Biochimica et Biophysica Acta 1808 (2011) 2527–2533

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Cholesterol regulates prokaryotic Kir channel by direct binding to channel protein

Dev K. Singh [a,*], Tzu-Pin Shentu [a], Decha Enkvetchakul [b], Irena Levitan [a]

[a] Section of Respiratory, Critical Care and Sleep Medicine, Department of Medicine, University of Illinois, Chicago, IL 60612, USA
[b] Department of Pharmacological and Physiological Science, Saint Louis University, Saint Louis, MO 63104, USA

ARTICLE INFO

Article history:
Received 27 January 2011
Received in revised form 7 July 2011
Accepted 9 July 2011
Available online 26 July 2011

Keywords:
Cholesterol
KirBac1.1
Lipid–protein interaction
Lipid-bilayer
Ion channel

ABSTRACT

Cholesterol is a major regulator of a variety of ion channels but the mechanisms underlying cholesterol sensitivity of ion channels are still poorly understood. The key question is whether cholesterol regulates ion channels by direct binding to the channel protein or by altering the physical environment of lipid bilayer. In this study, we provide the first direct evidence that cholesterol binds to prokaryotic Kir channels, KirBac1.1, and that cholesterol binding is essential for its regulatory effect. Specifically, we show that cholesterol is eluted together with the KirBac1.1 protein when separated on an affinity column and that the amount of bound cholesterol is proportional to the amount of the protein. We also show that cholesterol binding to KirBac1.1 is saturable with a K_D of 390 μM. Moreover, there is clear competition between radioactive and non-radioactive cholesterol for the binding site. There is no competition, however, between cholesterol and 5-Androsten 3β-17 β-diol, a sterol that we showed previously to have no effect on KirBac1.1 function. Finally, we show that cholesterol–KirBac1.1 binding is significantly inhibited by trifluoperazine, known to inhibit cholesterol binding to other proteins, and that inhibition of cholesterol–KirBac1.1 binding results in full recovery of the channel activity. Collectively, results from this study indicate that cholesterol-induced suppression of KirBac1.1 activity is mediated by direct interaction between cholesterol and the channel protein.

Published by Elsevier B.V.

1. Introduction

A variety of ion channels are regulated by changes in the level of membrane cholesterol, a major lipid component of the plasma membrane (reviewed by [1,2]). The mechanisms, however, of cholesterol regulation of ion channels are still poorly understood. The prevailing hypothesis is that changes in membrane cholesterol regulate ion channel activity by affecting the integrity of the signaling platforms that form within the environment of cholesterol-rich membrane domains and by disrupting the interactions of the channels with various signaling or regulatory molecules [3–8]. Alternatively, it has also been suggested that ion channels may be regulated by cholesterol as an annular lipid [9] or by cholesterol-induced changes in the elastic properties of the membrane lipid bilayer [10,11]. In this study, we investigate the mechanism of cholesterol regulation of a prokaryotic inwardly-rectifying K^+ channel, KirBac1.1, and provide the first direct evidence for binding between cholesterol and a purified ion channel.

Inwardly-rectifying K^+ channels (Kir) is a major class of K^+ channels known to play critical roles in the regulation of multiple cellular functions including membrane excitability, heart rate and vascular tone [12–14]. Kir channels are classified into seven sub-families (Kir1-7) identified by distinct biophysical properties and sensitivities to different regulators and differentially expressed in different tissues [13–15]. Cholesterol regulation of Kir channels was first demonstrated in our earlier studies for Kir channels in endothelial cells [16] that express predominantly Kir2.1 and Kir2.2 [17]. The same effect was observed when Kir2 channels were heterologously expressed in a null cell line [18,19]. Furthermore, Kir channels are suppressed by plasma hyper-cholesterolemia in vivo in aortic endothelial cells and in bone-marrow derived progenitor cells [20,21]. More recently, we have shown that members of all the sub-families of Kir channels are sensitive to cholesterol with a predominant effect being cholesterol-induced suppression [22]. In terms of the mechanism, our recent studies provided the first insights into the structural determinants of cholesterol regulation of Kir channels by demonstrating that it critically depends on a specific region in the C-terminus of the cytosolic domain, the CD loop [22,23], as well as several other residues in the cytosolic domain that form a "cholesterol sensitivity belt" around the channel pore [24].

However, in the complex environment of the plasma membrane, it was not possible to rule out the possibility that cholesterol regulates the channels through other intermediates. To resolve this difficulty, we have turned to a bacterial homologue of Kir channels, KirBac1.1 that has high sequence homology with mammalian Kirs. Specifically, the overall similarity between KirBac1.1 and different Kir2 channels ranges between 47% (21% identity, 17% strong similarity, 9% weak

* Corresponding author at: Section of Respiratory, Critical Care and Sleep Medicine, Department of Medicine University of Illinois at Chicago, IL 60612–7323, USA. Tel.: +1 312 413 8502; fax: +1 312 355 0748.
E-mail address: dksingh@uic.edu (D.K. Singh).

0005-2736/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.bbamem.2011.07.006

similarity for Kir2.3) and 52% (20% identity, 21% strong similarity, 11% weak similarity for Kir2.1). Indeed, in recent years, KirBac channels have been extensively used as a structural model of mammalian Kir in order to understand the general principles of the channel function and the mechanisms by which the channels are regulated by different agents [12,25,26]. The strength of this approach is that bacterial Kir channels can be easily purified and reconstituted into liposomes of well-defined lipid composition [27,28]. Using this system, we have demonstrated that cholesterol suppresses purified KirBac1.1 channels ruling out the possibility that any intermediate signaling molecules are required to mediate cholesterol sensitivity of these channels [29]. Moreover, the homology between KirBac1.1 and Kir2 channels in the sequence of their CD loops, the region that we have recently shown to be critical for cholesterol sensitivity of the channels [23] is remarkably high: the similarly between all Kir2 channels and KirBac1.1 channels in the CD loop is 77% (22% identity, 22% strong similarity and 33% weak similarity) further supporting the notion that KirBac1.1 represents an appropriate model to study cholesterol regulation of Kir channels. It is important to note, however, that while mutations of the residues in the CD loop abrogates the sensitivity of Kir2.1 channels to cholesterol, this does not mean that cholesterol binds to this domain directly. Furthermore, more recently, we showed that while multiple potential binding sites can be identified in the Kir protein using docking analysis, none of them overlap with the CD loop or with more extended "cholesterol sensitivity belt" leading us to the conclusion that these domains are regulatory sites rather than cholesterol binding sites [24]. Moreover, the question of whether cholesterol binding is required for cholesterol sensitivity of the channels remained open.

