Chapter 15
Physiological Roles and Cholesterol Sensitivity of Endothelial Inwardly-Rectifying K^+ Channels: Specific Cholesterol-Protein Interactions Through Non Annular Binding Sites

Irena Levitan, Sang Joon Ahn, Ibra Fancher, and Avia Rosenhouse-Dantsker

Abstract Inwardly-rectifying K^+ channels (Kir) have been implicated to play a major role in endothelial sensation of shear stress forces and suggested to constitute a primary flow sensor. The studies of our group focused on elucidating the impact of hypercholesterolemia on endothelial Kir channels and elucidating molecular, biophysical and structural basis of cholesterol-induced Kir suppression. In this chapter, we first review briefly what is known about expression of Kir channels in different types of endothelial cells and their role in endothelial function and then discuss in detail the mechanisms of cholesterol-Kir interactions. Briefly, endothelial Kir channels are suppressed by loading the cells with cholesterol and by exposing them to atherogenic lipoproteins *in vitro* and by plasma hypercholesterolemia *in vivo*. A series of studies revealed that cholesterol interacts with the channels directly stabilizing them in a long-lived closed "silent" state and that multiple structural features of the channels are essential for conferring their cholesterol sensitivity. There is also a significant cross-talk between cholesterol, caveolin-1 and a regulatory phospholipid $PI(4,5)P_2$ in the regulation of these channels. Further studies are needed to determine the impact of cholesterol-induced suppression of Kir on endothelial function.

Keywords Potassium channels • Cholesterol • Cholesterol binding motifs • Hypercholesterolemia • Endothelial cells

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Introduction

Multiple studies established that dyslipidemia-induced endothelial dysfunction plays a key role in the early stage of the development of atherosclerosis (e.g. [1–3]. The mechanisms, however, that underlie dyslipidemia-induced endothelial dysfunction are still poorly understood. Our studies demonstrated that an increase in cellular cholesterol *in vitro* and plasma hypercholesterolemia *in vivo* result in strong suppression of endothelial K^+ channels belonging to a class of inwardly-rectifying K^+ channels (Kir) [4, 5]. These channels are known to play a major role in regulating resting membrane potential and excitability in a variety of cell types [6, 7] and were shown to be sensitive to fluid shear stress, a frictional force generated by blood flow [5, 8]. Furthermore, since activation of Kir channels is one of the fastest responses of endothelial cells to shear stress, it was proposed that shear stress-sensitive Kir channels may constitute a primary shear stress sensor responsible for the ability of endothelial cells to respond to their hemodynamic environment and thus play a major role in endothelial physiology [9]. Our observations that endothelial Kir channels are sensitive to plasma dyslipidemia led us to hypothesize that cholesterol-induced suppression of these channels may play a significant role in endothelial dysfunction under hypercholesterolemic conditions. In this chapter, we summarize what is currently known about the role of Kir channels in endothelial physiology and provide an in depth discussion of cholesterol interaction with these channels.

Expression of Kir Channels in Endothelial Cells

In general, endothelial cells (ECs) express two major classes of K^+ channels; inwardly-rectifying K+ (Kir) channels, which as described above, maintain stable membrane potential and are sensitive to fluid shear stress [5, 8] and Ca^{2+}-activated K^+ (K_{Ca}) channels that are sensitive to the level of intracellular Ca^{2+} [10, 11]. Among Kir channels that are divided into seven sub-families (Kir1-7) that differ in their biophysical properties and sensitivities to different regulators [6], endothelial cells express Kir2 channels, strong rectifiers with high basal open probability [12–15] and ATP-dependent Kir6 channels [16–20]. Among Ca^{2+}-sensitive K^+ channels, endothelial cells were shown to express mainly small and intermediate conductance K_{Ca} channels (SK and IK, respectively), [21–23] with some evidence for the expression of large-conductance (BK) channels as well [24, 25].

Kir2 channels or strong rectifiers were found in both conduit and microvasculature endothelial cells across multiple species. Specifically, functional Kir2 channels are expressed in human [14], bovine [4] and porcine [14] aortic endothelial cells, as well as in bovine pulmonary endothelium [26, 27], but were not found in rabbit [28] and mouse [29] aortic endothelium. Kir2 channels were also

found in endothelial cells isolated from rat brain microvasculature [15, 30] and from bovine cornea [13]. Two studies also looked at the differential distribution of Kir channels between the luminal and the abluminal surfaces of the endothelium using a "stamp method" that allows to isolate endothelial cells with their abluminal side up. The study by Manabe et al. [31] revealed that in freshly-isolated cardiac endothelium functional Kir channels are expressed exclusively on the luminal side of the endothelium with no detectable expression on the subluminal side. In contrast, in cultured bovine aortic endothelial cells, there was no difference in Kir expression between the basal and the apical endothelial surfaces [32]. It is possible that these differences may be related to the hemodynamic conditions.

The subfamily of Kir2 channels consists of four members (Kir2.1-2.4) [6] but only Kir2.1 and Kir2.2 were found to be functionally expressed in endothelial cells. Our study showed that while all four of Kir2 channels are expressed in human aortic endothelial cells on the molecular level (mRNA), single-channel analysis and differential expression of specific dominant-negative Kir2.x subunits (dnKir2.1-2.4) revealed that only Kir2.1 and Kir2.2 are functionally expressed with a strong contribution of Kir2.2 channels [14]. Furthermore, our observations suggest that Kir2.1 and Kir2.2 subunits may interact in aortic endothelium. Equivalent expression of Kir2.1 and Kir2.2 channels on the mRNA levels was also found in bovine and rat brain capillary ECs but no functional analysis was performed to discriminate between relative contributions of these two channels [15, 30].

Kir6 or ATP-dependent Kir channels that are regulated by an auxiliary subunit of ATP-binding cassette (ABC) sulfonylurea receptors and thus sensitive to the metabolic state of the cells are also expressed in multiple types of endothelial cells. The two members of the Kir6 family (Kir6.1 and Kir6.2) were also found in both conduit and microvascular endothelial cells: specifically Kir6.1 and 6.2 were found in the endothelium of heart capillaries with a predominant expression of Kir6.2 [16] and in coronary conduit arteries [33]. Kir6.2 were also found in microvascular pulmonary endothelial cells where these channels were also shown to be shear stress sensitive [17, 20]. Furthermore, Chatterjee et al. demonstrated that Kir6.2 channels play an important role in endothelial response to the cessation of flow that may occur during lung ischemia.

