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Large conductance, calcium- and voltage-gated potassium (BK) channels: regulation by cholesterol

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Abstract

Cholesterol (CLR) is an essential component of eukaryotic plasma membranes. CLR regulates the membrane physical state, microdomain formation and the activity of membrane-spanning proteins, including ion channels. Large conductance, voltage- and Ca^{2+} -gated K^{+} (BK) channels link membrane potential to cell Ca^{2+} homeostasis. Thus, they control many physiological processes and participate in pathophysiological mechanisms leading to human disease. Because plasmalemma BK channels cluster in CLR-rich membrane microdomains, a major driving force for studying BK channel-CLR interactions is determining how membrane CLR controls the BK current phenotype, including its pharmacology, channel sorting, distribution, and role in cell physiology. Since both BK channels and CLR tissue levels play a pathophysiological role in human disease, identifying functional and structural aspects of the CLR-BK channel interaction may open new avenues for therapeutic intervention. Here, we review the studies documenting membrane CLR-BK channel interactions, dissecting out the many factors that determine the final BK current response to changes in membrane CLR content. We also summarize work in reductionist systems where recombinant BK protein is studied in artificial lipid bilayers, which documents a direct inhibition of BK channel activity by CLR and builds a strong case for a direct interaction between CLR and the BK channel-forming protein. Bilayer lipid-mediated mechanisms in CLR action are also discussed. Finally, we review studies of BK channel function during hypercholesterolemia, and underscore the many consequences that the CLR-BK channel interaction brings to cell physiology and human disease.

Keywords

MaxiK channel; cholesterol; membrane lipids; lipid raft; hypercholesterolemia; alcohol

1. Introduction

Cholesterol (CLR) is an essential component of the plasma membrane in animal cells. Lipids in general and CLR in particular do not distribute randomly in the membrane but cluster in “domains” that may expand one or the two bilayer leaflets (Devaux & Morris,

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Conflict of Interest Statement

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2004; Dopico & Tigyi, 2007; Simons & Gerl, 2010). These domains are usually enriched in not only CLR but also sphingolipid species, which emboldens the domain with physical properties that differ from those of the bulk bilayer (Dopico & Tigyi, 2007; Lingwood, 2011). Remarkably, numerous cell membrane signaling molecules and receptors, as well as chaperone and cytoskeletal elements seem to preferentially localize and interact within these membrane lipid microdomains, leading to optimization of membrane signaling. Thus, cell membrane-associated signaling and protein function are framed within a tightly controlled, CLR-enriched microenvironment (Dopico & Tigyi, 2007; Dart, 2010; Levental et al., 2010; Contreras et al., 2011). Functional assays, proteomics and, more recently, structural biology converge to indicate that the function of membrane-spanning proteins is critically controlled by their lipid microenvironment, CLR local presence being a major modulator of membrane proteins, including ion channels (Dart, 2010; Levitan et al., 2010; Ohno-Iwashita et al., 2010).

Ion channels are membrane-spanning proteins that contain an aqueous central cavity or pore through which specific ions are conducted following their electrochemical gradient. The pore, however, is gated in a rather specific manner by many signals, including light, temperature, mechanical tension, electrical potential, and chemical ligands (Hille, 2001). Large conductance, Ca^{2+} - and voltage-gated K^+ (BK) channels are defined by both ion conduction and gating properties. Their permeation signature is given by the combination of a large unitary conductance (typically >150 pS in symmetric 100 mM K^+) and very high selectivity for K^+ over other ions. BK channels' other signature is given by their dual gating: the open state of the pore is favored as the transmembrane voltage is made more positive and/or Ca^{2+} levels in the vicinity of the plasmalemma BK channel cytosolic side are increased (Section 2.1). Thus, BK channels link changes in transmembrane voltage to Ca^{2+} -mediated signaling. Given the role of Ca^{2+} as universal second messenger and the critical importance of BK channels in providing negative feed-back on intracellular Ca^{2+} levels in excitable and nonexcitable cells, it is not surprising that BK channels are found in cell membranes across different tissues and species where they regulate cell homeostasis and play an important role in pathophysiology (Section 2.2).

Given BK channel participation in multiple biological processes and ubiquitous distribution, a possible modulation of BK ionic currents by membrane CLR levels received early recognition (Bregestovski & Bolotina, 1989; Bolotina et al., 1989). Since then, CLR regulation of BK currents has received increasingly focused attention regarding several issues. The most obvious is determining the mechanism(s) that allows membrane CLR to control the BK ionic current phenotype, including its pharmacology, channel sorting and distribution and, eventually, the impact of this control on cell physiology. In particular, CLR has been conceptualized as a type II lipid: it promotes negative monolayer curvature, and hexagonal H_{II} phase formation in phospholipid bilayers (Tilcock et al., 1984). Cholesterol and other type II lipids play a critical role in endocytosis, exocytosis, and neurotransmitter release (Cullins & de Kruijff, 1980; Wasser & Kavalali, 2009; Chasserot-Golaz et al., 2010). Notably, BK channels cluster in presynaptic membranes and play a key role in regulating exocytotic neurotransmitter release (Section 2.2). In addition, BK channels participate in major pathophysiological mechanisms leading to disease (Sections 2.2 and 8). In turn, CLR levels are modified by genetic and environmental factors leading to a wide spectrum of disorders (Papakostas et al., 2004; Saini et al., 2004; Palinski et al., 2007; Tuñón et al., 2007; Boyer et al., 2010; Laloux et al., 2010; Ong et al., 2010). Thus, identifying functional and structural aspects of CLR-BK channel interactions will bring new insights on our understanding of human diseases (Section 8) and even potentially lead to new therapeutic interventions. From a more basic research standpoint, since CLR levels found in natural membranes modify several physical properties of the lipid bilayer, CLR-BK channel interactions can be used as a model to gain insights on how specific bilayer physical

properties modify the function of transmembrane ion channel complexes, as previously reported with ion channel-forming model peptides (Lundbæk et al., 1994; 1996; 2004; 2010; Morris & Juranka, 2007).

Cholesterol-BK channel functional interactions have been documented in both excitable and nonexcitable cells. This phenomenology is presented to the reader in Section 3. Given the complexity of intervening elements in the CLR-BK channel functional interaction (ion channel subunits, associated proteins, nearby organelles, local lipid microenvironment, cytosolic signaling, local ionic medium, etc.), it is not surprising that a given modification of membrane CLR levels (e.g., CLR depletion) has been reported to potentiate, inhibit or even fail to affect BK currents. Most studies, however, demonstrate that membrane CLR depletion and associated membrane domain disruption lead to potentiation of BK current, raising the speculation that membrane CLR exerts an inhibitory control on native BK channels. Studies on BK channel subunits reconstituted into artificial, two-three species phospholipid bilayers demonstrate that CLR presence in the bilayer depresses single channel activity (Crowley et al., 2003; Bukiya et al., 2008c; 2011c; Yuan et al., 2011). Thus, we next elaborate on CLR modulation of single channel function, dissecting out the role of channel subunit composition in CLR action (Section 4.1), and CLR-induced modifications in unitary conductance (Section 4.2) vs. gating and steady-state activity (Section 4.3). Generally speaking, modulation of BK channel function has been framed within two schools of thought: one considers that CLR insertion into the bilayer and direct interaction with bilayer lipids lead to modification of bilayer physical properties and, eventually, modified BK channel function. Modification of bilayer physical properties as mediators of CLR alteration of BK channel function is presented in Section 4.3.1. A second point of view puts forward the idea that CLR directly interacts with a protein surface, likely provided by the BK channel protein itself, and thus modifies BK channel function. This interpretation is supported by recent data from our laboratory and presented in Section 4.3.2. In addition, the possibility that CLR acts at a protein-lipid interface is contemplated.