In this study, we present first direct evidence that cholesterol binds to purified KirBac1.1 channel protein and that cholesterol–Kir binding is required for cholesterol suppression of the channel activity.

2. Methods

Dowex 50×4-100, Sephadex g50, N-methyl D-glucamine (NMG) and cholesterol were obtained from Sigma Chemical Co. and 3-[(3-Cholamidopropyl) dimethyl-ammonio]-propanesulfonate hydrate (CHAPS) were purchased from Aldrich USA. 1-Palmitoyl-2-oleoyl-Sn-glycero-3-[phospho-rac-(1-glycerol) (sodium salt)] (POPG) and 1-Palmitoyl-2-oleoyl-sn-glycero-3[phosphoethanolamine) (POPE) were obtained from Avanti lipids. Polystyrene column bodies were purchased from Pierce Chem. Inc. USA. $^{86}Rb^{+}$ was obtained from Perkin ELMER Life and Analytical Sciences, USA and ^{3}H-cholesterol was obtained from American Radiolabeled Chemicals, Inc.

2.1. Purification of KirBac1.1 proteins

Protein purification was performed, as previously described [27]. Briefly, transformed *E. coli* BL21 GOLD (DE3) pLysS cells were grown in a shaker at 37 °C until an OD600 of 1.0 was reached. Protein expression was induced with 1 mM isopropyl-D-thiogalactopyranoside and cells were grown for an additional 3 h at 37 °C. The cells were harvested, lysed by a freeze–thaw cycle, and resuspended in 50 mM Tris–HCl pH 8.0, 150 mM KCl, 10 mM imidazole, 30 mM decylmaltoside and one EDTA-free protease inhibitor cocktail 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 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. This procedure yields purified KirBac1.1 proteins, as was verified using SDS-PAGE [27,29].

2.2. Cholesterol binding assay

Cholesterol binding assays were performed as described earlier [30] with minor modification. In brief: desired concentration of ^{3}H-cholesterol or sterols were dried under nitrogen and then solublized in 100 μl of buffer A (50 mM Tris, pH 7.5 containing 150 mM NaCl and 0.1% of CHAPS) then pre-incubated with KirBac1.1 (1.5 μg) at 37 °C. After pre-incubation for 2 h, the assay mixture was diluted to 1 ml and loaded on a column prepacked with 0.5 ml of Ni-NTA-Agarose beads pre-equilibrated with wash buffer (Buffer A with no CHAPS). After 1 h of incubation, unbound cholesterol was washed seven times (1 ml each time) with washing buffer. And finally, the bound cholesterol was eluted in buffer A containing 0.25 M imidazole. The amount of bound cholesterol was calculated after subtracting the respective blank (no protein) and by converting the radioactivity of ^{3}H-cholesterol to cholesterol concentration using specific radioactivity as specified by the manufacturer.

2.3. Measurement of $^{86}Rb^{+}$ uptake

Rubidium flux assay was also performed as described earlier [27,29]. In brief, disposable polystyrene columns (Pierce Chemical Co) 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 proteins (2.5–10 μg per mg of total lipid) were added to CHAPS (37 mM) solubilized mixture of phosphatidylethanolamine: phosphatidylglycerol (9:1, Avanti Polar Lipids, Inc., 10 mg total lipid per ml) in buffer A and incubated for 30 min. Cholesterol was mixed with the other lipids. The mixture was dried under nitrogen, then solubilized in 37 mM CHAPS before adding the 10 mM HEPES buffer containing 450 mM KCl, 4 mM NMG pH 7.4 and was incubated at 37 °C for 1 h to swell the lipids. Column A (with Sephadex beads in buffer A) was partially dehydrated by spinning at 3000 rpm in a Hermle Z283 centrifuge. Liposomes were formed by spinning 100 μl of detergent-solubilized lipid/protein mixture through the partially dehydrated column A at 2500 rpm. Extraliposomal solution was exchanged for buffer B by centrifugation through a partially dehydrated column B. The assay was initiated by adding 400 μl of buffer B with 1–5 μM $^{86}Rb^{+}$. 50 μl aliquots of the radioactive mixture were taken at indicated time points, and extraliposomal $^{86}Rb^{+}$ removed by passage over a 0.5 ml Dowex cation exchange column in the $NMGH^{+}$ form. Samples were mixed with scintillation fluid and counted in a liquid scintillation counter.

2.4. Statistical 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 5% level ($p<0.05$). The fitting of the curves was done using Origin software.

3. Results

3.1. Binding of [^{3}H]Cholesterol to purified His-KirBac1.1

Earlier studies have shown that direct cholesterol binding assays can be performed by solubilizing cholesterol in a detergent and then incubating cholesterol detergent mixture with purified His-tagged proteins that allow separation of free and bound cholesterol by affinity chromatography [30–33]. Using the same strategy, we show here that cholesterol solubilized in CHAPS binds to KirBac1.1 protein and is eluted with the protein from the Ni-NTA agarose affinity column. A typical [^{3}H]cholesterol elution profile (Fig. 1A) consists of two clear peaks: the first peak corresponds to the unbound