Other Kir channels: In addition, there is molecular/histological evidence for the expression of Kir1 and G protein-coupled Kir3 channels in pulmonary vein endothelial cells but their functional role in these cells has not been studied [34].

Other K^+ channels: There are also multiple studies demonstrating the expression and functional roles of Ca^{2+}-sensitive K^+ channels in endothelial cells, particularly small-conductance (SK) channels that are expressed most abundantly in caveolin-rich domains [35] and endothelial cell gap junctions [36], and intermediate-conductance (IK) channels that are localized along endothelial projections adjacent to myoendothelial gap junctions [36, 37]. The roles of SK and IK channels in endothelial function are described in detail in the chapter by Kohler et al.

Flow Sensitivity and Functional Roles of Kir Channels in Endothelial Cells

Flow-sensitivity of endothelial Kir channels was first discovered in bovine aortic endothelial cells and this response was shown to underlie flow-induced endothelial hyperpolarization [8, 38]. It was proposed, therefore, that these channels may play a major role in endothelial responses to their hemodynamic environment [39]. Furthermore, Kir2.1 channels were shown to be sensitive to a mechanical stimulus when over-expressed in *Xenopus* oocytes [40]. Similar flow-induced activation of Kir channels was also observed later in our studies in human aortic endothelial cells [5, 41]. We also identified Kir2 channels as the dominant K^+ conductance in aortic endothelial cells under resting conditions [14]. However, due to a lack of appropriate genetic models, the evidence for a direct role of Kir2 channels in flow-induced endothelial responses is still rather sparse.

Endothelial Kir channels in control of vascular tone: Flow-induced vasodilation is a hallmark of endothelial response to flow and one of the essential vascular functions. Cooke et al. [42] were first to demonstrate that endothelial K^+ channels contribute significantly to flow-induced vasodilation by exposing pressurized rabbit iliac arteries to an array of K^+ channel blockers including Ba^{2+} that blocks Kir channels [6], as well as charybdotoxin and iberiotoxin that block Ca^{2+}-sensitive K^+ channels [43]. All blockers were shown to inhibit flow-induced vasodilatation, and it was proposed that the channel that is responsible for the flow response is a Ca^{2+}-sensitive K^+ channel [42]. The role of Kir channels was not established. A further study addressed the role of Kir channels in flow- and acetylcholine-induced vasodilation of cerebral arteries as measured by isometric force recordings of arterial rings [44]. In this study, Ba^{2+} was shown to inhibit the relaxation induced by intraluminal perfusion but not by acetylcholine suggesting that Kir channels are important for flow-induced but not for muscarinic-dependent vasodilation. In both studies, the effect of Ba^{2+} was eliminated by vessel denudation. In contrast, a recent study demonstrated that endothelial Ba^{2+}-sensitive Kir currents contribute to acetylcholine-induced vasodilation in rat mesenteric artery [45]. It was also shown that application of acetylcholine resulted in an increase in Kir currents in these cells but it was not established what sub-types of Kir channels were responsible for this effect [45]. Kir channels were also implicated in the regulation of myogenic response in renal arterioles as demonstrated by blunting the myogenic response by Ba^{2+} [46]. Interestingly, blocking Kir channels with Ba^{2+} was also shown to inhibit flow-induced Ca^{2+} response and augment flow-induced downregulation of endothelin-1 expression [47] suggesting that Kir channels may facilitate vasodilation by inhibiting endothelin-1 synthesis.

Notably, Kir channels are also implicated in the control of blood flow in human subjects. First, Dawes et al. [48] showed that infusing $BaCl_2$ (4 μM/min) into the brachial artery via a catheter to create a local increase in Ba^{2+} concentration resulted in a significant decrease in resting flow rate in the forearm of healthy subjects. Moreover, using the same approach, it was also shown that Ba^{2+} infusion inhibits

both reactive and exercise-induced hyperemia (increase in blood flow) [49, 50]. Taken together, these studies suggest that Kir channels may play an important role in flow-induced vasodilation.

Endothelial Kir channels in cell proliferation and migration. Kir channels were also shown to play a role in the regulation of endothelial proliferation by basic Fibroblast Growth Factor (bFGF), one of the well-known regulators of endothelial cell proliferation [51]. Specifically, bFGF was shown to significantly increase endothelial Kir current in human umbilical vein endothelial cells, whereas exposing cells to Ba^{2+} abrogated bFGF-induced proliferation. In addition, Ba^{2+} was also shown to inhibit bFGF-induced NO release. In terms of migration, the role of Kir was tested in the wound-healing model, also in human umbilical vein ECs (HUVECs) [52]. Several ion channel blockers, including Ba^{2+} were shown to significantly inhibit the velocity of cell migration but since the effect was not specific, it is hard to determine the role of Kir in this process. Clearly, a lack of genetic models for Kir2 channels, a major class of endothelial Kir channels, presents a major constraint in determining the physiological roles of these channels in endothelial function.

Endothelial Kir channels in lung ischemia. A series of studies from Chatterjee and colleagues provided significant insights into the roles of endothelial Kir, specifically Kir6 channels, in endothelial response to the cessation of flow that occurs during lung ischemia leading to membrane depolarization and production of reactive oxygen species [17, 20, 53]. This topic is described in detail in several excellent reviews [53, 54].

Suppression of Endothelial Kir Channels by Cholesterol In Vitro *and* In Vivo

Endothelial Kir: As described above, since plasma hypercholesterolemia is known to play a major role in causing endothelial dysfunction and since cholesterol is a major lipid component of the plasma membrane in all mammalian cells, our studies focused on determining the impact of cholesterol on mechanosensitive endothelial ion channels. Our studies showed that endothelial Kir channels are suppressed by an increase in cellular cholesterol (Fig. 15.1a, [4]), and by the exposure to elevated levels of pro-atherogenic very low density lipoproteins (VLDL) (Fig. 15.1b, [5]). In the first study [4], bovine aortic endothelial cells (BAECs) that were either depleted of or enriched with cholesterol using the cholesterol carrier methyl-β-cyclodextrin (MβCD) resulting in significant increase or decrease of endothelial Kir current without affecting cell capacitance or biophysical characteristics of the current. In a later study, we used human aortic endothelial cells (HAECs) that also express Kir channels even though typical Kir current densities in HAECs are significantly lower than those in BAECs. In fact, most of our studies in HAECs were performed in high K^+ extracellular solution to increase Kir current density. The effect of MβCD on Kir current in BAECs and HAECs were very similar. Exposure to 5–50 μg/ml VLDL