After addressing underlying mechanisms that may play a role in CLR modification of single channel function, we touch upon the more complex scenario of natural membranes, where changes in BK channel expression and/or distribution contribute to the overall effect of CLR on macroscopic BK current (Section 5). Modifications in current reflect alterations in channel functional expression and individual BK protein conformation. A logical consequence of a modification of channel expression and/or functional states by CLR is that such changes will also modify the response of BK channel populations to other biologically relevant ligands. Thus, a special section is devoted to recent studies on CLR-BK channel ligand interactions. Particular attention is paid to the CLR-alcohol interaction on BK channels as changes in CLR membrane distribution and content have both been reported after protracted alcohol exposure, and these two modulators of BK channels may contribute to pathophysiology *via* BK channels (Section 6).

In a final integrative step, we consider the CLR-BK channel interaction at the organ-organismal level. Because hypercholesterolemia alters membrane lipid composition, CLR levels in particular (Tulenکو et al., 2001; Vayá et al., 2008), we discuss the modifications in BK current in response to manipulation of CLR levels in serum (Section 7). For hypercholesterolemia to alter BK current, modification of individual BK channel function and/or number of functional channels must occur. In most cases, however, the mechanisms (genetic, epigenetic, etc.) leading to modification of BK channel-mediated cell function by changes in circulating CLR levels remain unknown. Finally, we conclude by elaborating on the possible consequences for cell and tissue function that result from modification of BK channel function by CLR (Section 8).

We consider that comprehension of the different topics on the CLR-BK channel interaction outlined above requires an introduction to the BK channel world. Thus, for readers that are not “BKologists”, we present two introductory sections on BK channel structure and gating (Section 2.1) and role in physiology and disease (Section 2.2). In these introductory sections, the reader will be mainly referred to review articles where the original articles can be found. Thus, we apologize to many colleagues for not being able to quote their original work on BK channels.

2. Basic concepts on BK channels

2.1. Structure and gating

Fully functional BK channels result from the tetrameric association of 125–140 kDa polypeptides termed α , slo or slo1 subunits. The peptide is encoded by a single gene, *Slo* (first cloned from the *Drosophila Slowpoke* locus; Atkinson et al., 1991), or its mammalian ortholog *Slo1* or *KCNMA1*. In several species, the gene coding for the BK channel-forming protein is spliced at several sites, with splicing significantly contributing to the channel phenotypic diversity (Fettiplace & Fuchs, 1999; Fury et al., 2002; Yan et al., 2008; Fodor & Aldrich, 2009). For example, the STREX variant, which includes a string of 59 additional residues in the cytosolic C-end, differs from STREX insert-free isoforms in ionic current phenotype, response to protein kinase A (PKA) and functional interaction with BK channel auxiliary proteins (Xie & McCobb, 1998; Tian et al., 2001; Petrik & Brenner, 2007). All slo1 subunits share significant homology with voltage-gated K_v channels of the six transmembrane domain (TM6) family (K_v channels) from which they likely evolved, with S1-S4 contributing to voltage-sensing and S5-S6 conforming the activation gate-pore region (Latorre & Brauchi, 2006; Salkoff et al., 2006; Lee & Cui, 2010). As found for other members of the K_v channel family, the slo1 subunit S4 helix contains a string of positively charged residues. However, only one of these (Arg213) has been demonstrated to contribute effectively to BK channel voltage-sensing (Ma et al., 2006) (Fig. 1).

In addition to the S1–S6 core, slo1 subunits contain a transmembrane (TM) S0 leading to an extracellular N-end (Wallner et al., 1996) and a large cytosolic tail domain (CTD) (Wei et al., 1994; Cox, 2005; Yuan et al., 2010), an architecture that is supported by electron cryomicroscopy (Wang & Sigworth, 2009). Disulfide cross-linking of the extracellular portions of BK TM helices has been used to determine the relative position of the slo1 helices and their sites of interaction with accessory subunits of the $\beta 1$ type. These studies, complemented with allosteric modeling of electrophysiological data, seem to indicate that S0 is needed for channel regulation by accessory β subunits and modulates the equilibrium between resting and active states of the channel voltage sensor (Rothberg, 2004; Morrow et al., 2006; Koval et al., 2007; Liu et al., 2008a,b).

The CTD includes two Regulator of Conductance of K^+ (RCK) domains, which include sites for sensing Ca^{2+} , allowing BK channels to increase channel steady-state activity (i.e., channel open probability; P_o) in response to rises in Ca^{2+}_i within physiological levels (Wei et al., 1994; Cox, 2005). The crystal structure of the CTD shows that the two RCKs from each slo1 subunit form an octameric Ca^{2+} -gating ring (Yuan et al., 2010). Ca^{2+} -sensing by this ring appears to increase the gating ring diameter (Ye et al., 2006; Yuan et al., 2010). This favors channel opening by “tugging” on the pore domain *via* the S6-RCK1 linker, which acts like a spring (Niu et al., 2004) (Fig. 1). Functional and structural data suggest that the CTD is close to the voltage-sensor during channel gating (Yang et al., 2008; Wang & Sigworth, 2009; Yuan et al., 2010). Voltage-sensor movement, Ca^{2+} -binding to RCK domains and pore opening are conceptualized as independent equilibria that interact allosterically with each other (Latorre et al., 2010).

The tight interaction between CTD regions involved in divalent recognition and voltage-sensing transmembrane regions seems to be particularly relevant for Mg^{2+} -driven activation of BK channels, which is coupled to the voltage-sensor *via* electrostatic interaction with Arg213 (Lee & Cui, 2010). Aqueous crevices, however, exist between the CTD and the membrane-spanning core, providing an area for accessory β subunit or small ligand interactions (Lee & Cui, 2010). The CTD contains several regions subject to phosphorylation/dephosphorylation (Schubert & Nelson, 2001) and sequences for specific interactions with physiological signals, such as carbon monoxide and heme (Tang et al., 2003; Jaggar et al., 2005; Hou et al., 2008), caveolin-1 (Alioua et al., 2008), and membrane lipids, including phosphatidylinositol 4,5-bisphosphate (Vaithianathan et al., 2008) and, likely, CLR itself (Section 4.3.2).

