Our earlier studies have shown that channel activity of Kir2 subfamily of inward rectifiers is strongly suppressed by the elevation of cellular cholesterol. The goal of this study is to determine whether cholesterol suppresses Kir channels directly. To achieve this goal, purified prokaryotic Kir (KirBac1.1) channels were incorporated into liposomes of defined lipid composition, and channel activity was assayed by $^8$Rb$^+$ uptake. Our results show that $^8$Rb$^+$ flux through KirBac1.1 is strongly inhibited by cholesterol. Incorporation of 5% (mass cholesterol/phospholipid) cholesterol into the liposome suppresses $^8$Rb$^+$ flux by $>50\%$, and activity is completely inhibited at 12–15%. However, epicholesterol, a stereoisomer of cholesterol with similar physical properties, has significantly less effect on KirBac-mediated $^8$Rb$^+$ uptake than cholesterol. Furthermore, analysis of multiple sterols suggests that cholesterol-induced inhibition of KirBac1.1 channels is mediated by specific interactions rather than by changes in the physical properties of the lipid bilayer. In contrast to the inhibition of KirBac1.1 activity, cholesterol had no effect on the activity of reconstituted KscA channels (at up to 250 $\mu$g/mg of phospholipid). Taken together, these observations demonstrate that cholesterol suppresses Kir channels in a pure protein-lipid environment and suggest that the interaction is direct and specific.

Inwardly rectifying potassium channels (Kir) are known to play critical roles in the regulation of multiple cellular functions including membrane excitability, heart rate, and vascular tone (1–3). Kir channels are classified into seven subfamilies (Kir1–7) identified by distinct biophysical properties and sensitivities to different regulators (2). Our earlier studies have shown that Kir2 channels, one of the major subfamilies of Kir that are responsible for maintaining membrane potential in a variety of cell types, are strongly suppressed by the elevation of membrane cholesterol (4, 5). Kir2 channel-induced suppression of Kir2 was first observed in aortic endothelial cells (4), in which resting K$^+$ conductance is dominated by Kir2.1 and Kir2.2 channels (6), and then when channels were heterologously expressed in Chinese hamster ovary cells (5, 7). Furthermore, the same effect was observed ex vivo in endothelial cells and bone marrow-derived progenitor cells isolated from hypercholesterolemic pigs (8, 9).

In terms of the mechanism, the first insights came from comparing the effects of cholesterol and of its chiral analogue, epicholesterol. Although the two sterols are known to have almost identical effects on the biophysical properties of the lipid bilayer (10, 11), their impact on Kir activity is completely different; partial substitution of endogenous cholesterol with epicholesterol resulted in significant increase in Kir current in endothelial cells (4). These observations suggest that specific sterol-protein interactions may be involved in the cholesterol sensitivity of Kir2 channels. However, in the complex environment of the plasma membrane, cholesterol may interact not only with the channels themselves but also with other proteins, which in turn may regulate the activity of the channels. In the cellular environment, therefore, it is impossible to discriminate between direct channel-cholesterol interactions and indirect effects. Moreover, it is impossible to define the actual concentrations of cholesterol in any given membrane compartment. To quantitatively test direct cholesterol-protein interactions, it is necessary to examine sensitivity of pure Kir channels to membrane cholesterol in a defined lipid composition. To date, only the cytoplasmic domains of several mammalian Kir channels have been purified (Kir2.1, Kir3.1, and Kir3.2) (12–15). We therefore concentrate in this study on the effect of cholesterol on two bacterial K$^+$ channels that differ in the level of their homology to mammalian Kir channels, KirBac1.1 and KcsA. KirBac channels have high sequence homology with mammalian Kirs (e.g. 52% homology between KirBac1.1 and Kir2.1; see Fig. 7A) and have now been extensively used as structural models of mammalian Kir channels (3, 16, 17). The sequence similarity between KcsA and mammalian K channels lies mainly in the transmembrane domain (18). The overall sequence homology of KcsA to mammalian Kir channels is relatively low (e.g. 22% homology between KcsA and Kir2.1; see Fig. 7A), with an entirely different cytoplasmic domain structure.