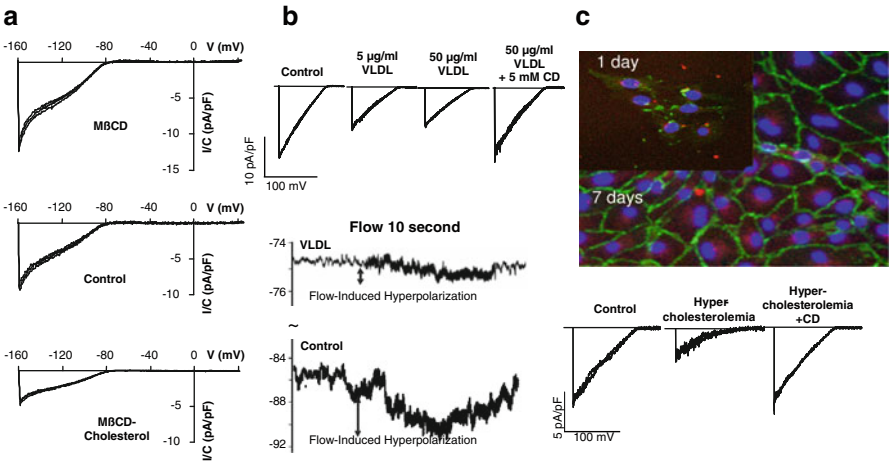

Fig. 15.1 Suppression of endothelial Kir channels by an increase in cellular cholesterol *in vitro* and *in vivo*. (**a**) Typical Kir currents recorded from BAECs exposed to 2.5 mM MβCD or MβCD-cholesterol for 1 h. Three superimposed traces are shown for each cell (recordings are performed in low K^+ solutions). **This research was originally published in Biophysical Journal** 83: p. 3211–3222. (**b**) *Upper panel* Typical Kir currents recorded from HAECs exposed to 5 or 50 μg/mL VLDL for 24 h or treated with 50 μg/mL VLDL for 24 h followed by 5 mM MβCD for 1 h (recordings are performed in high K^+ solutions). *Lower panel* Typical voltage traces recorded from a control and a VLDL-treated cells exposed to 2 dyn/cm^2 shear stress. (**c**) *Upper panel* Immunostaining of PAECs for PECAM (*Green*) and von-Willebrand Factor (*Red*). The staining was performed immediately after cell isolation, and repeated after cells were maintained in culture for 7 days to form a monolayer. *Lower panel* Representative recordings of Kir currents in freshly-isolated PAECs from control and hypercholesterolemic pigs, and in PAECs freshly-isolated from hypercholesterolemic pigs followed by a treatment of 5 mM MβCD for 1 h after isolation. **This research was originally published in Circulation Research** 2006. 98(8): p. 1064–1071

also resulted in current inhibition, which was completely reversible by MβCD, indicating that VLDL-induced suppression of Kir is mediated by an increase in the level of free cholesterol in endothelial membranes (Fig. 15.1b, upper panel). Similar effects were also observed for acetylated LDL (acLDL). Exposure to VLDL also significantly decreased flow-induced membrane hyperpolarization in aortic endothelium (Fig. 15.1b, lower panel). Most importantly, we showed that endothelial Kir channels were also suppressed by plasma dyslipidemia in a diet-induced porcine model of atherosclerosis. Endothelial cells were isolated by gentle mechanical scraping of porcine aortas immediately after animal sacrifice and harvesting of the aortas, and cells were identified by typical endothelial markers, PECAM and vWF (Fig. 15.1c, upper panel). Electrophysiological recordings were performed on freshly-isolated cells on the day of the sacrifice (Fig. 15.1c, lower panel). These recordings demonstrate that Kir currents recorded in aortic endothelial cells isolated from hypercholesterolemic animals were significantly lower than

currents recorded in cells isolated from control animals. Furthermore, the effect was fully abrogated by exposing cells isolated from hypercholesterolemic animals to MβCD after the isolation [5]. These observations demonstrate that endothelial Kir channels are suppressed by plasma dyslipidemia *in vivo*.

Cholesterol sensitivity of Kir in progenitor cells and cardiomyocytes: Dyslipidemia-induced suppression of Kir channels was observed not only in mature endothelial cells but also in endothelial progenitor cells isolated from the bone marrow of hypercholesterolemic pigs [55]. This study was performed with side-population cells, a sub-type of progenitor cells that can differentiate into endothelial cells [56, 57]. These cells express strongly-rectifying K+ channels and the current density in freshly isolated cells from the bone marrow was almost tenfold higher than in mature endothelial cells, and the current was decreased upon cell differentiation [55]. Similarly to dyslipidemia-induced suppression of Kir channels in aortic endothelium, reduced Kir activity was observed in side-population cells freshly isolated from hypercholesterolemic pigs. Dyslipidemia-induced Kir suppression was also observed in cardiomyocytes isolated from hypercholesterolemic rats [58].

The effect of cholesterol on endothelial Kir currents is mediated by Kir2.1 and Kir2.2 channels which, as described above, constitute the dominant Kir conductance in aortic endothelium [14]. Indeed, both Kir2.1 and Kir2.2 are highly cholesterol sensitive as demonstrated by expressing the channels in cell lines that lack endogenous Kir channels, such as Chinese Hamster Ovary (CHO) cells or HEK293 cells [59, 60]. In contrast, Kir2.3 and Kir2.4 show less cholesterol sensitivity, which may result in differential effects of cholesterol on the membrane potential of different cell types. Cholesterol was also shown to suppress the activity of Kir6.2 [61], which as described briefly above plays an important role in microvascular endothelial cells. The effects of cholesterol on Kir channels in cardiomyocytes are complex: while basal currents that are underlined by Kir2 channels are suppressed by cholesterol, acetylcholine-sensitive Kir channels (GIRK or Kir3) are actually enhanced by cholesterol, an effect that is also confirmed in a heterologous expression system [58]. These opposite effects of cholesterol on basal and acetylcholine-sensitive currents in the same cells might be the basis of complex electrophysiological patterns under high cholesterol conditions, but this is beyond the topic of the current book chapter.