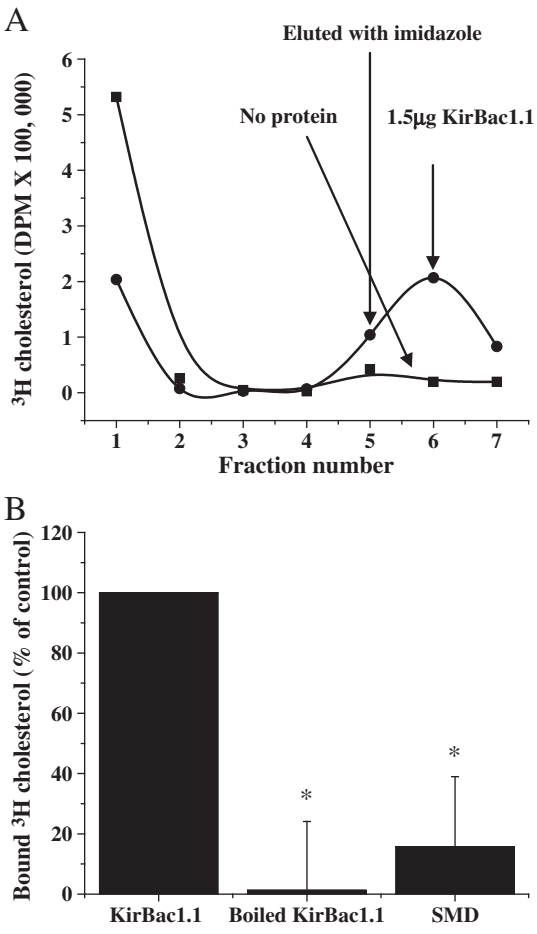

Fig. 1. [^{3}H]-cholesterol–KirtBac1.1 elution profiles. A: Typical elution profiles of [^{3}H]-cholesterol with and without 1.5 µg His$_6$-KirBac1.1 protein from Ni-NTA-agarose affinity column. The unbound cholesterol appears in fractions 1–4 and cholesterol bound to the KirBac1.1 protein appears in fractions 5–7 after the addition of imidazole-HCl. B: Total amount of bound cholesterol eluted with the imidazole buffer (fractions 5–7) in the presence of 1.5 µg His$_6$-KirBac1.1, denatured His$_6$-KirBac1.1 protein or His$_6$-SMD. The data are normalized to the amount of total cholesterol eluted with KirBac1.1. Data represent means ± SE, *$p<0.05$, at least 3 independent experiments.

cholesterol that is washed off with an excess of Tris–HCl buffer until the level of unbound cholesterol was practically zero (fractions 1–4 of the elution profile), then the protein with the *bound cholesterol* is eluted with 0.25 M imidazole (fractions 5–7). As expected, in the absence of the protein only one peak is observed with [^{3}H]cholesterol found almost exclusively in the unbound fractions (lower curve). Similarly, no binding was observed when KirBac1.1 was denatured by boiling (Fig. 1B). However, since denaturing of KirBac1.1 interferes with its ability to bind to the affinity column, we also tested whether under these experimental conditions cholesterol binds to an unrelated His-tagged protein Sphingomyelinase D (SMD). Fig. 1B shows that no significant binding was observed for the SMD protein. Significant KirBac1.1 cholesterol binding was also observed when cholesterol was dissolved in Fos-Choline 13 (not shown).

The time–course of cholesterol binding to KirBac1.1 protein is shown in Fig. 2. As was demonstrated in earlier studies for other cholesterol-binding proteins [30], the equilibrium for [^{3}H]cholesterol–KirBac1.1 binding was achieved after approximately 2 h. When the exposure was prolonged to 12 h, however, the binding was decreased, most likely because of partial denaturation of the channel protein at 37 °C. The rest of the experiments, therefore, were performed using CHAPS as cholesterol delivery system and the protein was incubated with [^{3}H] cholesterol for 2 h to allow the system to reach an equilibrium. Fig. 3 shows that, as expected, an increase in the amount of KirBac1.1 protein

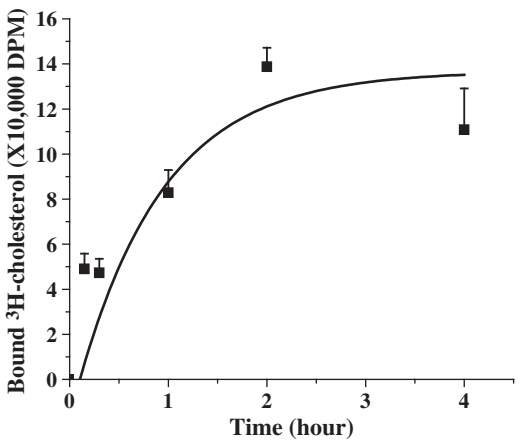

Fig. 2. Time course of [^{3}H]-cholesterol binding to His$_6$-KirBac1.1. Total amount of bound cholesterol eluted with KirBac1.1 at different time points. Each assay contained 200nM [^{3}H]-cholesterol and 1.5 µg of His$_6$-KirBac1.1. The experiment was performed at 37 °C. All data points represent means ± S.E, at least 3 independent experiments.

is linearly proportional to the amount of cholesterol being found in the bound form. In this series of experiments, increasing amounts of KirBac1.1 (0.45–3 µg) were incubated with the same amount of [^{3}H] cholesterol (200 nM). A series of typical [^{3}H]cholesterol elution profiles