Here we show that, similarly to Kir2 channels, prokaryotic Kir channels incorporated into liposomes are strongly suppressed by an increase in membrane cholesterol. Furthermore, the sensitivity of prokaryotic Kir to cholesterol is stereo-selective to cholesterol optical analogues. In contrast, KscA channels are insensitive to membrane cholesterol. These observations suggest that cholesterol directly suppresses Kir channels.
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**EXPERIMENTAL PROCEDURES**

Dowex 50x-4-100, Sephadex g50, N-methyl-d-glucamine (NMG), and cholesterol were obtained from Sigma, and CHAPS was purchased from Aldrich. POPG and POPE were obtained from Avanti lipids. All other sterols were purchased from Steraloids Inc. Polystyrene column bodies were purchased from Pierce, and $^{86}$Rb$^+$ was obtained from PerkinElmer Life Sciences and Analytical Sciences.

**Purification of KirBac1.1 and KcsA Proteins—**Protein purification was performed, as previously described (19). Transformed *Escherichia coli* BL21 GOLD (DE3) pLysS cells were grown in a shaker at 37 °C until an $A_{600}$ of 1.0 was reached. Protein expression was induced with 1 mM isopropyl-D-thiogalactopyranoside, and the cells were grown for an additional 3 h at 37 °C. The cells were harvested, lysed by a freeze-thaw cycle, and resuspended (50 mM Tris-HCl, pH 8.0, 150 mM KCl, 10 mM imidazole, 30 mM decylmaltoside, and one EDTA-free protease inhibitor mixture tablet). The suspension was gently rocked for 2–4 h at room temperature and then centrifuged at 30,000 × g for 45 min. The supernatant was mixed with cobalt affinity beads for 2 h. The supernatant/bead mixture was moved to an empty column, and the beads were extensively washed with 20–30 bed volumes of wash buffer (50 mM Tris-HCl, pH 8.0, 150 mM KCl, 10 mM imidazole, 5 mM decylmaltoside). Target protein was eluted with 1–2 ml of wash buffer containing 500 mM imidazole. The purity of KirBac1.1 and KcsA isolations was verified using SDS-PAGE (supplemental Fig. S1). Both KirBac1.1 and KcsA show a single major band corresponding to the expected molecular weight. In addition, KirBac1.1 has a minor band of slightly lower molecular weight, which we have shown previously to be recognized by an anti-His antibody, a tag that is attached to the channels, indicating that a minor band is also a modification of KirBac1.1 (19). There is also a minor band of higher molecular weight corresponding to a dimer of KirBac1.1 subunit. KcsA also shows two minor bands corresponding to a dimer and a tetramer, as reported previously (20).

**Measurement of $^{86}$Rb$^+$ Uptake—**Rubidium flux assay was also performed as described earlier (19) with minor modifications. In brief, disposable polystyrene columns (Pierce) were packed with Sephadex G-50 (fine) beads (1 ml), swollen overnight in buffer A or B (buffer A: 450 mM KCl, 10 mM HEPES, 4 mM NMG, pH 7; buffer B: 450 mM sorbitol, 10 mM HEPES, 4 mM NMG, 50 μM KCl, pH 7.0). Purified KirBac1.1 or KcsA proteins (2.5–10 μg/ml of total lipid) was added to CHAPS (37 mM) solubilized mixture of phosphatidylethanolamine:phosphatidylglycerol (9:1, Avanti Polar Lipids, Inc., 10 mg of total lipid/ml) in buffer A and incubated for 30 min. The choice of lipids was based on a well established protocol for assaying the activity of purified KirBac1.1 channels, as described in earlier studies (19, 21–23). The ratio of 9:1 PE/PG was also based on previous studies, in which it was established that this lipid composition yields the highest stability of the liposomes, as determined by the maximal uptake of $^{86}$Rb$^+$ (23).