Biophysical Basis of Cholesterol Regulation of Kir Channels: The Silent Channel Hypothesis

Silencing of Kir by cholesterol: Earlier studies have shown that cholesterol may regulate Ca^{2+}-sensitive K^+ channels by decreasing their open probability, the ability of the channels to undergo the transformation between closed and open states [62]. This is not the case, however, for Kir2 channels: analysis of single channel activity of both endothelial Kir channels and Kir2.1 channels expressed in a null cell showed

that enriching the cells with or depleting them of cholesterol results only in a small effect on the open probability of the channels (Fig. 15.2). In particular, following cholesterol enrichment of the cells, we observed only ~5 % decrease in the open probability of the channels [4], [59]. This small decrease in the open probability cannot account for the 2- to 3-fold decrease in the Kir current density observed in whole cell currents under the same experimental conditions. Moreover, since the basal open probability of Kir2.1 channels is very high as measured in the on cell configuration at negative voltages (−140 to −60 mV range) (>90 %), an increase in open probability would be impossible to account for a twofold elevation of the whole cell Kir current that is induced by cholesterol depletion. Changes in cellular cholesterol also had no effect on the unitary conductance of the channels indicating that cholesterol-Kir2 protein interaction does not affect the pore structure of the channels. An alternative possibility was that cholesterol might affect the expression of the channel protein or its trafficking to the plasma membrane. However, this was

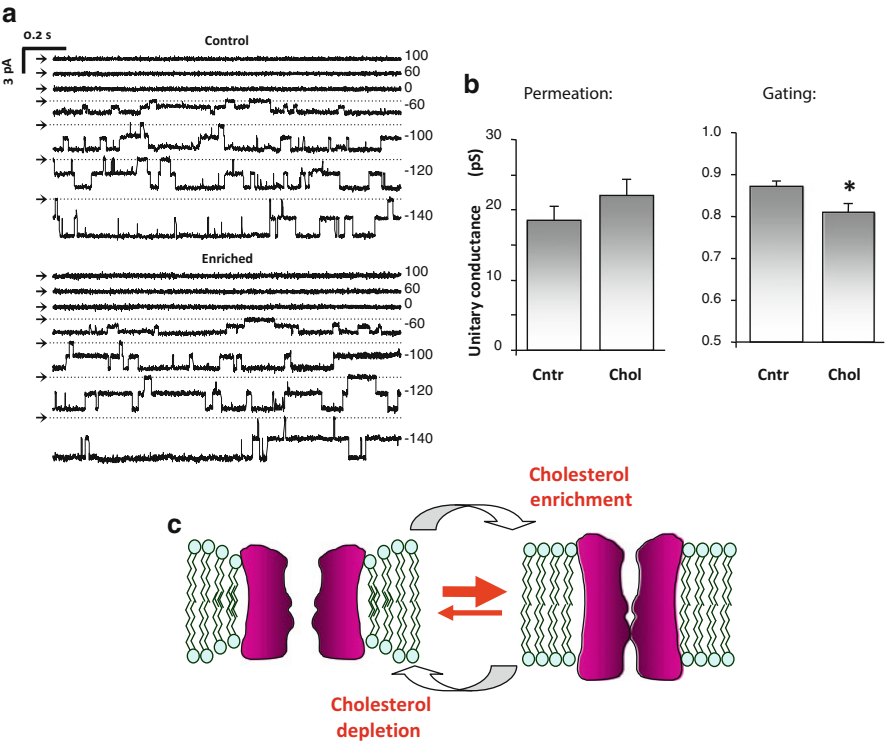

Fig. 15.2 Evidence for silent channel hypothesis. (**a**) Single channel recordings of Kir2.1 expressed in CHO cells under control and cholesterol-enriched conditions. (**b**) Average unitary conductance open probability for the two conditions. (**c**) An increase in membrane cholesterol shifts the conformation of the channels between "active" and "silent" states. **This research was originally published in Biophysical Journal**, 2004. 87: p. 3850–61

also not the case for Kir2 channels: there was no effect neither on the expression of the channels nor on their trafficking to the plasma membrane [59]. To resolve this apparent controversy, we proposed that an increase in membrane cholesterol stabilizes the channels in a *long-lived closed "silent" state* that decreases the number of "active" channels on the membrane and which cannot be therefore detected on a level of single channel.

Silencing of Kir by caveolin: More recently, the "silent channel" hypothesis was supported by demonstrating the same pattern of inhibition in caveolin-induced suppression of Kir current [60]. In this study, we showed that, Kir2.1 physically interact with cavelin-1 (Cav-1) (Fig. 15.3a) and that similarly to cholesterol, an increase in caveolin-1 (Cav-1) expression resulted in a significant decrease in Kir current density (Fig. 15.3b) without any detectable effect on the single channel properties of the channels or their expression on the plasma membrane [60]. Furthermore, a putative Cav-1 consensus binding motif that was previously defined as φXφXXXXφ, where φ is the aromatic amino acid Trp, Phe, or Tyr, [63], was identified at the interface between the outer transmembrane helix and the N-terminus of Kir2.1 channel (Fig. 15.3c, [60]. Comparative analysis of the closed and open conformation states of the channels based on the crystal structures reported earlier [64, 65] revealed that the putative caveolin-1 binding site of Kir2.1 channels would be more accessible to caveolin-1 when the channels are closed than when they are open and become partially obscured in the open state [60]. We proposed, therefore, that caveolin-1 binds preferentially to the closed conformation of Kir2 channels and stabilizes the

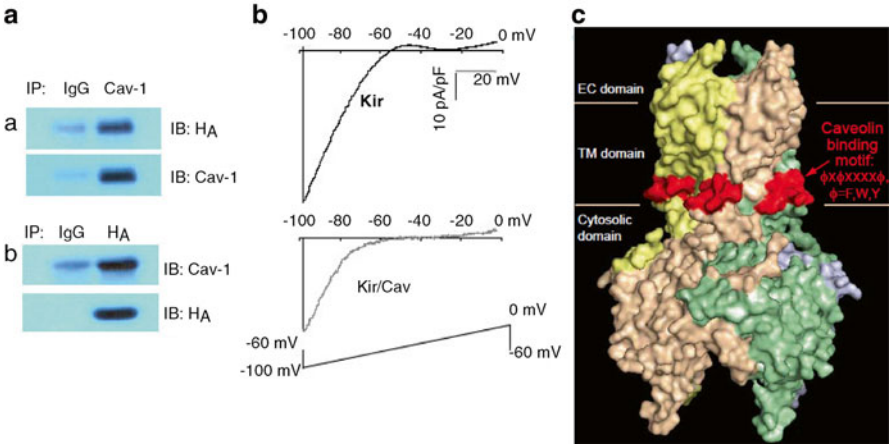

Fig. 15.3 Caveolin-1 is a negative regulator of Kir2.1 channels. (**a**) Co-immunoprecipitation of caveolin-1 with Kir2.1 channels. (**b**) Current traces for Kir2.1 with and without co-expression with Cav-1. (**c**) Location of the caveolin φXφXXXXφ binding motif (*red*) at the interface between the outer transmembrane helix and the N-terminus of the channels (residues 81–88 in Kir2.1) in a surface presentation of the crystal structure of Kir2.2. **This research was originally published in Journal of Physiology** 2014, 592:4025–38

channels in the closed "silent" state. As discussed in more detail below, however, our computational and site-directed mutagenesis analysis suggests that cholesterol and caveolin-1 do not compete for the same binding sites of Kir2 channels but regulate the channels through different binding sites with the two signals converging to the same intramolecular pathway mediated by common residues. These observations suggest that stabilization of Kir2 channels in a silent state is an important mechanism in the regulation of these channels by several effectors.