BK channel-forming subunits associate with partner proteins that modify ionic current phenotype, control ion gradients nearby the pore and/or regulate signaling that gates the channel (Weiger et al., 2002; Berkefeld et al., 2010). In cell membranes from vertebrates, native BK channels usually result from the association of slo1 proteins with auxiliary subunits generally termed β (Orio et al., 2002; Lu et al., 2006). Four types of BK β subunits have been identified, each being encoded by a different gene: *KCNMB1*, *KCNMB2*, *KCNMB3*, and *KCNMB4*. Furthermore, alternative splicing of *KCNMB3* results in four different BK $\beta 3$ isoforms (Orio et al., 2002; Torres et al., 2007). All BK β s share an overall topology of two TM segments joined by a large extracellular loop, plus two short intracellular N and C termini (Orio et al., 2002) (Fig. 1). The areas of contact between α and β subunits and subunit stoichiometry in native channels remain to be fully resolved (Berkefeld et al., 2010). However, it seems that a full set of four $\beta 1$ subunits is required for maximal modulation of slo1 channel function (Wang et al., 2002).

The association of BK β proteins with slo1 subunits constitutes a major mechanism leading to channel functional diversity. Briefly, $\beta 1$ slows down current activation kinetics and increases the apparent Ca^{2+} sensitivity of the channel. In addition, $\beta 1$ regulates trafficking from endosomal regions and membrane distribution of slo1 subunits (Toro et al., 2006). In turn, $\beta 2$ and several $\beta 3$ isoforms introduce rapid inactivation while $\beta 4$ (and in less degree $\beta 2$ and $\beta 3$) reduces the channel response to rather specific peptide blockers such as charybdotoxin and iberiotoxin, and slows down activation kinetics (Orio et al., 2002; Wang et al., 2006; Torres et al., 2007). The pharmacology of selective BK channel blockers and modulators has been reviewed elsewhere (Kaczorowski et al., 1996; Weiger et al., 2002; Nardi & Olesen, 2008).

BK β type expression is tissue-specific: $\beta 1$ and $\beta 4$ prevail in smooth muscle and neuronal tissue, respectively. In turn, $\beta 2$ and $\beta 3$ show a wider expression profile, the former being identified in chromaffin cells, brain and lung, and the later in testis, pancreas and spleen (Behrens et al., 2000; Brenner et al., 2000; Orio et al., 2002; Salkoff et al., 2006; Wang et al., 2006). Major efforts have been conducted to identify ligands that selectively modulate BK complexes containing a single class of BK β , as those ligands would allow modification of physiology in a tissue-specific manner (Weiger et al., 2002; Torres et al., 2007). Our group recently showed that micromolar levels of lithocholate and related steroids activate BK channels that include $\beta 1$ but not those including $\beta 2$ –4. Lithocholate action requires recognition by T169, L172 and L173 in the $\beta 1$ TM2 domain (Bukiya et al., 2008b,c; 2009b; 2011b). BK β subunits, however, are not necessary for sensing CLR (Bukiya et al., 2008c; 2011b; see also Section 4.1).

In the plasma membrane of non-excitabile LNCaP prostate cancer cells, slo1 subunits associate with another type of auxiliary subunit, the leucine-rich repeat-containing protein LRRC26, which enhances coupling between voltage sensor-activation and the closed-to-

open transition (Yan & Aldrich, 2010). In the plasmalemma of many cell types, the slo1 protein has been found associated with chaperone and other membrane proteins. These include ion channel types that control Ca^{2+} signaling, such as voltage-gated Ca^{2+} (Ca_v) channels (Marrion & Tavalin, 1998) and N-methyl-D-aspartate (NMDA) receptors (Isaacson & Murphy, 2001). In some cases, such functional associations seem to involve direct protein-protein interaction, such as BK channel associations with $\text{Ca}_v1.2$, $\text{Ca}_v1.3$, $\text{Ca}_v2.1$ and $\text{Ca}_v2.2$ channels (Dai et al., 2009). In the dynamic fluid state of cell membranes, even more complex arrays link functionally the BK subunits with $\text{Ca}_v1.2$ channels, the β_2 -adrenergic receptor, PKA and the A-kinase anchor protein (AKAP) 150 (Berkefeld et al., 2010). All these proteins are complex oligomers, making it particularly challenging to determine whether CLR modulation of BK function results from steroid-sensing by the BK proteins themselves or is secondary to CLR-sensing by proteins that participate in the functional “supercomplex” that includes the BK channel (see also Sections 4.2, 4.3.2 & 5).

Functional diversity of BK channels is also achieved by posttranslational modification of BK subunits. Channel regulation by phosphatases and kinases *via* de/phosphorylation of BK subunits and/or direct protein-protein interactions has been studied intensively. In general, protein kinase C (PKC) inhibits BK current (Schubert & Nelson, 2001; Barman et al., 2004) whereas CamKII (Liu et al., 2006) and Src tyrosine kinase (Ling et al., 2000) increase BK channel activity. Inhibition *vs.* activation of BK current by PKA, however, is isoform-specific (Schubert & Nelson, 2001; Tian et al., 2001; 2003; Yan et al., 2008; Braun, 2009; Zhou et al., 2010). The involvement of BK CTD in anchoring different AKAPs for PKA interaction with slo1 subunits has been reviewed elsewhere (Dai et al., 2009).

2.2. Channel distribution and physiological roles

BK channels are expressed ubiquitously across species, tissues and cell membranes. The vast majority of BK channels present in the plasma membrane of vertebrate cells are complexed with auxiliary β subunits (see previous section). However, BK β has not been identified in invertebrates (Orio et al., 2002; Berkefeld et al., 2006; Wu & Marx, 2010). Consistent with the ubiquitous expression of slo1 subunits, knock-down of *KCNMA1* in mouse results in a wide variety of abnormalities in the nervous, cardiovascular, endocrine and excretory systems (Meredith et al., 2004; Rüttiger et al., 2004; Sausbier et al., 2004, 2005; Rieg et al., 2007). In humans, decrease in BK channel-forming transcripts has been linked to schizophrenia (Zhang et al., 2006), and reduced channel expression has been related to mental retardation and autism (Laumonnier et al., 2006). BK-forming proteins, however, are not limited to the plasmalemma. In internal Ca^{2+} stores, BK channel activity negatively feeds back on Ca^{2+} release (Yamashita et al., 2006). In mitochondria, BK channels contribute to K^+ homeostasis and respiration (Skalska et al., 2009) while participating in anti-apoptotic mechanisms (Cheng et al., 2011).