**RESULTS**

**Cholesterol Inhibits Purified KirBac1.1 Channels—**Earlier studies have shown that purified KirBac1.1 protein incorporated into liposomes forms a functional K$^+$ channel, as assessed by selective uptake of Rb$^+$ (19). In a typical experiment, liposomes with and without the protein are exposed to $^{86}$Rb$^+$ in the external medium at time 0, and the uptake is monitored over time. Maximal uptake is measured after the addition of a K$^+$

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4 The abbreviations used are: NMG, N-methyl-d-glucamine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac(1-glycerol) (sodium salt); POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; PL, phospholipid.
Bac1.1 protein after the addition of valinomycin, indicating that incorporation of the channels does not interfere with liposome formation or integrity. At high protein concentration (10 μg/mg of PL), the uptake was similar to that observed after the addition of valinomycin, and therefore, an intermediate concentration of 5 μg of KirBac1.1/mg of PL was used for the rest of the experiments.

The first finding of this study is that incorporation of cholesterol strongly suppresses KirBac1.1-mediated 86Rb+ flux (Fig. 1B), indicating that the addition of cholesterol per se is sufficient to suppress the channels and excluding the possibility that additional protein or other cellular intermediates are required (Fig. 1B). In this experiment, incorporation of 50 μg of cholesterol/mg of PL (5%) cholesterol resulted in a more than 3-fold decrease in KirBac-mediated 86Rb+ uptake but had no effect on 86Rb+ uptake in liposomes containing no protein. The latter observation indicates that the addition of cholesterol at this concentration does not affect nonspecific 86Rb+ background counts. No effect was also observed on maximal 86Rb+ uptake after the addition of valinomycin, verifying that incorporation of cholesterol does not interfere with liposome formation.

In the next series of experiments, we compared KirBac1.1 activity in liposomes containing 4–120 μg/mg of PL (Fig. 2). A significant decrease in KirBac1.1-mediated 86Rb+ uptake was observed above 25 μg/mg of PL with complete inhibition of channel activity at 80–120 μg/mg of PL. To compare multiple cholesterol conditions across independent experiments, 86Rb+ uptake in each experiment was normalized to the uptake level at 240 s measured in liposomes containing no cholesterol in the same experiment (Fig. 2B). It is important to note that KirBac1.1 is inhibited by levels of cholesterol that are significantly lower than normal cholesterol levels in the plasma membranes of mammalian cells, which range between 30 and 50% of total membrane lipids (25, 26). Thus, KirBac1.1 channels would be expected to be completely closed at normal cholesterol levels of mammalian cells. As pointed out before, an increase in membrane cholesterol in this range (4–120 μg/mg of PL) had no effect on the amount of uptake after the addition of valinomycin, excluding the possibility that a cholesterol-induced decrease in 86Rb+ flux could be partially due to a decrease in the number and/or size of the liposomes (Fig. 2B, inset). Similar effects were observed when cholesterol was added to the lipid mixture before or after incorporation of channel protein. Specifically, when cholesterol was incorporated into liposomes after reconstitution with KirBac1.1 protein, it also significantly inhibited KirBac1.1-mediated 86Rb+ flux (supplemental Fig. S2). These observations exclude the possibility that cholesterol inhibition could be attributed to decreased incorporation of KirBac1.1 protein into the liposomes.

### Sensitivity of KirBac1.1 to Cholesterol Chirality—
We next asked whether cholesterol-induced inhibition of KirBac1.1 might be attributed to changes in the physical properties of the lipid bilayer. It is well known that an increase in membrane cholesterol decreases membrane fluidity (25, 26), increases membrane stiffness (27, 28), and alters the packing of phospholipids (29, 30). Furthermore, a variety of membrane proteins have been shown to be sensitive to changes in the physical properties of the membrane (26, 31). Alternatively, channels may be