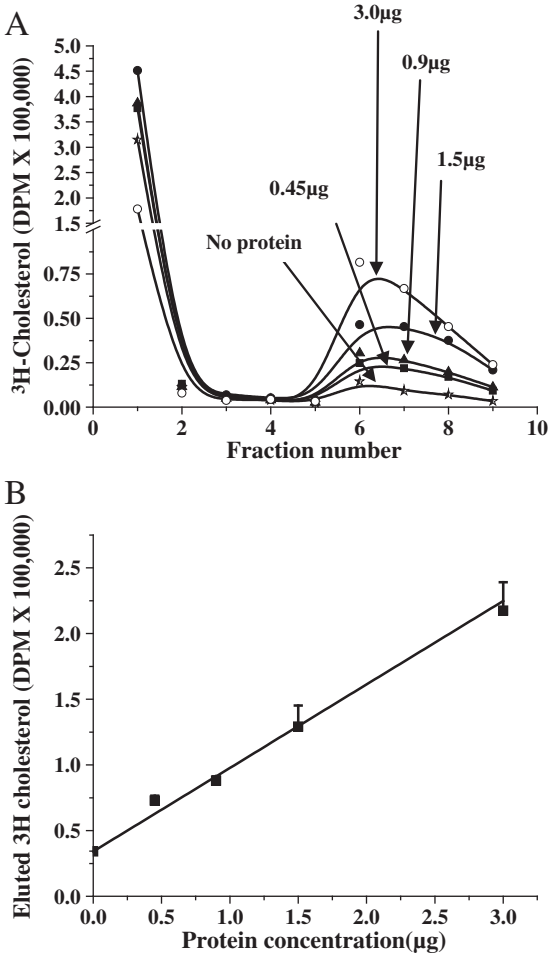

Fig. 3. Dependence of [^{3}H]-cholesterol binding on the amount of KirBac1.1 protein. A: Typical elution profiles of [^{3}H]-cholesterol obtained in the presence of increasing amounts of His$_6$-KirBac1.1. All the profiles shown in this panel were obtained in the same experiment B: Total bound [^{3}H]-cholesterol as a function of His$_6$-KirBac1.1 concentration. Each data point represent means ± SE of 5 independent experiments.

obtained with the increasing amount of the protein is shown in Fig. 3A. The total amount of [^{3}H]cholesterol found in the bound form as a function of protein concentration is shown in Fig. 3B.

3.2. Saturability and competition

Next, we tested whether cholesterol binding to KirBac1.1 saturates with increasing concentration of the ligand. In the first series of these experiments, cholesterol concentration was varied between 50 and 600 nM, a range that was shown earlier to have saturable binding for SCAP1 protein [30] but no saturation was observed in this range for KirBac1.1 protein (not shown). However, at higher cholesterol concentrations (100 μM–1.3 mM), cholesterol–KirBac1.1 dose response was clearly saturable with the K_D value of 390 μM, as is apparent both from the saturation isotherm of cholesterol binding and from the linearization of the data shown as Scatchard plot (Fig. 4A). We also estimated the stoichiometry between cholesterol and the protein but the molar ratio of cholesterol to the protein was unrealistically high (60:1 mol/mol). The most likely explanation for this observation is that since cholesterol has to be dissolved in a detergent, it interacts with a protein as micelle rather than as an individual molecule and it is impossible to completely get rid of any trace amounts of the detergent in the system. The estimated molar ratio, therefore, is unlikely to reflect the true molar ratio between cholesterol and the protein. Furthermore, the value of the Kd may also be overestimated because of the same problem. These experiments were performed by adding tracer amounts of [^{3}H] cholesterol to unlabeled cholesterol in 1:1000 ratio. In all the experiments, the measurements were performed with and without KirBac1.1 proteins for all cholesterol concentrations and the blank values have been subtracted. Furthermore, we also tested whether any detectable amounts of phospholipids could be found in the purified protein preparation used in these experiments using gas–liquid chromatography but no phospholipids were detected (not shown). The high K_D value suggests that most likely cholesterol interacts with the channel protein by weak hydrophobic association.

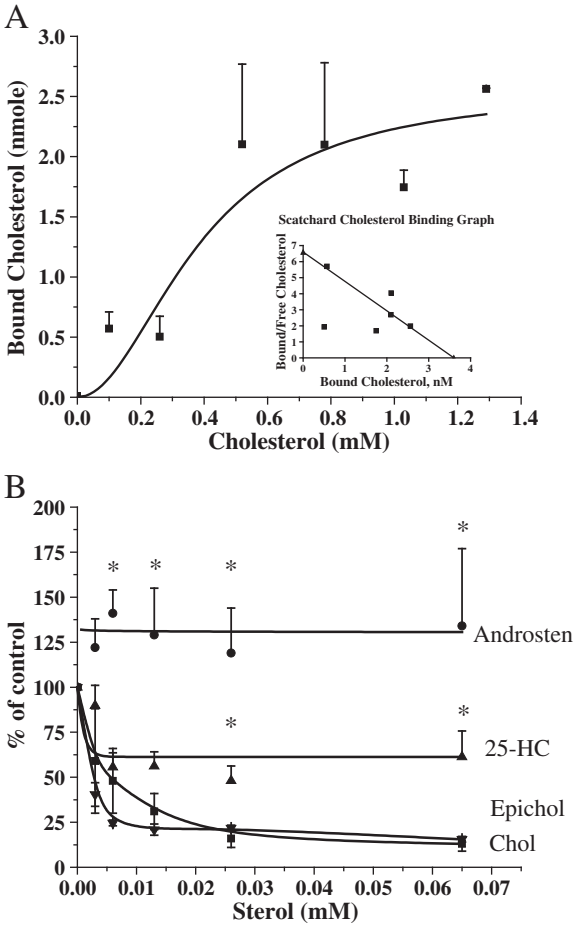

Fig. 4. Saturability and Competition of Cholesterol–His_6-KirBac1.1 binding. A: Saturable dose response of cholesterol binding to 1.5 μg His_6–KirBac1.1. In this series of experiments, unlabeled cholesterol was added to ^{3}H-cholesterol to obtain the indicated concentrations while maintaining the specific radioactivity for ^{3}H-cholesterol constant. All the experiments were performed with appropriate blanks that contained no KirBac1.1 protein and the blanks were subtracted. Each data point represents mean ± S.E of 3–5 independent determinations. A Scatchard plot of the binding data is shown as inset. B: Competition between ^{3}H-cholesterol and unlabeled cholesterol, epicholesterol, 25-Hydroxycholesterol and, 5-Androsten 3β-17 β-diol. All the experiments were performed with the appropriate blanks and the blanks have been subtracted. Each data point is mean of 3–5 independent determinations.