Because of its dual activation by membrane depolarization and increased Ca^{2+} level in the vicinity of its cytosolic Ca^{2+} sensors, the BK channel plays critical roles in the physiology of excitable tissues. In neurons, slo1 subunits are primarily located in axons and presynaptic terminals (Knaus et al., 1996; Misonou et al., 2006). Thus, BK channels have been found to regulate action potential (AP) repolarization, sharpening individual spikes (Storm, 1987), and give rise to I_c , the current underlying the fast afterhyperpolarization that follows an AP (Sah & Faber, 2002; Berkefeld & Fakler, 2008). In spite of these generalizations, functional diversity arises from distinct ionic current phenotypes associated to differential BK subunit expression. Thus, in hippocampal pyramidal cells, the inactivating BK channel, which likely results from slo1+BK β_2 subunits, promotes frequency-dependent AP broadening along a train of spikes (Shao et al., 1999). In hippocampal granule cells, however, BK currents show slowed activation as a result of slo1 association with BK β_4 . This ionic current phenotype

seems to function as a “low-pass filter” that prevents high-frequency AP firing and spike sharpening. BK $\beta 4$ subunits are highly expressed in neurons (Section 2.1). Consistently with decreased BK function, knocking-down *KCNMB4* in mice leads to increased number of APs evoked by current injection, as reported with hippocampal neurons (Brenner et al., 2005) and striatal medium spiny neurons (Martin et al., 2008). *KCNMB4* knock-out (K/O) mice are also characterized by modifications in central nervous system (CNS)-mediated electrophysiological and behavioral responses to alcohol (Martin et al., 2008), and increased susceptibility to temporal lobe seizures (Brenner et al., 2005). A truncation in the $\beta 3$ subunit, however, has been related to generalized epilepsy (Lorenz et al., 2007). The role of BK channels in seizure generation has been recently reviewed elsewhere (N’Gouemo, 2011). In the thalamus, BK channel activation participates in regulating the tonic spike firing that operates during wakefulness (Pape et al., 2004). Finally, BK current appears to be involved in antinociception in response to muscarinic receptor activation (Ocaña et al., 2004).

Plasmalemma BK channels have been found in close proximity to Ca_v channels (L, N, P/Q, or R types) (Marion & Tavalin, 1998; Fakler & Adelman, 2008; Kato et al., 2009) and thus regulate neurotransmitter and hormone release (Yazejian et al., 2000), and even shape dendritic Ca^{2+} spikes (Golding et al., 1999). Moreover, in both neurons and neuroendocrine cells, BK and $\text{Ca}_v 1.3$ channels have been located within specific membrane nanodomains (Section 5, and Vandael et al., 2010). As described for central neurons, differential subunit expression renders plurality of BK current phenotypes and function in hormone-releasing cells. In adrenochromaffin cells, two BK phenotypes have been identified: a) noninactivating and b) inactivating, slowly deactivating currents, which likely result from assembly of *slo1* homotetramers and *slo1*+BK $\beta 2/\beta 3$ heteromers, respectively. Cells where the inactivating, slowly deactivating current phenotype prevails feature a robust afterhyperpolarization that relieves voltage-dependent Na^+ channel inactivation and thus, promotes repetitive AP firing (Lingle et al., 1996; Xia et al., 1999; Orio et al., 2002; Wang et al., 2002).

In auditory sensory hair cells, serial and repetitive activation of L-type, Ca_v and BK channels results in depolarization-repolarization cycles that are fundamental for hearing (Art et al., 1995). Cycle frequency is modulated gradually by the differential expression of BK $\beta 1$ subunits along the main axis of the hearing organ: BK currents from cells where BK $\beta 1$ is poorly associated with the channel-forming protein activate rapidly and enable high frequency cycles. In contrast, increased association between BK $\beta 1$ and α subunits slows current deactivation and increases the channel Ca^{2+} sensitivity, which promotes sustained activity and suppression of high-frequency cycles (Fettiplace & Fuchs, 1999; Ramanathan & Fuchs, 2002).

In smooth muscles, BK channels may be activated by intracellular, local Ca^{2+} signals (sparks) and/or Ca^{2+} influx *via* depolarization-activated Ca_v channels. In vascular smooth muscle, BK channel opening leads to spontaneous transient outward currents (STOCs) that tend to hyperpolarize the cell membrane and, thus, oppose depolarization-induced smooth muscle contraction (Brayden & Nelson, 1992; Brenner et al., 2000; Jaggar et al., 2000; Pérez et al., 2001). Likewise, Ca^{2+} -spark regulation of STOCs plays a physiological role in controlling the myogenic tone in the urinary bladder (Heppner et al., 2003), and BK channels play a critical role in regulating airway smooth muscle tone (Kotlikoff, 1993; Semenov et al., 2006).

Vascular myocyte BK channels are additionally targeted by endothelial factors that regulate vasomotion, such as nitric oxide (NO), prostaglandin I₂, and epoxyeicosatrienoic acid (Tanaka et al., 2004; Félétou & Vanhoutte, 2006). Modification of vascular smooth muscle

BK channel function has been reported in several experimental models of human vascular disease. In rodent models of hypertension, different groups have reported modifications in smooth muscle BK channel function resulting from changes in subunit expression and/or individual channel sensitivity to Ca^{2+} (Cox & Rusch, 2002; Amberg et al., 2003). In streptozotocin-induced diabetic rats, the inhibitory effect of angiotensin II on coronary artery BK channels is enhanced by increased caveolar targeting of the channel (Lu et al., 2010). In insulin-resistance rats, BK channel function is diminished in macro- and microvessels, probably due to reduced $\beta 1$ subunit expression (Li et al., 2011). Consistent with the high expression of BK $\beta 1$ subunits in smooth muscle (Section 2.1), targeted deletion of *KCNMB1* results in BK channels with decreased Ca^{2+} sensitivity and Ca^{2+} spark-STOC coupling, leading to impaired smooth muscle relaxation. Thus, *KCNMB1* K/O mice have increased arterial tone and elevated systemic blood pressure (Brenner et al., 2000; Plüger et al., 2000; Patterson et al., 2002). In contrast, a *KCNMB1* gain-of-function mutation that leads to the $\beta 1$ E65K substitution has been associated with low prevalence of diastolic hypertension, severe hypertension and myocardial infarction (Fernández-Fernández et al., 2004; Köhler, 2010). In turn, the coding polymorphism C818T, which leads to the $\beta 1$ R140W substitution, is linked to increased bronchial asthma severity (Seibold et al., 2008).

The importance of BK channels in renal physiology, pathophysiology and contribution to systemic hypertension have been addressed more recently. BK α and $\beta 1$ subunits are found in glomerular cells and most segments of the nephron. In the distal segment, BK channels mediate flow-induced K^+ secretion, which may partially result from shear stress-induced increase in cell Ca^{2+} levels. Data from *KCNMA1.1* and *KCNMB1* K/O mice suggest that flow-induced K^+ secretion requires both channel subunits (Grimm & Sansom, 2007). In turn, by applying siRNA against BK $\beta 4$ in C11 cells, it has been recently shown that $\beta 4$ -containing BK complexes play a role in shear-induced loss of K^+ from kidney intercalated cells, suggesting that an α - $\beta 4$ complex regulates intercalated cell volume during high-flow (Holtzclaw et al., 2010). Both *KCNMB1* K/O and *KCNMB4* K/O mice exhibit abnormal K^+ homeostasis and hypertension. However, the latter is more severe in the *KCNMB1* K/O mouse due to summation of altered vasodilation and hyperaldosteronism with high fluid retention (Grimm & Sansom, 2010; Köhler & Ruth, 2010).