Cholesterol-rich domains: The partitioning of proteins into cholesterol-rich domains is believed to regulate their function by providing scaffolds to signaling platforms and protein-protein interactions. However, this process does not seem to be critical for cholesterol sensitivity of Kir2 channels. We found that Kir2 channels indeed do partition into cholesterol-rich membrane domains [59, 66] and co-precipitate with caveolin-1 [60], but there is only a small shift in the channel distributions between the domains upon cholesterol depletion or enrichment [66] suggesting that disengaging from these domains upon cholesterol depletion or a small increase in channel association with the domains upon cholesterol enrichment is unlikely to have a major impact on Kir2 function. Furthermore, the presence of caveolins is not a pre-requisite of cholesterol sensitivity of Kir channels, as demonstrated in cells devoid of caveolins. Specifically, we showed that while genetic deletion of caveolin-1 results in an increase in Kir currents in macrophages [60] and endothelial cells (not shown), the currents remain cholesterol sensitive in both cell types. Notably, since genetic deletion of caveolin-1 was shown to result in the loss of caveolae structure [67], preservation of cholesterol sensitivity of Kir2 channels in Cav-1 knock out mice indicates that neither the integrity of caveolae nor partitioning of the channels into these domains play a critical role in their sensitivity to cholesterol.

Direct Cholesterol-Kir Interactions: Identification of Cholesterol-Binding Kir2 Domains

Comparative sterol analysis: The first indication that cholesterol regulates Kir2 channels by specific cholesterol-protein interactions and not by changing the physical properties of the lipid bilayers, as was primarily believed earlier, came from a comparison between cholesterol and its chiral analogue epicholesterol [4]. The two sterols differ in an angle of a single OH group and are known to have similar though not identical effects on membrane fluidity [68, 69]. Cells cannot survive without cholesterol and it is also impossible to substitute it completely with epicholesterol. However, we found that removing ~50 % of the membrane cholesterol from aortic endothelial cells and substituting it with a similar amount of epicholesterol maintains the cells' viability and membrane integrity. We could, therefore, test how this substitution affects endothelial Kir channels. To our surprise, substituting endothelial cholesterol with epicholesterol resulted in a significant increase in Kir current that was even stronger than the increase induced by cholesterol depletion [4]. These

observations indicated that cholesterol-induced regulation of endothelial Kir channels depends on specific cholesterol-protein interactions and suggested that the two sterols compete for a binding site either in the Kir protein itself or in an auxiliary protein that might mediate cholesterol effects on the channels. Interestingly, this is not the case for volume-activated anion channels (VRAC) in the same cells. VRAC are not sensitive to the chiral nature of cholesterol, and are regulated instead by changes in the physical properties of the membrane [70, 71].

Insights from purified channels: To discriminate between direct and indirect effects of cholesterol on Kir channels, we reconstituted a purified bacterial analogue of Kir2 cannels, KirBac1.1, into lipid vesicles and found that cholesterol can regulate Kir channels in a purified system without any intermediates. We also found that there is no correlation between KirBac1.1 function and membrane fluidity when the two parameters were compared for an array of different sterols [69]. Importantly, the same conclusion was reached in a later study that used purified mammalian Kir2.1 channels which were reconstituted in lipid vesicles that contained either cholesterol or ent-cholesterol, another chiral analogue of cholesterol whose physical properties are virtually identical to that of cholesterol [72]. Furthermore, we also demonstrated that cholesterol binds to KirBac1.1 channels in a specific and saturable way, and that inhibition of cholesterol-KirBac1.1 binding abrogates the inhibitory effect of cholesterol on KirBac1.1 function [73]. As expected from our studies on cholesterol/epicholesterol substitution in endothelial cells, epicholesterol was shown to compete with cholesterol for KirBac1.1 binding. Thus, clearly Kir2 channels are regulated by specific and direct cholesterol-protein interactions.

First insights into the structural determinants of cholesterol sensitivity of Kir channels: Our quest to identify the structural determinants of cholesterol sensitivity in Kir2 channels started [74] with testing the residues on the protein-lipid interface of the transmembrane domains [75] and on the interface between the transmembrane and cytoplasmic domains, specifically focusing on the residues that were identified earlier to confer the sensitivity of the channels to a regulatory phospholipid PI(4,5)P_2 [76]. These studies led to the identification of the first residues that are essential for cholesterol sensitivity of Kir2.1, which surprisingly were found on the C-terminus of the cytosolic domain of the channels and not on the lipid-protein interface between the channel and the lipid bilayer [74]. More specifically, we found that cholesterol sensitivity of Kir2.1 critically depends on a set of specific residues within the CD loop of the C-terminus with one of the residues, leucine 222 (L222) having the most profound effect (see Fig. 15.2 for the position of L222 in the channel). L222 was also found earlier to play a critical role in the sensitivity of Kir2.1 channels to PI(4,5)P_2 [76]. A loss of cholesterol sensitivity was not associated with a loss of the ability of the channels to partition into cholesterol-rich lipid domains [74]. Importantly, identifying a residue that confers cholesterol sensitivity to the channels does not mean that this residue is part of a cholesterol-binding site. Indeed, we proposed that the CD loop may be important for maintaining the channels in a conformation susceptible to cholesterol modulation.

Cholesterol-sensitivity belt: Further analysis based on the differential cholesterol sensitivities of Kir2.1 and Kir2.3 channels revealed that the cytosolic CD loop is part of a regulatory belt of residues that surrounds the cytosolic pore of the channels in proximity of the inner leaflet of the membrane (Fig. 15.4a shows the side and top of view [77], Fig. 15.4b shows the side view of two opposite subunits indicating the position of the CD loop). However, docking analysis of the cholesterol molecule to the channel protein [78–80] showed that cholesterol does not dock to the residues that comprise the cholesterol sensitivity belt, suggesting that this structure does not represent a cholesterol-binding site of Kir2 channels. Instead, based on a database of crystallographic structures of Kir channels we found that the residues of the cholesterol sensitivity belt were correlated with the residues known to be critical for gating of these channels suggesting that the cholesterol sensitivity belt constitutes a regulatory site that couples cholesterol-binding to channel gating [77].