Furthermore, Fig. 4B shows that unlabeled cholesterol competes with [^{3}H]cholesterol for binding to KirBac1.1 protein. In these experiments, KirBac1.1 protein was incubated with a constant amount of [^{3}H]cholesterol (200nM) and increasing amounts of unlabeled cholesterol. As expected, the amount [^{3}H]cholesterol–KirBac1.1 binding decreases significantly and goes practically to zero, as the concentration of unlabeled cholesterol increases. It was surprising, however, that relatively low levels of cold cholesterol compete efficiently with radioactive cholesterol even though there supposed to be sufficient binding sites available. This observation, however, was very robust and consistent through all the experiments. One possibility would be that the Kd is overestimated, as suggested above. To examine the sterol specificity of this effect, we tested three sterols: epicholesterol, a chiral isomer of cholesterol that was also shown to suppress KirBac1.1 activity but to a lesser degree than cholesterol and two other sterols, 5-Androsten 3β-17β-diol and 25-hydroxycholesterol that do not have significant effect on KirBac1.1 activity [29]. As described above, all the experiments were performed with blank controls. These experiments show that there is significant difference in the ability of the three sterols to compete with cholesterol for KirBac1.1 binding: epicholesterol was similar to cholesterol, 25-hydroxycholesterol was a significantly weaker competitor and 5-Androsten 3β-17β-diol did not compete with cholesterol at all. Since epicholesterol efficiently competed with cholesterol for the channel binding, it suggests that epicholesterol also binds to the KirBac1.1 proteins, which is not completely unexpected since epicholesterol was shown earlier to suppress channel activity even though to a lesser degree than cholesterol. A weaker competition of 25-hydroxycholesterol suggests that it may also bind to the channels to some degree but clearly significantly less than cholesterol. A lack of any competition between [^{3}H]cholesterol and 5-Androsten 3β-17β-diol indicates that the latter does not bind to KirBac1.1 protein.

3.3. Direct binding is required for cholesterol-induced suppression of KirBac1.1 channels

To determine whether inhibition of cholesterol binding abrogates its inhibitory effect on KirBac1.1 function, we used trifluoperazine, a cationic amphiphilic compound that was shown to inhibit cholesterol binding to SCAP1 [30]. Here we show that, similarly to its effect on cholesterol–SCAP1 binding, trifluoperazine also significantly inhibits cholesterol binding to KirBac1.1 channels in a dose dependent way (Fig. 5A). However, the inhibitory effect of trifluoperazine on cholesterol–KirBac1.1 binding was observed at significantly higher concentrations than its effect on cholesterol–SCAP1 binding. Specifically, whereas the inhibitory effect on KirBac1.1 cholesterol binding was observed only at concentrations of at least 100 μM, significant inhibition of cholesterol–SCAP1 binding was observed already at 5 μM [30]. It is interesting to note, though, that affinity of KirBac1.1 to cholesterol is also significantly lower than that of SCAP1. Thus, there is a correlation between the affinity of these proteins to cholesterol and to the inhibitor.

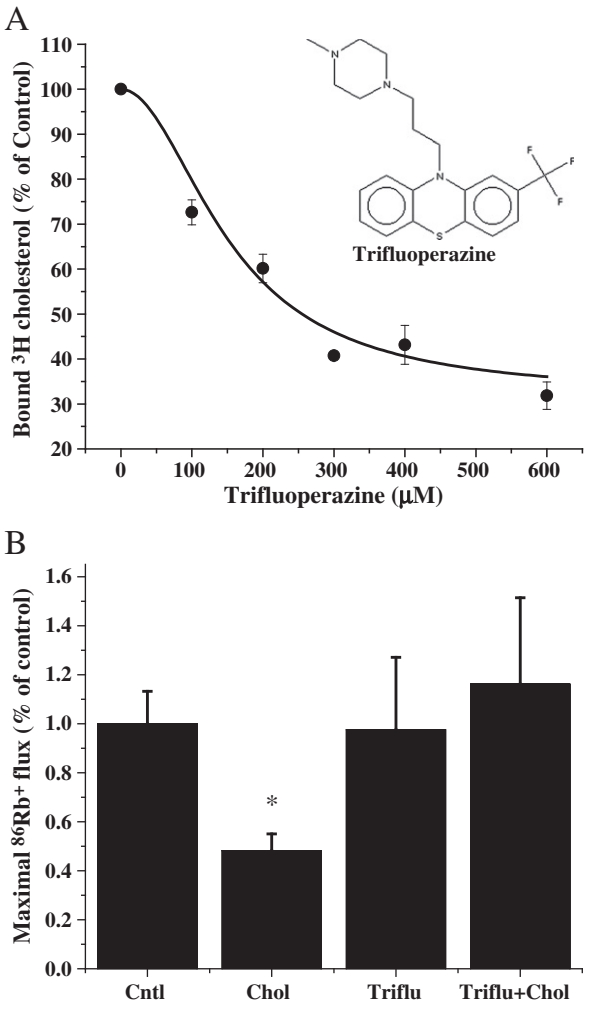

Fig. 5. Cholesterol–His$_6$-KirBac1.1 binding regulates KirBac1.1 function. A: Inhibition of ^{3}H-cholesterol binding to KirBac 1.1 by trifluoperazine. Each assay contained the varying amounts of trifluoperazine, 200nM of solublized ^{3}H-cholesterol and 1.5 μg of purified His-tagged KirBac1.1 and incubated for 2 h at 37 °C. The structure of trifluoperazine is shown in the inset. Each data point is mean of 3–5 independent determinations. B: Recovery of cholesterol-induced suppression of KirBac1.1-mediated ^{86}Rb$^+$ uptake by trifluoperazine. Normalized maximal uptake of ^{86}Rb$^+$ after 240 s in liposomes (POPE: POPG, 9:1) containing: (i) no cholesterol, (ii) 50 μg cholesterol per mg total lipid, (iii) 600 μM trifluoperazine, (iv) 50 μg cholesterol + 600 μM trifluoperazine. All liposomes contained 1.5 μg of KirBac1.1 protein per mg phospholipids. Data represent means ± SE, *$p<0.05$, 3 independent experiments performed for each condition.