BK channels also mediate K^+ secretion in the distal colon (Sausbier et al., 2006) and its modulation by aldosterone (Sørensen et al., 2008) and adrenaline (Sørensen et al., 2010). BK channel function is altered in colon pathology: BK channel expression is increased in colon biopsies from subjects with ulcerative colitis (Sandle et al., 2007) and labeling of BK protein is enhanced in enterocytes in end-stage renal disease (Mathialahan et al., 2005).

Roles of BK channels in immunity and cancer cell biology have been explored recently. BK channels participate in microbial killing in leukocytes (Ahluwalia et al., 2004), invasion of glioma cells and brain metastasis (Sontheimer, 2008). *KCNMA1* amplification favors human prostate cancer proliferation (Bloch et al., 2007) whereas siRNA block of *KCNMA1* significantly reduces breast cancer invasion (Khaitan et al., 2009). Finally, BK channel activity increases as a response to the down-regulation of inwardly rectifying potassium (Kir) channels that occurs during gliosis (Bringmann et al., 2000).

3. Phenomenology of BK current responses to manipulation of membrane cholesterol

The sensitivity of native BK currents to treatments that modify membrane CLR levels was first reported in cultured myocytes from human and rabbit aortas (Bregestovski & Bolotina, 1989; Bolotina et al., 1989) and since then in a wide variety of species and tissues, including mouse colonic epithelial cells (Lam et al., 2004), rat ureteric (Babiychuk et al., 2004),

uterine (Shmygol et al., 2007) and cerebrovascular myocytes (Bukiya et al., 2011c), pituitary tumor GH3 cells (Lin et al., 2006) and cardiomyocytes (Prendergast et al., 2010), bovine aortic endothelial cells (Wang et al., 2005), chicken hair cells (Purcell et al., 2011), human myometrial myocytes in culture (Brainard et al., 2005), melanoma IGR39 cells (Tajima et al., 2011), and glioma cell lines (Weaver et al., 2007). Considering the wide variety of BK subunit compositions and the tissue-specific distribution of BK accessory subunits (Section 2.1) the ubiquitous finding of BK current modulation by CLR seems to indicate that CLR action does not require a specific BK subunit combination. BK current sensitivity to CLR has been reported after transfection of Madin-Darby canine kidney (Bravo-Zehnder et al., 2000) and human embryonic kidney (HEK293) (Yuan et al., 2011) cells with BK channel-forming subunits cloned from human brain (hslo1), suggesting that BK β subunits are not necessary for CLR modulation of BK current (the role of subunit composition in BK channel sensitivity to membrane CLR is further discussed in Section 4.1).

Manipulation of CLR levels in native membranes has been usually achieved by acutely exposing cells to CLR-depleting (e.g., methyl- β -cyclodextrin; M β CD) or CLR-enriching (M β CD complexed with CLR) medium, which does not allow accurate determination of the actual CLR molar fraction in the membrane. However, CLR modulation of BK current has also been reported using channel reconstitution into artificial planar phospholipid bilayers where the CLR molar fraction can be titrated. These studies indicate that CLR at 30–50 mol % (e.g., molar fractions present in plasma membranes; Gennis, 1989; Sackmann, 1995) modulates BK channel activity in absence of the complex proteolipid organization that is characteristic of natural membranes (Chang et al., 1995; Crowley, 2003; Bukiya et al., 2008c; 2011a). All studies of BK protein reconstitution into simple, 1–3 species lipid bilayers, whether using native BK (Chang et al., 1995) or recombinant slo1 proteins (Crowley et al., 2003; Bukiya et al., 2008c; 2011a; Yuan et al., 2011), concur that: 1) elevation in bilayer CLR content at molar fractions found in natural membranes leads to significant reduction in BK Po; 2) this inhibition is concentration-dependent; 3) decreased Po results from multiple changes in channel dwell times by CLR, which lead to both reduction of mean open and lengthening of mean closed times; 4) decreased Po is accompanied by minor, if any, reduction in channel unitary conductance (Section 4.2).

Cholesterol modulation of BK Po has been conceptualized within two types of interaction: 1) CLR insertion into a membrane alters the bilayer physical properties (Fig. 2, bottom left), which secondarily modifies BK channel protein conformation and function, and 2) CLR insertion into the bilayer leads to a direct recognition of the sterol by a protein surface present in the BK channel protein (Fig. 2, top right). These two types of interactions will be discussed in Sections 4.3.1 and 4.3.2, respectively. However, they should not be seen as mutually exclusive: first, a CLR recognizing domain could be contributed to by a BK channel surface and the bulk bilayer lipid (Fig. 2, bottom right), and CLR presence could alter both protein conformation and physical properties of the lipid that surround the channel membrane-spanning regions. Second, protein-mediated and lipid-mediated interactions could *independently* contribute to CLR modulation of BK channel function, as reported for the oxytocin receptor and its structurally related brain cholecystokinin receptor (Gimpl et al., 1997).

In native membranes, however, BK channels are clustered in membrane domains that are enriched in CLR and sphingolipids and act as platforms for clustering of cell signaling molecules. Thus, in addition to the BK channel-forming protein and a simple lipid bilayer, the final outcome of acutely manipulating membrane CLR on BK current results from the orchestration of a variety of factors. These include BK partner ion channels, chaperone proteins, signaling molecules, and the local ionic medium (Section 5).

Changes in BK current to *protracted* modification of CLR levels have been addressed by evaluating BK channel function in tissues from animals subject to treatments that lead to hypercholesterolemia. In most studies, however, CLR levels in the membranes where the BK channel was evaluated have not been determined. Thus, it becomes impossible to establish whether modification of BK current in response to hypercholesterolemia is, indeed, a direct consequence of altered membrane CLR levels. The relations among hypercholesterolemia, modified BK current, pathophysiology and disease are provided in Sections 7 and 8.

4. Sites and mechanisms underlying cholesterol regulation of BK single channel function

4.1. Role of subunit composition in channel response to cholesterol

BK channel α subunits are usually accompanied by accessory subunits (β 1–4), which provide distinct ionic current phenotype and pharmacological responses to the BK complex (Sections 2.1 and 2.2). In particular, BK subunit composition drastically affects the BK current sensitivity to acute application of steroids, whether from the estrane (C18 steroidal nucleus), androstane (C19), pregnane (C21) or cholane (C24) series. From several studies, the pattern that emerges is that the presence of distinct BK β subunits either is necessary for steroid action or modifies the final BK current response to steroid application (Valverde et al., 1999; Dick & Sanders, 2001; King et al., 2006; Bukiya et al., 2009b).