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**FIGURE 1.** Cholesterol suppresses KirBac1.1-mediated 86Rb+ uptake. A, time courses of 86Rb+ uptake (disintegrations per minute (DPM)) into liposomes (POPE:POPG, 9:1) containing no cholesterol for different levels of KirBac1.1 protein (0.0, 2.5, 5.0, or 10.0 μg/mg of PL). Valinomycin (0.1 μg/mg of PL) was added at 240 s, and 86Rb+ was measured 1 min following the addition of valinomycin. The curves represent the time courses obtained in the same experiment. B, time courses of 86Rb+ uptake into liposomes reconstituted with 50 μg of cholesterol/mg of PL and as compared with liposomes containing no cholesterol (control). Both batches of liposomes contained 5 μg of KirBac1.1/mg of PL. Blank liposomes contain no protein. The points represent the averages of three independent experiments (means ± S.D.) with each experiment normalized to control liposomes that contain no cholesterol and that were measured on the same day. Note that 86Rb+ is a short life radionuclide with half-life of 18.66 days, which results in a large variability in counts between different days. Each experiment, therefore, includes a double control set of liposomes, the first with channels but no cholesterol and the second with blank liposomes that do not include either channels or cholesterol.

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regulated by specific cholesterol-protein interactions (26, 31).

To discriminate between these possibilities, we compared KirBac1.1 activity in liposomes containing cholesterol or its chiral analogue, epicholesterol. The only difference between the two sterols is the orientation of the hydroxyl group in position 3 (see the structures in Fig. 4), and both have similar effects on membrane physical properties (10, 11). However, epicholesterol is significantly less efficient than cholesterol in inhibiting KirBac1.1-mediated \[^{86}\text{Rb}^+\] uptake (Fig. 3). At lower concentrations (25 \(\mu\text{g/mg}\)), cholesterol significantly suppressed KirBac1.1 activity, but epicholesterol had no effect. There was also a significantly lower maximal effect of epicholesterol. Different effects of cholesterol and epicholesterol suggest that channel inhibition is unlikely to be fully accounted for by changes in the physical properties of the lipid bilayer. It is also possible that epicholesterol can bind directly to the channel protein but with a lower affinity than cholesterol, as was shown previously for Scap-1 protein (32). A relationship between KirBac1.1 activity and membrane fluidity is explored further in the next part of the study.

**Structural Sterol Analysis of KirBac1.1 Inhibition**—To obtain further insights into the mechanism of cholesterol-induced inhibition of KirBac1.1 channels, we compared the effects of multiple sterols that differ from cholesterol either in the structure of the side chain (group A), or of the rings (group B), or both (group C) (Fig. 4). In group A, we tested five different sterols: 25-hydroxycholesterol (5-cholesten-3\(,25\)-diol), which...
differs from cholesterol by having an additional hydroxyl group in position 25; desmosterol (5, 24-cholestadien-3β-ol), which has an additional double bond at position 24; and three sterols that have an additional ethyl, ethylene, or methyl group in position 24: β-sitosterol (5-cholesten-24β-ethyl-3β-ol), fucosterol (5-cholesten-24(28)-ethylene-3β-ol), and campesterol (5-cholesten-24α-methyl-3β-ol) respectively (Fig. 4). Of these five sterols, only 25-hydroxycholesterol did not have an effect on KirBac1.1-mediated 86Rb− uptake, whereas desmosterol and β-sitosterol had partial effects, and fucosterol and campesterol were indistinguishable from cholesterol (Fig. 5, A and B). In group B, five sterols were tested: 19-hydroxycholesterol (5-cholesten-3β,19-diol), which has an additional hydroxyl group in position 19; epicholesterol (5-cholesten-3α-ol), which, as described above, differs from cholesterol (5-cholesten-3α-ol) by the orientation of the hydroxyl group at position 3; and coprostanol (5β-cholestan-3β-ol), epicoprostanol (5β-cholestan-3α-ol), and cholestanol (5α-cholestan-3β-ol), which lack a double bond at position 5. In addition, epicoprostanol also differs from cholesterol in the orientation of the hydroxyl group at position 3, and cholestanol differs from coprostanol in the orientation of the hydrogen at position 5. In this group, the only sterol that did not inhibit KirBac1.1-mediated 86Rb+ flux was 19-hydroxycholesterol, whereas epicholesterol, as described above, had an intermediate effect, and coprostanol and cholestanol had the same effect as cholesterol (Fig. 5, C and D). Finally, in group C, we tested three more sterols: 5-adrosten 3β-17β-diol, which has no side chain but has an extra hydroxyl group in the ring structure at position 17; ergosterol, which, similarly to campesterol, has an additional methyl group at position 24 and two additional double bonds at positions 22 (side chain) and 7 (rings); and stigmastanol, which has an ethyl group at position 24 (side chain) and a lack of double bond at position 5 (rings). In this group, 5-adrosten 3β-17β-diol had no effect on KirBac1.1, ergosterol had a partial effect, and stigmastanol was identical to cholesterol (Fig. 5, E and F). In summary, the three sterols without effect on KirBac1.1 function all have additional hydroxyl groups at positions 25, 19, or 17, suggesting that the addition of a hydroxyl group significantly interferes with sterol-channel interactions.