Finally, we tested whether addition of trifluoperazine results in recovery of KirBac1.1 function from cholesterol-induced suppression. To address this question, we compared the activity of KirBac1.1 channels in the presence and in the absence of the inhibitor using a functional assay described in our previous studies [27–29]. Briefly, KirBac1.1 protein is incorporated into POPE: POPG (3:1) liposomes and the activity of the channels is quantified by the flux of ^{86}Rb$^+$ from the external medium into the liposomes through the channel pore. Liposomes that have no protein are used as controls. 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$^+$ ionophore, valinomycin. Fig. 5B shows that KirBac1.1-mediated ^{86}Rb$^+$ flux was not affected by trifluoperazine alone indicating that the channels are fully functional. Most importantly, we show here that addition of trifluoperazine (600 μM) that significantly inhibits cholesterol–KirBac1.1 binding fully abrogates its inhibitory effect on the channel activity.

4. Discussion

A key question in elucidating the mechanisms that underlie cholesterol-induced regulation of ion channels is to determine whether the effect of cholesterol is mediated by direct binding to the channel proteins or whether it is mediated by changing the physical properties of the lipid bilayer. Earlier studies suggested that cholesterol regulates Kir channels through specific sterol–protein interactions based on differential effects of an array of sterols on the channel function [16,29,34] but direct binding of cholesterol to the channel has not been demonstrated. In this study, we show that cholesterol binds to purified KirBac1.1 channels and that cholesterol–KirBac1.1 binding is essential for the inhibitory effect of cholesterol on channel activity. Thus, this study provides the first evidence of the direct interaction between cholesterol and an ion channel protein.

Cholesterol binding has been demonstrated so far for only a handful of proteins, such as SCAP1 and NPC1 [30,31,33]. The main constraint in establishing an in vitro cholesterol binding essay, particularly for membrane proteins, is delivering cholesterol to the protein in a solubilized form without disrupting the binding ability of the protein [30]. Indeed, most major organic solvents, including ethanol and chloroform, were shown to interfere with specific sterol–protein interactions of membrane proteins [30]. This problem was resolved for Scap1 and NPC1 proteins by solubilizing cholesterol in detergent micelles of either Fos-Choline 13 or Nonidet P-40 [30–32]. In this study, we used the same approach but our first choice of the detergent was CHAPS because we have already shown previously that cholesterol can be efficiently solubilized in 0.1% CHAPS and that at this concentration CHAPS does not affect neither KirBac1.1 function nor its sensitivity to cholesterol when incorporated into liposomes [29]. Here, we show that when solubilized in CHAPS, cholesterol binds to KirBac1.1 protein in a highly reproducible way indicating that the binding properties of KirBac1.1 are retained. Furthermore, multiple lines of evidence indicate that cholesterol interacts with KirBac1.1 directly: (i) the binding is clearly saturable; (ii) non-radioactive cholesterol efficiently competes with [^{3}H]cholesterol for binding, whereas 5-Androsten 3β-17β-diol and 25-hydroxycholesterol, two sterols that do not affect KirBac1.1 function [29] show no or only partial competition with [^{3}H]cholesterol for binding; (iii) finally, cholesterol–KirBac1.1 binding can be abrogated by the same drug that inhibits cholesterol binding to SCAP1 [30]. Taken together, these observations provide strong evidence for direct cholesterol–KirBac1.1 binding.

We also show here, however, that there is no significant difference between cholesterol and epicholesterol in their ability to compete for KirBac1.1 indicating that both cholesterol and epicholesterol bind to the channel protein. Interestingly, Radhakrishnan et al. [30] also showed that epicholesterol can bind to a sterol-sensing domain of SCAP, a protein that controls the transport and proteolytic activation of sterol regulatory element, even though the affinity of epicholesterol to SCAP was lower than that of cholesterol. Furthermore, our previous studies showed that epicholesterol also inhibits KirBac channels although to a lesser degree than cholesterol [29]. Our observations raise an important question about the interpretation of differential effects of cholesterol chiral analogs on the channel activity. The most common interpretation is that if chiral analogs of cholesterol have differential effects on protein function, it means that cholesterol binds to the protein whereas its analog does not. However, in most cases, this conclusion is not tested directly because the binding data is unavailable. Our current observations suggest an alternative possibility that chiral analogs may bind to the channel protein but fail to elicit the inhibitory effect. This conclusion is fully consistent with our earlier studies showing that partial substitution of cholesterol with epicholesterol enhanced Kir currents in endothelial cells [16], leading us to propose a hypothesis that epicholesterol competes with cholesterol for a binding site in Kir2 channel protein but does not induce an inhibitory effect. Similarly, we propose here that while both

cholesterol and epicholesterol bind to KirBac1.1 protein, only cholesterol induces channel conformation that decreases channel activity. In terms of the nature of cholesterol–KirBac1.1 interaction, our observations suggest that cholesterol interacts with the channels by hydrophobic associations that are not sensitive to the position of the hydroxyl group which constitutes the difference between cholesterol and epicholesterol. More specifically, we propose that multiple cholesterol molecules interact with the hydrophobic regions of the channel to suppress channel activity. More studies are needed to elucidate the mechanism of this effect.

Most importantly, a full recovery of KirBac1.1 function from cholesterol-induced inhibition by TFP, a drug that inhibits cholesterol–KirBac1.1 binding, indicates that cholesterol binding to the KirBac1.1 protein is essential for the inhibitory effect. Furthermore, while it is known that TFP is a highly lipophilic drug that easily interacts with membrane lipids [35–37], our observations show that addition of TFP to liposomes containing no cholesterol has no effect on KirBac1.1 activity. We conclude, therefore, that the effect of TFP is to prevent cholesterol from binding and suppressing KirBac1.1 channels. These observations are also fully consistent with our previous studies showing that there is no correlation between the effects of different sterols on KirBac1.1 function and changes in membrane fluidity, as assayed by fluorescence anisotropy of two membrane dyes, DPH and TMA-DPH [29]. In the earlier study, however, it was not possible to exclude the possibility that the general estimate of global membrane fluidity may not reflect local changes in lipid packing, membrane elastic properties or in bilayer width, which might be more important determinants of ion channel function than membrane fluidity [10,11,38]. This concern is alleviated in the present study.

