \pm SE ($n = 4$ –8).



What mechanism is responsible for the regulation of the number of the Kir channels by cholesterol? It is unlikely that the Kir gene expression is under regulation by cellular cholesterol level because significant upregulation of the Kir current was observed in less than 2 h after substitution of endogenous cholesterol with epicholesterol, and within 3–4 h the current reaches its new steady-state level. This period is much shorter than typical time courses for gene expression of Kir 2.1 channels. When Kir2.1 is expressed in *Xenopus* oocytes or HEC-293 or CHO-K1 cells by injection of the channel mRNA or transfection with the cDNA, a 24–48-h incubation is typically necessary to attain maximal channel activity (Forsyth et al., 1997; Nehring et al., 2000; Liu et al., 2001; Shieh and Lee, 2001). In addition, regulation of expression of both inwardly rectifying (Kir 2.1) and voltage-gated K⁺ channel (Kv1.4) proteins by various physiological and pharmacological stimuli in their native cellular systems is observed after at least 5–6 h (Levitan and Takimoto, 1998; Oonuma et al., 2002), and the maximal effect (or steady state) is reached after 12–24 h and more (Levitan and Takimoto, 1998; Nakamura et al., 1998; Fischer-Lougheed et al., 2001). We conclude, therefore, that the relatively short time needed for Kir current to respond and to reach a new steady state suggests that the regulation of the Kir channels by sterols does not occur by regulation of the cellular protein level of the channel.

Another possibility is that the decrease in the number of channels may be a result of retrieval of the plasma membrane into the intracellular compartment. This mechanism has been proposed to underlie the modulation of Kir 2.1 channels by tyrosine phosphorylation because the tyrosine-kinase-induced decrease in Kir 2.1 current density is accompanied by a significant decrease in membrane capacitance (Tong et al., 2001). Our observations show, however, that neither the cholesterol-induced decrease in the number of the Kir channels nor the epicholesterol-induced increase in the number of the channels is accompanied by changes in cell capacitance, indicating that changes in the membrane sterol composition do not induce major changes in the membrane area. In addition, the effect of cholesterol on the number of the channels is specific for the Kir channels. Our earlier study showed that whereas an increase in cellular cholesterol suppresses activation of VRACs it has no effect on the maximal level of VRAC activity, indicating that the number of the active channels in the membrane is not affected (Levitan et al., 2000). Furthermore, changes in cellular cholesterol had no effect on the nonselective cation channels in the same cells (Fig. 12). Although we cannot completely exclude the possibility that the sterol composition of the membrane regulates the retrieval of the plasma membrane and the fusion of small intracellular vesicles, which contain only Kir but not the other types of ion channels, our data suggest that membrane retrieval is not likely to account for the cholesterol-induced regulation of Kir.

These observations led us to the hypothesis that endothelial Kir channels exist in two subpopulations: active channels that flicker between the closed and the open states and silent channels that are stabilized in the closed state. Regulation of the Kir channels by cholesterol, in this case, is mediated by a shift in the distribution between the active and silent subpopulations of the channels in the plasma membrane. Cholesterol-induced activation of the silent channels would provide a mechanism that allows the cells to dynamically respond to changes in membrane cholesterol without involving either metabolic or membrane-recycling machineries.

The substitution of cholesterol by epicholesterol provides additional insights into the mechanisms underlying the regulation of the endothelial Kir channels by cholesterol. Earlier studies have suggested that the regulation of membrane proteins by cholesterol could be accounted for by either cholesterol-induced changes in the physical properties of the membrane (Bolotina et al., 1989; George and McElhaney, 1992; Gimpl et al., 1997; Sooksaware and Simmonds, 2001) or by specific sterol-protein interactions (Cornelius et al., 2001; Scanlon et al., 2001). Comparison between the effects of cholesterol and epicholesterol on the properties of Kir current in BAECs allows discrimination between these possibilities. Cholesterol and epicholesterol have similar molecular shapes (inverted cone), and both molecules promote the formation of the H_{II} phase (Cheetham et al., 1989). Formation of the H_{II} phase is expected to induce a concave monolayer curvature and to increase the structural stress of the membrane (Andersen et al., 1999). These changes manifest themselves in the decreased fluidity of the bilayer (Yeagle, 1985). As expected, cholesterol and epicholesterol have the same effect on bulk membrane fluidity, as measured by fluorescence polarization anisotropy (Gimpl et al., 1997; Xu and London, 2000). The striking difference between the effects of these isomers on endothelial Kir channels indicates that the modulation of these channels by cholesterol is not caused by changes in the bulk membrane fluidity.

Depletion of membrane cholesterol with cyclodextrins, indeed, was shown to have a disordering effect on cellular membranes (Gidwani et al., 2001). Several lines of evidence, however, suggest that cholesterol and epicholesterol have similar effects on membrane ordering. First, the two analogs were shown to induce a strong condensation effect on the area of the phospholipid molecules, a direct measure of membrane ordering (Demel et al., 1972). Mean molecular area of a phospholipid lecithin was reduced from 78 Å² (lecithin alone) to 45.7 Å² by the addition of cholesterol and to 51.1 Å² by the addition of epicholesterol. Slight differences between the two analogs have been explained by different orientations of the stereoisomers within the bilayer (Murari et al., 1986). Second, epicholesterol was shown to have an effect similar to that of cholesterol on the water permeability of liposomes (Bittman and Blau, 1972), an-

other measure of membrane ordering. Third, more recently, Xu and London (2000) showed that the formation of sterol-rich ordered domains is equally promoted by the two analogs. We conclude, therefore, that changes in membrane ordering per se are not likely to account for the dependence of the endothelial Kir on membrane cholesterol.

The similarity of the effects of cholesterol and epicholesterol on the bulk lipid properties of the membrane suggests that the striking difference between the effects of the two sterols on Kir current is caused by specific sterol-protein interactions. Specific sterol-protein interactions may form between the channel protein itself and its annular lipids, the belt of lipids that constitute the immediate environment of the channel (Barrantes, 1992), or between the sterols and other membrane proteins that modulate channel function. Indeed, several membrane proteins are modulated by cholesterol but not by epicholesterol, suggesting that specific sterol-protein interactions are responsible for these effects (Mickus et al., 1992; Gimpl et al., 1997; Sooksaware and Simmonds, 2001). In all these cases, however, substitution of cholesterol by epicholesterol was shown to induce an effect similar to that of cholesterol depletion. The novel nature of the stereospecificity of the cholesterol effect on Kir is that cholesterol/epicholesterol substitution induces a significantly greater effect on the current than cholesterol depletion. We propose, therefore, that epicholesterol competes with cholesterol for the site of specific interaction with the channel or with an accessory regulatory protein. Future studies are needed to discriminate between these possibilities.

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