To examine whether there is a correlation between the inhibition of KirBac1.1 activity and membrane fluidity, we estimated membrane anisotropy for liposomes that incorporated each of the sterols used in this study. Even though earlier studies have already estimated the effects of these sterols on membrane anisotropy in different types of membranes, the exact effects may depend on other lipids in the membrane, and it is important, therefore, to perform these measurements in the same set of liposomes as used in the rest of the study. Fig. 6 shows the relationship between KirBac1.1 activity and membrane anisotropy measured in the same liposomes. As expected, incorporation of cholesterol, as well as of several other sterols, significantly increases the membrane anisotropy, indicating that membrane fluidity is decreased. However, most importantly, there is no correlation between the decrease in KirBac1.1 activity and anisot-
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KirBac1.1 activity. Conversely, sterols that are similar to cholesterol in their suppression of KirBac1.1 activity, such as coprosterol, fucosterol, campesterol, and stigmasterol, have very different effects on membrane anisotropy.

KcsA Activity Is Not Inhibited by Cholesterol—Finally, we tested whether cholesterol also inhibits KcsA channels similarly purified and incorporated into liposomes. Earlier studies have shown that KcsA can be incorporated into liposomes and form functional K⁺ channels (19, 33, 34). We observed significant KcsA-mediated ⁸⁶Rb⁺ flux (Fig. 7A), but, in contrast to KirBac1.1, KcsA-mediated ⁸⁶Rb⁺ uptake is insensitive to membrane cholesterol (Fig. 7B) up to 250 μg/mg of PL, double the concentration required to completely inhibit KirBac1.1.

DISCUSSION

Cholesterol Modulation of Ion Channels—A growing number of studies have shown that a variety of ion channels, including multiple types of K⁺ channels, are regulated by changes in membrane cholesterol (reviewed in Refs. 35 and 36), but the mechanisms responsible for these effects are still poorly understood. A fundamental—and unanswered—question is whether cholesterol regulates channels by direct interactions or indirectly via intermediary regulatory molecules. In the present study, we show that cholesterol suppresses purified KirBac1.1 channels incorporated into artificial liposomes of a defined composition, in the absence of additional proteins. Furthermore, KirBac1.1 channels have a highly differential sensitivity to an array of different sterols ranging from strong inhibition to lack of any detectable effect depending on the structure of the sterol. Finally, we show that although KirBac1.1 channels are highly sensitive to changes in membrane cholesterol, KcsA channels are insensitive. Taken together, these observations suggest that cholesterol directly interacts with KirBac1.1 channels but does not interact with KcsA channels.