Finally, high sequence similarity between KirBac1.1 and mammalian Kir channels suggests that KirBac1.1 may be used as a model to study the mechanisms that regulate eukaryotic Kirs. Indeed, we have previously demonstrated that KirBac1.1 channels exhibit many of the same key properties as eukaryotic Kir channels, including sensitivity to a regulatory phospholipid phosphatidylinositol 4,5-biphosphate and cholesterol [27–29,39]. The current observations provide the basis for identifying cholesterol binding sites of Kir channels in the future studies.

Acknowledgments

We thank Dr. Papasani V. Subbaiah and Dr. Avia Rosenhouse-Dantsker for multiple critical discussions. We also thank Dr. Avia Rosenhouse-Dantsker for performing the homology analysis between KirBac and Kir2 channels and Dr. Ninder Panesar for her help with the experiments involving protein measurement and recovery. This work was supported by NIH grants HL073965 and HL083298 (to IL) and DK069424 (to DE).

References

[1] A. Maguy, T.E. Hebert, S. Nattel, Involvement of lipid rafts and caveolae in cardiac ion channel function, Cardiovasc. Res. 69 (2006) 798–807.
[2] I. Levitan, Y. Fang, A. Rosenhouse-Dantsker, V.G. Romanenko, Cholesterol and ion channels, in: J.R. Harris (Ed.), Cholesterol Binding and Cholesterol Transport Proteins, 2010, pp. 509–549.
[3] T.P. Lockwich, X. Liu, B.B. Singh, J. Jadlowiec, S. Weiland, I.S. Ambudkar, Assembly of Trp1 in a signaling complex associated with caveolin-scaffolding lipid raft domains, J. Biol. Chem. 275 (2000) 11934–11942.
[4] J.R. Martens, R. Navarro-Polanco, E.A. Coppock, A. Nishiyama, L. Parshley, T.D. Grobaski, M.M. Tamkun, Differential targeting of shaker-like potassium channels to lipid rafts, J. Biol. Chem. 275 (2000) 7443–7446.
[5] I.S. Ambudkar, S.C. Brazer, X. Liu, T. Lockwich, B. Singh, Plasma membrane localization of TRPC channels: role of caveolar lipid rafts, Novartis Found. Symp. 258 (2004) 63–70 discussion 70–64, 98–102, 263–106.
[6] J.R. Martens, K. O'Connell, M. Tamkun, Targeting of ion channels to membrane microdomains: localization of KV channels to lipid rafts, Trends Pharmacol. Sci. 25 (2004) 16–21.
[7] B. Pani, H.L. Ong, X. Liu, K. Rauser, I.S. Ambudkar, B.B. Singh, Lipid rafts determine clustering of STIM1 in endoplasmic reticulum–plasma membrane junctions and regulation of store-operated Ca2+ entry (SOCE), J. Biol. Chem. 283 (2008) 17333–17340.
[8] C. Dart, Lipid microdomains and the regulation of ion channel function, J. Physiol. 588 (2010) 3169–3178.
[9] F.J. Barrantes, Structural basis for lipid modulation of nicotinic acetylcholine receptor function, Brain Res. Brain Res. Rev. 47 (2004) 71–95.
[10] J.A. Lundbaek, P. Birn, J. Girshman, A.J. Hansen, O.S. Andersen, Membrane stiffness and channel function, Biochemistry 35 (1996) 3825–3830.
[11] J.A. Lundbaek, P. Birn, A.J. Hansen, R. Sogaard, C. Nielsen, J. Girshman, M.J. Bruno, S.E. Tape, J. Egebjerg, D.V. Greathouse, G.L. Mattice, R.E. Koeppe II, O.S. Andersen, Regulation of sodium channel function by bilayer elasticity: the importance of hydrophobic coupling. Effects of Micelle-forming amphiphiles and cholesterol, J. Gen. Physiol. 123 (2004) 599–621.
[12] D. Bichet, F.A. Haass, L.Y. Jan, Merging functional studies with structures of inward-rectifier K(+) channels, Nat. Rev. Neurosci. 4 (2003) 957–967.
[13] Y. Kubo, J.P. Adelman, D.E. Clapham, L.Y. Jan, A. Karschin, Y. Kurachi, M. Lazdunski, C.G. Nichols, S. Seino, C.A. Vandenberg, International Union of Pharmacology. LIV. Nomenclature and molecular relationships of inwardly rectifying potassium channels, Pharmacol. Rev. 57 (2005) 509–526.
[14] H. Hibino, A. Inanobe, K. Furutani, S. Murakami, I. Findlay, Y. Kurachi, Inwardly rectifying potassium channels: their structure, function, and physiological roles, Physiol. Rev. 90 (2010) 291–366.
[15] I. Levitan, Cholesterol and Kir channels, IUBMB Life 61 (2009) 781–790.
[16] V.G. Romanenko, G.H. Rothblat, I. Levitan, Modulation of endothelial inward-rectifier K+ current by optical isomers of cholesterol, Biophys. J. 83 (2002) 3211–3222.
[17] Y. Fang, G. Schram, V.G. Romanenko, C. Shi, L. Conti, C.A. Vandenberg, P.F. Davies, S. Nattel, I. Levitan, Functional expression of Kir2.x in human aortic endothelial cells: the dominant role of Kir2.2, Am. J. Physiol. Cell Physiol. 289 (2005) C1134–C1144.
[18] V.G. Romanenko, Y. Fang, F. Byfield, A.J. Travis, C.A. Vandenberg, G.H. Rothblat, I. Levitan, Cholesterol sensitivity and lipid raft targeting of Kir2.1 channels, Biophys. J. 87 (2004) 3850–3861.