In contrast to results obtained with bile acids and related cholane steroids (Bukiya et al., 2009b), the CLR response of BK channels does not require the presence of accessory BK β subunits. Data obtained after reconstitution of homomeric hslol channels into 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) : 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphol-serine sodium salt (POPS) (3:1 w/w) lipid bilayers first demonstrated that CLR inhibition of BK channel steady-state activity, previously reported with native channel in natural membranes (Section 3) was sustained in a bare proteolipid environment nominally composed of two phospholipid species and the channel-forming hslol subunit (Crowley et al., 2003). In the same binary bilayer type, a similar CLR action was evoked on slo1 channels cloned from rat cerebral artery myocytes (cbv1 channels; Bukiya et al., 2008c). Cholesterol action on hslol and cbv1 channels is concentration-dependent: BK P_o progressively decreases as membrane CLR increases from 0 to 40 mol%) in the lipid mixture, with an $IC_{50} \approx 33$ mol% (Bukiya et al., 2008c). In addition, for both BK constructs, the reduced P_o results from shortening of mean open and lengthening of mean closed times (Crowley et al., 2003; Bukiya et al., 2011a). These similarities underscore that neither the complex cytoarchitecture of cell membranes nor the continuous presence of cell signaling is required for CLR-induced inhibition of BK channel activity. In addition, the similarities in dwell-time changes and sterol potency reported in these studies strongly suggest that CLR action on recombinant BK channels is mediated by a recognition site(s) that is common to the different experimental systems: the two phospholipid species, the channel α subunit, and/or some proteolipid contaminant from the cell membrane preparation where the recombinant channel was originally expressed.

A more detailed characterization of CLR action on cbv1 subunits from rat cerebral artery smooth muscle reveals that the channel under study generates a current phenotype that is characteristic of recombinant BK channels made of α subunit homotetramers (Crowley et al., 2003; Liu et al., 2008c) and native BK channels thought to result from α homotetramers (Bukiya et al., 2008c). Thus, any putative contaminant protein in the bilayer reconstitution system that could contribute to CLR sensing by BK channels should be selective enough to bind CLR and eventually alter cbv1 channel gating without altering cbv1 channel gating in

absence of CLR. A simpler and more plausible explanation is that *cbv1* itself contains a site(s) for CLR-sensing that is responsible for CLR reduction of BK channel activity (Section 4.3.2).

Electrophysiological data following *cbv1* channel reconstitution into POPE:POPS (3:1 w/w) bilayers reveal that the channel-forming subunit is sufficient to sustain CLR inhibition of BK channel steady-state activity. In addition, accessory $\beta 1$ subunits cloned from the same cell type (rat cerebral artery myocyte) fail to modify this CLR action (Bukiya et al., 2008c). These results from a highly reduced system made of recombinant BK channel subunits and a two-species phospholipid bilayer seem to be validated by patch-clamp electrophysiology on native BK channels in natural cell membranes: exposure of freshly isolated cerebral artery myocytes to CLR-depleting treatment by using M β CD leads to a similar increase in BK Po in C57BL/6 mouse (which possesses $\beta 1$ -containing BK channels) and *KCNMB1* K/O mouse (Bukiya et al., 2011c). Collectively, results from recombinant and native cerebrovascular BK channels strongly suggest that the presence of $\beta 1$ subunits in the BK complex does not significantly modify CLR effect on channel steady-state activity. However, detailed gating kinetic studies may be needed to rule out $\beta 1$ subunit modulation of specific aspects of BK channel gating altered by CLR.

The role of BK $\beta 2$ and $\beta 4$ subunits on CLR sensitivity of *hslol* channels has been probed with patch-clamp methods on transfected HEK293 cells (King et al., 2006). In this study, 10 μ M CLR failed to evoke any significant change in BK macroscopic current amplitude whether the channel included $\beta 2$ or $\beta 4$ subunits. This failure of CLR to modulate BK current can be attributed to a variety of reasons, most notably the fact that CLR in the aqueous phase was perfused onto the cells. Conceivable, cell perfusion with CLR-containing bath solution failed to deliver sufficient amount of sterol to a hydrophobic CLR-recognition site located deep into the proteolipid environment. Alternatively, *hslol* channels may be CLR-insensitive. However, the steady-state activity of *hslol* channels reconstituted into artificial phospholipid bilayers has been reported to be inhibited by 0–50 mol% CLR in a concentration-dependent manner (Crowley et al., 2003). Thus, it is possible that $\beta 2$ and $\beta 4$ subunits or some other proteolipid component native to cell membranes embolden the *hslol* channel with CLR-resistance.

4.2. Changes in unitary conductance by cholesterol

Changes in Po induced by manipulating the amount of CLR in membranes have been reported in a wide variety of systems, ranging from ion channel model peptides reconstituted into simple phospholipid bilayers to heterooligomeric ion channel protein complexes in their complex, native proteolipid environment (Section 4.3.1). In contrast, reports on modification of unitary channel conductance of membrane-spanning ion channel proteins in response to changes in membrane CLR levels are scarce. Moreover, it has been widely documented that ion channel proteins may respond to changes in membrane CLR by altering Po without noticeable changes in single channel conductance. For example, it has been shown that CLR alters the equilibrium between conducting and nonconducting states of Kir2 channels and, thus, drastically reduces macroscopic current, an effect that occurs in absence of modifications in unitary conductance (Romanenko et al., 2002; 2004a). Likewise, CLR drastically decreases Po of mechano-sensitive cationic channels in leukemia K562 cells (Morachevskaya et al., 2007) and volume-regulated anionic (VRAC) channels in bovine endothelial cells (Romanenko et al., 2004b) without altering unitary conductance. On the other hand, incubation of BC3H-1 cells or membrane patches excised there from with CLR-containing liposomes increases 4-fold the frequency of nicotinic acetylcholine receptor channel openings, yet again, this effect on channel gating seems to occur in absence of changes in unitary conductance (Barrantes, 1993).

Regarding the BK channel conduction response to changes in membrane CLR, refractoriness and very mild changes (whether increase or decrease) in unitary conductance have been reported. Cholesterol insertion into POPE:POPS (3:1 w/w) bilayers at molar fractions that match CLR levels in native membranes ($\approx 33\text{mol}\%$) decreases Po . This molar fraction is almost maximal for CLR-induced inhibition (IC_{90}). Again, such reduction of Po is not accompanied by any noticeable change in unitary slope conductance, which was obtained in $300/30\text{ mM } [\text{K}^+]_{\text{i}}/[\text{K}^+]_{\text{o}}$ within a wide voltage range (-80 to 70 mV) (Bukiya et al., 2011a). This outcome matches data from hslol channels reconstituted into the same bilayer type (Crowley et al., 2003; Yuan et al., 2004). These measurements of ion conduction in planar lipid bilayers somewhat differ from data obtained with rat brain BK channels reconstituted into POPE:POPS (55:45 w/w) bilayers where the presence of CLR at 11% of total lipid weight ($\approx 17\text{ mol}\%$) introduced a minor, yet statistically significant decrease (-7%) in slope conductance (Chang et al., 1995). This study was conducted using a $300/100\text{ mM}$ (cis/trans) KCl gradient while other authors (Crowley et al., 2003; Yuan et al., 2004; Bukiya et al., 2011a) used a $300/30\text{ mM}$ KCl gradient. Thus, the larger K^+ gradient could have created a driving force for K^+ flow through the channel pore that was large enough to mask subtle changes in slope conductance caused by CLR. Finally, a 7.5% increase in unitary conductance has been reported for BK channels in human melanoma IGR39 cells when membrane CLR is depleted with M β CD (Tajima et al., 2011).