FIGURE 5. Differential effects of sterol on KirBac1.1-mediated ⁸⁶Rb⁺ uptake. A, C, and E, time courses of Rb⁺ uptake in liposomes containing the sterols from groups A, B, and C as defined in the legend to Fig. 4, respectively. 50 μg of sterol/mg of PL were used for all the sterols. All of the time courses are normalized to control liposomes in the same experiment. B, D, and F, maximal uptake of ⁸⁶Rb⁺ for the conditions defined above. All of the data points represent the means ± S.D. of three to seven independent experiments performed for each sterol. *, p < 0.05. All of the experiments included control liposomes containing no sterol and liposomes containing 50 μg cholesterol/mg of PL as a positive control. 25-HC, 25-hydroxycholesterol; Desm, desosterol; β-Sito, β-sitosterol; Camp, campesterol; Fuco, fucosterol; Chol, cholesterol; Copro, coprosterol; 19-HC, 19-hydroxycholesterol; Epicopro, epicoprosterol; Epi-chol, epicholesterol; Andro, 5-androsten 3β-17β-diol; Ergo, ergosterol; Stigma, stigmastanol. *, significant difference (p < 0.05) between a specific sterol and control; #, between a specific sterol and cholesterol (p < 0.05).
Models for Cholesterol-Channel Interactions—The most common hypothesis to explain the sensitivity of ion channels to cholesterol involves the association of the channel with cholesterol-rich domains (lipid rafts) (35, 37). In the lipid raft model, it is generally proposed that cholesterol facilitates formation of membrane domains that segregate membrane proteins into signaling complexes, thereby providing a platform for specific protein-protein interactions. Indeed, multiple types of ion channels, including different types of Kir channels (5, 7, 38, 39), voltage-gated K\(^+\) channels (40–42), and large conductance Ca\(^{2+}\)-sensitive K\(^+\) channels (43) have been shown to partition into cholesterol-rich domains. It has been proposed, therefore, that interaction of the channels with other components of these domains, such as caveolin or the regulatory phospholipid phosphatidylinositol 4,5-bisphosphate, PI(4,5)P\(_2\), may be the underlying molecular basis for the apparent sensitivity of the channels to cholesterol. In this study we show that, at least for prokaryotic Kir channels, no other proteins or regulatory lipids are required and that changes in cholesterol per se are clearly sufficient to regulate the activity of KirBac channels.

Specific versus Nonspecific Channel-Cholesterol Interactions—In terms of how ion channels or other proteins may be directly regulated by their cholesterol environment, two general models have been proposed: 1) a hydrophobic mismatch model, in which ion channels are regulated by changes in the physical properties of membrane lipid bilayer (specifically, it is proposed that hydrophobic interactions between the channel protein and the surrounding lipids may regulate the ability of the protein to undergo conformational changes, e.g. between closed and open states (44–46)), or 2) direct specific interaction between cholesterol and the protein (47–49). Our observations strongly support the possibility that KirBac1.1 channels interact with cholesterol in a manner that is not dependent on the presence of other proteins or lipids.
cholesterol directly through specific sterol-protein interactions. First, we show that KirBac1.1 is significantly more sensitive to cholesterol than to its chiral analogue, epicholesterol. The two sterols were shown in earlier studies to have similar effects on the water permeability of the liposomes (50), a measure of membrane ordering, and on membrane fluidity (10, 11). However, it was also shown that cholesterol and epicholesterol reside in the lipid bilayer at different angles (51), which may result in some differences in the lipid packing and membrane fluidity. Therefore, we performed further structural analysis of multiple sterols that were shown earlier to have distinct effects on membrane fluidity ranging from virtually no effect to an effect similar to that of cholesterol (10). No correlation was found between the effects of the sterols on KirBac1.1 activity.

FIGURE 8. Comparison of KirBac1.1, KcsA, and Kir2.1 structures. A, alignment of Kir2.1, KirBac1.1, and KcsA. Identical residues are colored red, strongly similar residues are blue, and weakly similar residues are green. B, two opposite facing subunits of KirBac1.1 and KcsA showing the low similarity between the cytosolic domains of the channels. The figures are based on the crystallographic structure of KirBac1.1 (Protein Data Bank code 1P7B) and the full-length model of KcsA (Protein Data Bank code 1F6G) that was constructed from crystallographic and EPR data. C, the cytosolic domain of Kir2.1 and KirBac1.1 share a similar fold as can be seen following alignment of the crystallographic structures of KirBac1.1 (Protein Data Bank code 1P7B) and the cytosolic domain of Kir2.1 (Protein Data Bank code 1U4F).
and their effects on membrane fluidity, indicating that changes in membrane fluidity cannot account for the effects of the sterols on KirBac1.1 activity.