[19] S. Tikku, Y. Epshtein, H. Collins, A.J. Travis, G.H. Rothblat, I. Levitan, Relationship between Kir2.1/Kir2.3 activity and their distributions between cholesterol-rich and cholesterol-poor membrane domains, Am. J. Physiol. Cell Physiol. 293 (2007) C440–C450.
[20] Y. Fang, E.R. Mohler III, E. Hsieh, H. Osman, S.M. Hashemi, P.F. Davies, G.H. Rothblat, R.L. Wilensky, I. Levitan, Hypercholesterolemia suppresses inwardly rectifying K+ channels in aortic endothelium in vitro and in vivo, Circ. Res. 98 (2006) 1064–1071.
[21] E.R. Mohler III, Y. Fang, R.G. Shaffer, J. Moore, R.L. Wilensky, M. Parmacek, I. Levitan, Hypercholesterolemia suppresses Kir channels in porcine bone marrow progenitor cells in vivo, Biochem. Biophys. Res. Commun. 358 (2007) 317–324.
[22] A. Rosenhouse-Dantsker, E. Leal-Pinto, D.E. Logothetis, I. Levitan, Comparative analysis of cholesterol sensitivity of Kir channels: role of the CD loop, Channels (Austin) 4 (2010) 63–66.
[23] Y. Epshtein, A.P. Chopra, A. Rosenhouse-Dantsker, G.B. Kowalsky, D.E. Logothetis, I. Levitan, Identification of a C-terminus domain critical for the sensitivity of Kir2.1 to cholesterol, Proc. Natl. Acad. Sci. U.S.A. 106 (2009) 8055–8060.
[24] A. Rosenhouse-Dantsker, D.E. Logothetis, I. Levitan, Cholesterol sensitivity of KIR2.1 is controlled by a belt of residues around the cytosolic pore, Biophys. J. 100 (2011) 381–389.
[25] A. Kuo, J.M. Gulbis, J.F. Antcliff, T. Rahman, E.D. Lowe, J. Zimmer, J. Cuthbertson, F.M. Ashcroft, T. Ezaki, D.A. Doyle, Crystal structure of the potassium channel KirBac1.1 in the closed state, Science 300 (2003) 1922–1926.
[26] A. Kuo, C. Domene, L.N. Johnson, D.A. Doyle, C. Venien-Bryan, Two different conformational states of the KirBac3.1 potassium channel revealed by electron crystallography, Structure 13 (2005) 1463–1472.
[27] D. Enkvetchakul, J. Bhattacharyya, I. Jeliazkova, D.K. Groesbeck, C.A. Cukras, C.G. Nichols, Functional characterization of a prokaryotic Kir channel, J. Biol. Chem. 279 (2004) 47076–47080.
[28] D. Enkvetchakul, I. Jeliazkova, C.G. Nichols, Direct modulation of Kir channel gating by membrane phosphatidylinositol 4,5-bisphosphate, J. Biol. Chem. 280 (2005) 35785–35788.
[29] D.K. Singh, A. Rosenhouse-Dantsker, C.G. Nichols, D. Enkvetchakul, I. Levitan, Direct regulation of prokaryotic Kir channel by cholesterol, J. Biol. Chem. 284 (2009) 30727–30736.
[30] A. Radhakrishnan, L.P. Sun, H.J. Kwon, M.S. Brown, J.L. Goldstein, Direct binding of cholesterol to the purified membrane region of SCAP: mechanism for a sterol-sensing domain, Mol. Cell 15 (2004) 259–268.
[31] R.E. Infante, L. Abi-Mosleh, A. Radhakrishnan, J.D. Dale, M.S. Brown, J.L. Goldstein, Purified NPC1 protein. I. Binding of cholesterol and oxysterols to a 1278-amino acid membrane protein, J. Biol. Chem. 283 (2008) 1052–1063.
[32] R.E. Infante, M.L. Wang, A. Radhakrishnan, H.J. Kwon, M.S. Brown, J.L. Goldstein, NPC2 facilitates bidirectional transfer of cholesterol between NPC1 and lipid bilayers, a step in cholesterol egress from lysosomes, Proc. Natl. Acad. Sci. U.S.A. 105 (2008) 15287–15292.
[33] G. Gimpl, Cholesterol–protein interaction: methods and cholesterol reporter molecules, Subcell. Biochem. 51 (2010) 1–45.
[34] N. D'Avanzo, K. Hyrc, D. Enkvetchakul, D.F. Covey, C.G. Nichols, Enantioselective protein–sterol interactions mediate regulation of both prokaryotic and eukaryotic inward rectifier k channels by cholesterol, PLoS One 6 (2011) e19393.
[35] A. Broniec, A.U. Gjerde, A.B. Olmheim, H. Holmsen, Trifluoperazine causes a disturbance in glycerophospholipid monolayers containing phosphatidylserine (PS): effects of pH, acyl unsaturation, and proportion of PS, Langmuir 23 (2007) 694–699.

[36] A.B. Hendrich, K. Michalak, O. Wesolowska, Phase separation is induced by phenothiazine derivatives in phospholipid/sphingomyelin/cholesterol mixtures containing low levels of cholesterol and sphingomyelin, Biophys. Chem. 130 (2007) 32–40.
[37] O. Wesolowska, K. Michalak, A.B. Hendrich, Direct visualization of phase separation induced by phenothiazine-type antipsychotic drugs in model lipid membranes, Mol. Membr. Biol. 28 (2011) 103–114.
[38] J.A. Lundbaek, A.M. Maer, O.S. Andersen, Lipid bilayer electrostatic energy, curvature stress, and assembly of gramicidin channels, Biochemistry 36 (1997) 5695–5701.
[39] D. Enkvetchakul, I. Jeliazkova, J. Bhattacharyya, C.G. Nichols, Control of inward rectifier K channel activity by lipid tethering of cytoplasmic domains, J. Gen. Physiol. 130 (2007) 329–334.