Two-way molecular switch: Surprisingly, we found that the cholesterol sensitivity of Kir2 channels is also regulated by a two-way molecular switch that is comprised of two distant cytosolic residues, L222 of the cholesterol sensitivity belt that is located close to the interface between the C-terminus and the transmembrane domain, and N251 that is located further away from the transmembrane domain in the EF loop (Fig. 15.4b, c [81]). Specifically, the L222I and N251D mutations each render the channels to be cholesterol insensitive, but together they cancel each other, and the double mutants are cholesterol sensitive. Moreover, we showed that the same switch also regulates the sensitivity of the channels to $PI(4,5)P_2$. Again, each one of these two mutations alone reduced the strength of the interaction between the channel and $PI(4,5)P_2$, but together they cancel each other. Based on the crystal structure of the cytosolic domain of the channel, the distance between the two residues is ~24 Å excluding the possibility that these two residues interact directly with each other. Instead, molecular dynamics simulations of the Kir2.1-WT, L222I-Kir2.1 mutant and the L222-N251D-Kir2.1 double mutant showed that the effects of these mutations span extensive regions of the C-terminus suggesting that the two residues are connected through a reversal-residue-chain that seems to serve as the wiring between the two residues (Fig. 15.4c, lower panel, [81]). In addition, our data suggest that functional links between the N- and the C-termini that couple the intracellular domains of the four subunits of the channels during gating also play an important role in cholesterol sensitivity of Kir2 channels [82]. Accordingly, multiple cytosolic structures in Kir2 channels play a critical role in their sensitivity to cholesterol. Yet, neither of these structures has the characteristics of a cholesterol-binding site.

Identification of novel cholesterol-binding sites. Our next strategy was to test whether Kir2 channels have regions homologous with the known cholesterol-binding motifs: the cholesterol consensus motif (CCM) and the cholesterol recognition amino acid consensus motif (CRAC), two well-established cholesterol-binding motifs that have been found and characterized in other proteins. Indeed, recent studies showed that CRAC is found in TRPV1 channels [83] and in BK channels [84]. A variation of CRAC motif, CARC, an inverted sequence of CRAC was suggested

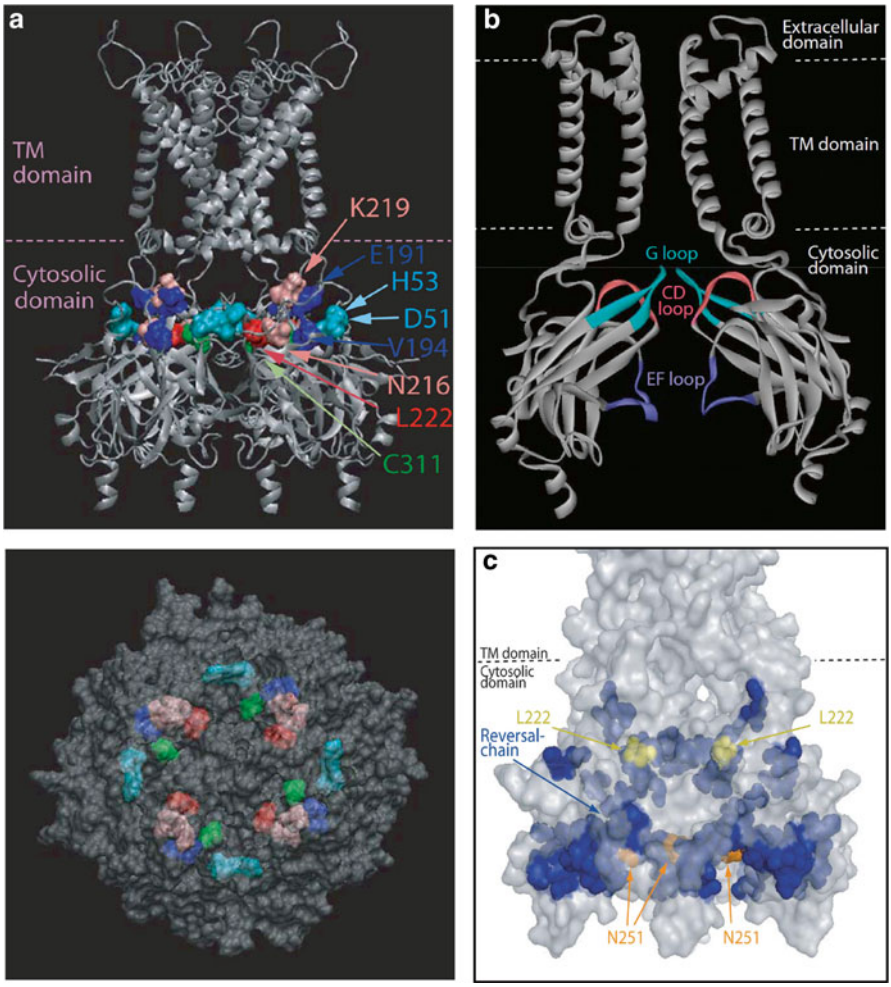

Fig. 15.4 Structural features of cholesterol sensitivity of Kir2.1 channels. (**a**) *Upper panel* Side view of a model of Kir2.1 that includes all four subunits. Shown in the model are the residues whose mutation affects cholesterol sensitivity: D51 and H53 (*cyan*), E191 and V194 (*blue*), N216 and K219 (*pink*), L222 (*red*) and C311 (*green*). *Lower panel*. Top view of the model of Kir2.1 from the membrane showing the cholesterol sensitivity belt formed by the residues whose mutation affects the cholesterol sensitivity of the channel. **This research was originally published in Biophysical Journal**, 2011:100:381–9. (**b**) Model of two opposite facing subunits of Kir2.1 showing the cytosolic G-loop, CD-loop and EF-loop; (**c**) Surface presentation of the cytosolic domain of Kir2.1 showing the reversal residue chain (*blue*), L222I (*yellow*), and N251 (*orange*). **This research was originally published in Journal of Biological Chemistry**. Rosenhouse-Dantsker, A., et al., *Distant Cytosolic Residues Mediate a Two-way Molecular Switch That Controls the Modulation of Inwardly Rectifying Potassium (Kir) Channels by Cholesterol and Phosphatidylinositol 4,5-Bisphosphate (PI(4,5)P2).* Journal of Biological Chemistry, 2012. 287(48): p. 40266-40278. **© the American Society for Biochemistry and Molecular Biology**

to underlie cholesterol sensitivity of AChR [85]. However, based on the sequence analysis, Kir2.1 channels have no CCM motif and no appropriate CRAC or CARC motifs that would be energetically favorable to bind a cholesterol molecule [86]. Therefore, we turned to an alternative approach of identifying cholesterol-binding regions in Kir2 channels using a combination of molecular docking, molecular dynamics (MD) simulations and site-directed mutagenesis that is not biased or limited only to the known cholesterol-binding motifs.