The mechanism(s) underlying decrease in BK slope conductance by the presence of CLR in membranes remains undetermined. The CLR molecule is primarily located in the hydrophobic core of lipid membranes. More precisely, the rigid hydrophobic steroid ring is placed perpendicular to the bilayer plane, with CLR polar single hydroxyl at C3 in the phospholipid head region (Villalán, 1995; Loura & Prieto, 1997; Ohvo-Rekilä et al., 2002) (for CLR molecular structure refer to Fig. 3A). Thus, it is unlikely that CLR acts as an open pore blocker, which would require CLR direct access and energetically-favored dwelling in the protein channel aqueous vestibulum and permeation pathway. Cholesterol insertion into phospholipid bilayers may thicken the bilayer (Tulenکو et al., 1998) and bilayer thickening diminishes BK channel unitary conductance (Yuan et al., 2007). However, while hslol channels decreased their channel slope conductance in response to bilayer thickening evoked by varying phospholipid acyl chain, their conductance remained unaltered by insertion of CLR (Yuan et al., 2004; 2007; 2011).

It should be underscored that results from most studies on recombinant slo subunits in artificial planar bilayers of simple lipid composition parallel results obtained with native BK channels studied in their natural environment: membrane CLR manipulation that drastically alters macroscopic BK current and/or Po occurs in absence of noticeable changes in channel unitary conductance, as first reported in rabbit aorta myocytes (Bolotina et al., 1989) and, more recently, in melanoma IGR39 cells (Tajima et al., 2011). Finally, CLR-depletion of GH3 pituitary tumor cell membranes leads to increased BK Po without altering unitary current amplitude (Lin et al., 2006). From all the studies considered, a common pattern emerges: CLR exerts a powerful influence on BK channel gating, which usually results in decreased Po (Sections 4.3.1 and 4.3.2) while evoking minor, if any, changes in unitary conductance. The dichotomy between single channel steady-state activity and conductance responsiveness to manipulation of membrane CLR is consistent with a structural design for BK-channel forming proteins in particular, and transmembrane ion channels in general, in which water-filled cavities and ion permeation pathways are isolated from the bilayer lipid core where CLR primarily resides (Ohvo-Rekilä et al., 2002) whereas the gating machinery is more peripheral, whether in direct contact with CLR-containing regions in the bilayer or coupled to the permeation pathway *via* membrane lipids (MacKinnon, 1995; 2003; Yuan et al., 2010).

4.3. Mechanisms and interactions underlying changes in single BK channel steady-state activity by cholesterol

4.3.1. Bilayer lipid-mediated mechanisms—Studies on BK channel-forming recombinant proteins reconstituted into artificial bilayers of simple composition demonstrate that a planar binary phospholipid bilayer suffices to support CLR modulation of BK channel steady-state activity (Crowley et al., 2005; Bukiya et al., 2008c; 2011a; Yuan et al., 2011). Thus, CLR modulation of BK channel function may occur in absence of cytosolic signaling, intracellular organelles, caveolae organization and even a complex bilayer lipid composition. At molar fractions found in natural membranes (~ 50 mol% CLR; Gennis, 1989; Sackmann, 1995) and used in most bilayer studies of BK channel function, however, CLR modifies a wide variety of physical properties of the bulk lipid bilayer, including broadening and eventual elimination of the gel-to-liquid crystalline phase transition, decrease in the cross sectional area occupied by the phospholipid in the liquid-crystalline state, modification of bilayer thickness, increase in the modulus of compressibility and mechanical strength, increased lateral stress and stiffness of the phospholipid monolayer or bilayer in the physiologically relevant fluid phase (Helfrich, 1973; Gruner, 1985; Evans & Needham, 1986; Epand & Bottega, 1987; Needham & Nunn, 1990; McMullen et al., 1999; McConnell & Radhakrishnan, 2003; Chong et al., 2009). Moreover, both model peptides and complex oligomeric ion channel complexes have been reported to change function in consonance with modification of one or several of the aforementioned bilayer physical properties (Lundbæk & Andersen 1994; Lundbæk et al. 1996, 2004; Bezrukov, 2000; Helfrich et al., 2006; Bruno et al. 2007; Lundbæk et al., 2010).

The possibility that CLR-induced reduction of BK channel steady-state activity is determined by changes in physical properties of the bulk membrane lipid that occur upon sterol insertion into the bilayer has been early recognized. Inhibition of BK Po in aortic myocytes by CLR-enrichment medium is coincidental with a decrease in the rotational diffusion coefficient of diphenylhexatriene. This decrease has been interpreted as reflecting a decrease in the cell membrane “fluidity” (Bolotina et al., 1989), that is, an increase in membrane order parameters. Data from a recent structure-activity relationship (SAR) study (Bukiya et al., 2011a), however, demonstrate that 1) CLR and epicholesterol, while having similar effects on bulk bilayer order as measured by fluorescence polarization anisotropy (Gimpl et al., 1997; Xu & London, 2000) are, respectively, effective and ineffective in reducing cbv1 Po, and 2) coprostanol, while having an “anti-CLR” effect on bilayer lipid order (Xu & London, 2000) is an effective inhibitor of channel activity. In addition, generalized polarization of Laurdan in large unilamellar vesicles where CLR was probed at small increments within a biologically relevant range (20–50 mol%) shows that fluorescence anisotropy does not change monotonically with increased CLR levels (Venegas et al., 2007). Moreover, CLR and related sterols can be distributed regularly into superlattices that coexist with CLR-phospholipid condensed (“packed”) complexes within the fluid lipid bilayer (Chong et al., 2009). A recent statistical mechanical model describing how CLR superlattices and CLR-phospholipid condensed complexes are interrelated leads to the conclusion that the extent and type of superlattices and thus, membrane-associated properties such as membrane packing, should vary with CLR molar fraction in a predictable, *nonmonotonic* manner (Sugár & Chong, 2012). In contrast to the nonmonotonic dependence on CLR molar fraction of superlattice formation and fluorescence anisotropy mentioned above, CLR inhibition of BK channels reconstituted into planar phospholipid bilayers by CLR (0–50 mol%) shows a monotonic dependence on CLR molar fraction (Crowley et al., 2003; Bukiya et al., 2008). These dichotomies and data from the SAR study question the idea that increased bilayer order/lipid packing is a significant player in CLR modulation of BK channel activity.