In contrast, there is a striking correlation between the effects of sterols on KirBac1.1 activity and their binding to a sterol-sensing domain of SCAP, a protein that controls the transport and proteolytic activation of sterol regulatory element-binding proteins (32). Specifically, the three sterols that have been identified in our study not to affect KirBac1.1 (25-hydroxycholesterol, 19-hydroxycholesterol, and 5-androsten 3β-17β-diol) were also shown not to bind purified SCAP protein. Conversely, desmosterol and β-sitosterol, which are similar to cholesterol in binding SCAP, also inhibit KirBac1.1, although less efficiently than cholesterol. Another interesting parallel between the two studies is the effect of epicholesterol. Similarly to our study, Radhakrishnan et al. (32) showed a partial effect, indicating that epicholesterol may also bind to proteins and compete with cholesterol. Therefore, although it is impossible to fully exclude the possibility that sterols may affect KirBac1.1 activity through subtle changes in membrane structure unrelated to fluidity, taken together, our data strongly point to a direct cholesterol-channel interaction as the underlying basis for channel inhibition.

Specific Interaction of Cholesterol with Kir—Comparison between highly cholesterol-sensitive KirBac1.1 and cholesterol-insensitive KcsA provides some clues to the potential structural basis of cholesterol-Kir interaction. Both KirBac1.1 and KcsA have two transmembrane domains, M1 and M2, that flank the pore-forming P segment (16, 18). The sequence homology between the transmembrane domain of KirBac1.1 and KcsA is quite high (~74%; see Fig. 8A), and the overall transmembrane topology of the two channels is very similar (Fig. 8B). The eight-residue signature sequence of the selectivity filter, TXXTXGYG, (52) also has substantial sequence identity (75%; see Fig. 8A), being the most conserved region in potassium channels in general and in these two channels in particular. The cytosolic domains of KirBac1.1 and KcsA channels, however, are dramatically different (Fig. 8B). Although KcsA has only a short helix-structured cytosolic domain, with no resemblance to the cytosolic domains of mammalian Kir channels, KirBac1.1 is defined by a cytosolic domain with significant homology to mammalian Kirs. Fig. 8C depicts an alignment of the cytosolic domain of Kir2.1 with the cytosolic domain of KirBac1.1. We recently showed that cholesterol sensitivity of Kir2.1 channels depends critically on the CD loop of the C-terminus cytoplasmic domain (53). Furthermore, it has been shown that a residue on the boundary between a transmembrane helix (helix 6) and a cytosolic loop, Asp<sup>143</sup>, in SCAP affects its sensitivity to sterols (54). Together, these data suggest a critical role for the cytosolic domain of Kir channels in determining their cholesterol sensitivity.

Based on the high sequence similarity between the bacterial KirBac channels and mammalian Kir channels, crystallographic structures of KirBac channels have been used to construct homology models of several members of the eukaryotic Kir channel family (54–57). Furthermore, using a system of purified proteins incorporated into liposomes, we have previously demonstrated that KirBac1.1 channels exhibit many of the same key properties as eukaryotic Kir channels (19, 21, 22). Specifically, KirBac1.1, like eukaryotic channels, is highly sensitive to membrane phosphatidylinositol 4,5-bisphosphate (22). However, in contrast to mammalian Kir channel activity, which is enhanced by phosphatidylinositol 4,5-bisphosphate (56, 58–60), KirBac1.1 activity is inhibited, possibly because of subtle differences in the channel structure in the regions linking the transmembrane and cytosolic domains (22). In our current study, we show that the general impact of cholesterol on KirBac1.1 is the same as on Kir2 channels, although KirBac1.1 appears to be more sensitive, which may also be due to small differences in channel structure. It is important to note that cholesterol effects on different Kir channels may vary (61). For example, Kir4 channels were shown to be inhibited by cholesterol depletion rather than by cholesterol enrichment (38). In addition, Kir6 channels may also be regulated by the effects of cholesterol on cAMP-dependent protein kinase and protein kinase C (39, 62).

The present study demonstrates a direct effect of cholesterol on the activity of pure KirBac1.1 channels in a membrane of defined composition. This suggests that cholesterol may regulate the function of inwardly rectifying potassium channels directly.

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