These studies led to the identification of two novel non-annular hydrophobic cholesterol-binding regions in Kir2.1 [86] (See Fig. 15.5a, b).

One binding region was located in the center of the transmembrane domain of the channel (region 1, Fig. 15.5d) [86]. Within this region, we identified six residues whose mutation abrogated the sensitivity of the channel to cholesterol. In addition, mutations of two other residues resulted in a significant decrease in the channel's cholesterol sensitivity. The majority of these eight residues were bulky hydrophobic residues (isoleucine, leucine and valine), and the remaining two residues were smaller residues (alanine and serine). Notably, the known cholesterol-binding motifs (CCM, CRAC and CARC) include in addition to a bulky hydrophobic residue also an aromatic residue (tyrosine, tryptophan or phenylalanine) and a positively charged residue (arginine or lysine). However, mutations of several aromatic and positively charged residues located in region 1 of Kir2.1 resulted in a non-functional channel or did not affect the sensitivity of the channel to cholesterol. Since mild mutations may not affect the interaction of the channel with the cholesterol molecule, the role of these residues in cholesterol binding cannot be excluded.

The second binding region was located at the interface between the transmembrane and cytosolic domains of the channel (region 2) [86]. Within this region, we identified five hydrophobic residues (alanine, leucine, valine and methionine) that affected the sensitivity of the channel to cholesterol. Among these, three mutations abrogated the sensitivity of the channel to cholesterol. As noted above, our earlier studies have shown that mutations of residues located at the lipid-protein interface between the channel and the lipid bilayer do not affect the sensitivity of the channel to cholesterol [74] implying that cholesterol does not bind to annular or boundary sites located on the transmembrane surface of the channel protein. In contrast, in both of the putative cholesterol-binding regions described above, the residues whose mutation affected the sensitivity of the channel to cholesterol were distributed among α-helices of two adjacent subunits of the channel suggesting that the cholesterol molecule would bind in between the α-helices. Furthermore, MD simulations demonstrated that due to the matching between the cholesterol molecule and the hydrophobic and aromatic moieties in the two putative cholesterol-binding regions, these regions prefer cholesterol to phospholipids. Together, this suggests that these two cholesterol-binding regions are non-annular surfaces that are occluded from phospholipid binding (Fig. 15.3c) [86].

In order to compare the strength of the interactions between the cholesterol molecule and the channel in each of the two putative cholesterol-binding regions we assessed the binding energy and binding affinity of cholesterol to the channel.

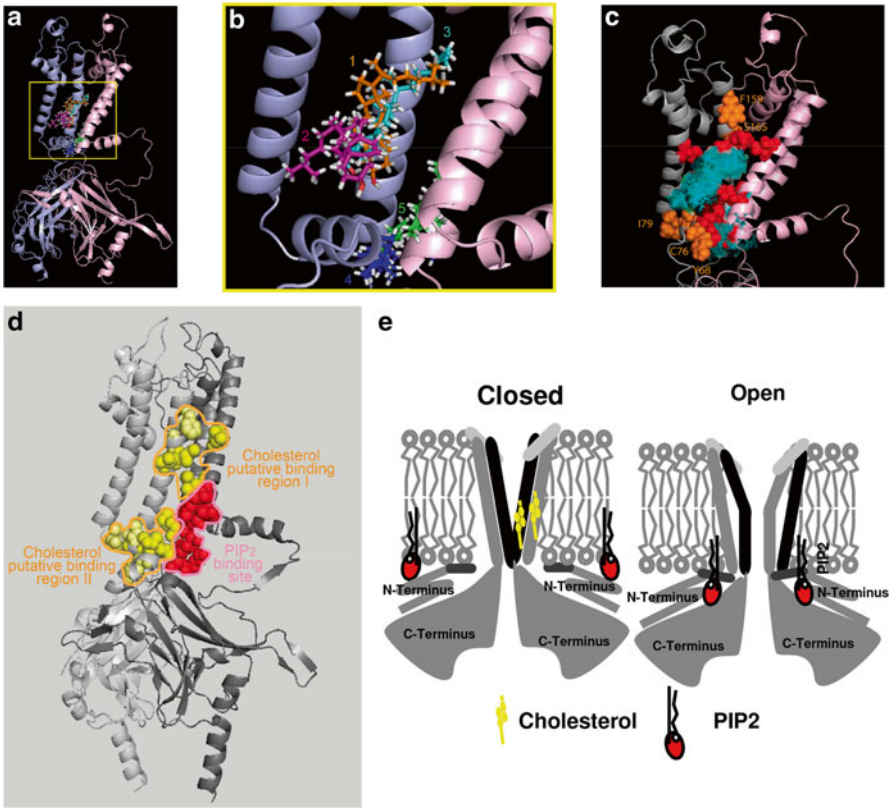

Fig. 15.5 Proximity of the cholesterol and PI(4,5)P_2 binding sites in Kir2 channels. (**a**) Location of two putative cholesterol-binding regions in Kir2.1 that were obtained from 50-ns all-atom full-membrane MD simulations. The starting points of the simulations were the centers of five clusters that were obtained from docking analysis. (**b**) E*nlargement* of the channel region that includes the two putative cholesterol-binding regions depicted as a *yellow rectangle* in **a**. In both **a** and **b**, the two adjacent subunits of the channel that interact with the cholesterol molecule are shown in a *ribbon representation*. (**c**) *Ribbon representation* of the transmembrane and extracellular domains of two adjacent subunits of Kir2.1 (in *gray* and *pink*) depicting the locations of primary (*red*, directly interacting residues) and secondary (*orange,* within 4 Å from the primary residues) cholesterol sensitive residues. Also shown are the locations of the cholesterol molecules at the two putative cholesterol-binding regions (*cyan sticks* and *surface representations*). (**a–c**) **This research was originally published in Journal of Biological Chemistry**. Rosenhouse-Dantskeret al., *Identification of Novel Cholesterol-binding Regions in Kir2 Channels.* Journal of Biological Chemistry, 2013. 288(43): p. 31154–31164 **© the American Society for Biochemistry and Molecular Biology**. (**d**) A ribbon presentation of two adjacent subunits (*Gray* and *light gray*) of the crystal structure of Kir2.2 (PDB ID 3spi) showing the binding site of PI(4,5)P_2 in the channel (*red balls*). Also shown are the corresponding Kir2.2 residues to Kir2.1 residues that form two putative cholesterol-binding regions in Kir2.1 based on functional data and molecular modeling (*yellow balls*—direct interaction; *light yellow balls*—secondary effect). (**e**) Schematic model of cholesterol and PI(4,5)P_2 interactions with Kir2 channels. (**d–e**) **This research was originally published in Computational Structural Biotechnology J**. Rosenhouse-Dantsker, A., Y. Epshtein, and I. Levitan, *Interplay Between Lipid Modulators of Kir2 Channels: Cholesterol and PIP2*. Comput Struct Biotechnol J, 2014. 11: p. 131–7

To that end, we calculated the binding enthalpy, which is a quantitative indicator of changes in the binding energy, and the equilibrium free energy of the process that enables to assess binding affinity and stability. Our calculations showed that the average binding enthalpy of cholesterol at region 1 (−6 ± 2 kcal/mol) was slightly stronger than the average binding enthalpy to the cholesterol molecule at region 2 (−4 ± 2 kcal/mol). Our calculations also indicated that the equilibrium free energy was favorable in region 1 and unfavorable in region 2. Yet, even for the favorable region 1, the absolute value of the free energy was small suggesting that the binding affinity of cholesterol to this region is weak. Overall, these results suggest preference for binding region 1 in the center of the transmembrane domain of the channel. However, due to the small absolute values and the typical standard error, we cannot rule out the possibility of weak cholesterol binding to region 2 as well. Since mutations of residues in both regions abrogated the sensitivity of the channels to cholesterol, both regions may form cholesterolophilic surfaces. Moreover, region 2 may represent a transient cholesterol-binding site necessary for cholesterol to have an effect on channel function. Alternatively, it is also possible that transient binding to the interface of region 2 is necessary for cholesterol to access the more stable binding region (region 1) in the transmembrane domain of the channel [86]. The same strategy was used in a later study leading to a very similar conclusion [87].

Importantly, based on the locations of the cholesterol-binding regions we suggested that cholesterol stabilizes the channels in the closed configuration by opposing the hinging motion of the transmembrane domain that underlie the gating mechanism of the channels, thereby providing the first mechanistic explanation for how cholesterol shifts Kir channels into the "silent state" [86].

Interplay between cholesterol and PI(4,5)P2 in the modulation of Kir2 channels. As noted above, our first studies of the structural determinants of cholesterol sensitivity in Kir2 channels led to cytosolic residues [74] that have been previously shown to affect the sensitivity of the channels to a regulatory phospholipid $PI(4,5)P_2$ [76]. Furthermore, our subsequent studies [81] resulted in the identification of a two-way molecular cytosolic switch that regulates the sensitivity of Kir2.1 to both cholesterol and $PI(4,5)P_2$. We thus explored whether cholesterol regulates Kir channels by regulating their access to $PI(4,5)P_2$. This, however, was not the case because sequestering $PI(4,5)P_2$ had no effect on cholesterol sensitivity of Kir2.1 channels [74]. Moreover, examining the role of cholesterol in channel-$PI(4,5)P_2$ interactions, we then showed that whereas cholesterol depletion resulted in strengthening of Kir2-$PI(4,5)P_2$ interactions, cholesterol enrichment had no effect on Kir2-$PI(4,5)P_2$ interactions [88]. Taken together, these data suggest that cholesterol and $PI(4,5)P_2$ act through overlapping regions of the channel. However, since residues that have been shown to bind directly to $PI(4,5)P_2$ do not affect the sensitivity of the channel to cholesterol [74], it is likely that the two lipids bind to distinct binding sites. This notion is further corroborated by comparing the locations of the two putative cholesterol binding regions described above [86] with the binding site of $PI(4,5)P_2$ in Kir2.2 [65, 88]. Accordingly, these

two lipids bind to different adjacent non-overlapping sites in the channel (Fig. 15.5d). This structural proximity between the sites gives rise to the possibility that in addition to the common mechanism that the two lipids share, functional interplay between cholesterol and $PI(4,5)P_2$ may also originate from interactions between the binding sites. Figure 15.5e summarizes our current working hypothesis of the mechanism that underlies cholesterol regulation of Kir channel. Cholesterol binding in between the transmembrane helices of the channel stabilizes the closed state of the channel by interfering with the hinging motion of the inner transmembrane of the channel. Removal of cholesterol strengthens channel-$PI(4,5)P_2$ interactions and stabilizes the channel in its open state.

Conclusions: What Is Now Known and What Is Unknown

It is well established today that Kir channels are expressed in several types of endothelial cells and that their activity is strongly suppressed by the elevation of cellular cholesterol *in vitro* and *in vivo*. There is also no doubt that cholesterol interacts with the channels directly via specific sterol-protein interactions. Moreover, the preponderance of evidence suggests that cholesterol binds to the channels not at the protein-membrane interface but at non-annular sites in hydrophobic pockets formed by the helices of the transmembrane domains of the channels. The binding of cholesterol to these sites affects the gating machinery of the cytosolic pore. What is not clear, however, is how binding of the cholesterol molecule to a hydrophobic pocket in the transmembrane domain of the channels is transduced to the cytosolic domain to affect channel gating. This question should be addressed by a combination of molecular dynamic simulations, site-directed mutagenesis and biophysical analysis of the channel activities. The physiological impact of cholesterol-induced suppression of Kir on endothelial function remains to be explored. The only evidence so far that suppression of Kir plays a significant role in endothelial function is a correlation between the loss of channel activity and impairment of flow-induced vasodilation in a porcine model of diet-induced hypercholesterolemia. Clearly, this is not sufficient to establish the role of Kir. Structural studies provide novel and unique tools to address this question. Specifically, our studies identified multiple mutants of Kir2.1 channels that lose their cholesterol sensitivity. Creating transgenic models that substitute native Kir channels with their cholesterol-insensitive mutants will allow to establish the impact of cholesterol sensitivity of these channels on vascular function.

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