Cholesterol insertion into phospholipid bilayers modifies bilayer elastic properties, introducing negative monolayer curvature and increasing lateral stress in the hydrocarbon chain region (Bezrukov, 2000; Lundbæk et al., 2004). These actions translate into decreased channel open times, as demonstrated for gramicidin A (Andersen et al., 2007). Remarkably, in an artificial bilayer where the lipid component can be tightly controlled, addition of CLR at 11% of total lipid weight into the POPE:POPS (55:45 w/w) mixture significantly decreases BK channel mean open time and thus, overall Po (Chang et al., 1995). At CLR molar fractions ~33 mol%, modification of bilayer properties by CLR results primarily from CLR-phospholipid interactions, as CLR-CLR interactions become truly significant at higher sterol molar fractions (Huang, 2009). According to the “umbrella model” CLR relies on polar phospholipid headgroup coverage to avoid contact with the aqueous phase (Huang, 2009). Individual CLR molecules are considered to be inserted as a “wedge” between phospholipid molecules (Demel & De Kruyff, 1976; Loura & Prieto, 1997; Hayakawa et al., 1998; Preston Mason et al., 2003). Arrhenius plots allowed Chang et al. (1995) to calculate the activation energy for BK channel transitions from the open to the closed state(s), which is reduced by CLR. Because the computed lateral stress energy that results from the insertion of CLR as a wedge between the phospholipids is significantly larger than the BK activation energy, it has been advanced that a CLR-induced increase in lateral stress favors the BK channel to be deflected back to the closed state, leading to the decrease in mean open time (Chang et al., 1995). Remarkably, CLR-induced inhibition of hslol Po reconstituted into POPE:POPS (3:1 w/w) bilayers is reduced in pure POPE bilayers, a reduction that cannot be explained simply by dilution of negatively charged POPS (Crowley et al., 2003). Cholesterol and POPE are nonlamellar phase-preferring type II lipids: they possess a relatively large hydrophobic nucleus-tail region compared with the small polar “head”. Upon insertion into bilayer, type II lipids increase lateral pressure in the hydrocarbon chain area of the bilayer and thus, introduce a negative monolayer curvature (Bezrukov, 2000). Eventually, type II lipids modify ion channel function (Lundbæk et al., 1996), including decrease in hslol Po (Crowley et al., 2005). Conceivably, a pure POPE bilayer with a high initial degree of lateral stress could mask CLR modulation of this parameter.

Several lines of evidence, however, indicate that increase in lateral stress is neither the only nor the main mechanism underlying CLR inhibition of BK Po. Entropy changes secondary to BK channel open to closed transitions do not tightly follow predictions should lateral stress be the only contributor to CLR reduction of BK Po (Chang et al., 1995). In addition, bilayer lateral stress is controlled by the sterol ability to evoke lipid “condensation” (or tight lipid packing), in turn depending on sterol molecular volume and shape (Demel et al., 1972, Kessel et al., 2001). A systematic SAR study on CLR and related monohydroxysterols (Bukiya et al., 2011a) demonstrates that the differential efficacies of these sterols to reduce cbv1 Po (CLR > coprostanol > cholestanol >> epicholesterol) do not follow molecular area rank (coprostanol >> epicholesterol > CLR > cholestanol). In addition, the computationally predicted energies for CLR (effective BK inhibitor) and epicholesterol (ineffective) to adopt a planar conformation are similar. Finally, CLR and coprostanol both reduce Po, yet these sterols have opposite effects on tight lipid packing and, likely, on lateral stress (Xu & London, 2000).

Cholesterol insertion also modifies bilayer thickness, as reported with artificial phospholipid bilayers (Ohki, 1969) and natural membranes (Tulenکو et al., 1998). In addition, bilayer thickness regulates BK (hslol) channel mean open time (Yuan et al., 2007), which is reduced by CLR presence in the bilayer (Chang et al., 1995; Crowley et al., 2003). However, P-P distances in dimyristoylphosphatidylcholine (DMPC) bilayers that contain epicholesterol are intermediate between those of CLR-containing and sterol-free DMPC bilayers (Róg & Pasenkiewicz-Gierula, 2003), yet epicholesterol is totally ineffective in

reducing BK Po (Bukiya et al., 2011a). Therefore, it is very unlikely that changes in bilayer thickness provide a major mechanism underlying CLR inhibition of BK channel activity.

Cholesterol inhibition of BK channel activity has been consistently reported in artificial bilayers of varied lipid composition: POPE:POPS (55/45 w/w) (Chang et al., 1995), POPE:POPS (3:1 w/w) (Crowley et al., 2003; Bukiya et al., 2011a), and 1,2-dioleoyl-3-phosphatidylethanolamine (DOPE):sphingomyelin (SPM) (3:2 molar ratio) (Yuan et al., 2011), such inhibition being drastically reduced in pure POPE bilayers (Crowley et al., 2003). This result might reflect a relatively low miscibility of CLR in pure PE (McMullen et al., 1999), which would preclude CLR from accessing a distinct site of action in this bare proteolipid system. Alternatively, the BK channel protein in a pure POPE bilayer might acquire a conformation that prevents it from directly interacting with CLR (Section 4.3.2). Differential phospholipid solubility between sterols could explain, or at least contribute to, the differential effect of CLR *vs.* epicholesterol on cbv1 channels, as the β monohydroxysterol is more soluble in lecithin than its α epimer (Demel et al., 1972). However, cbv1 Po values in CLR-containing bilayers are similar to those in cholesterol-containing bilayers (Bukiya et al., 2011a), yet cholesterol is more soluble in lecithin than CLR is (Demel et al., 1972). Thus, while poor CLR-PE miscibility could contribute to the low CLR sensitivity of slo1 channels in pure POPE bilayers, different phospholipid-sterol miscibility is unlikely the single mechanism leading to differential inhibition of BK channels by CLR and related monohydroxysterols.

In synthesis, it is extremely unlikely that modification of a single physical property of the lipid bilayer is the only mechanism determining CLR inhibition of BK channel activity. Alternatively or complementary, CLR action may result from CLR-specific recognition by a protein site(s) (see next section).

4.3.2. A case for a BK channel protein-cholesterol direct interaction—SAR studies of CLR and related sterols have been widely used to discriminate between direct sterol-ion channel protein interaction *vs.* perturbation of bulk bilayer lipid properties in CLR modulation of ion channel function (Harrison et al., 1987; Gimpl et al., 1997; Paradiso et al., 2000; Romanenko et al., 2002, 2004a; Addona et al., 2003; Akk et al., 2007). Moreover, predictions from SAR studies have often been verified by structural data. Indeed, a SAR study first identified the structural constraints for steroids to modulate Kir2 channels, leading to the postulation of a selective CLR-sensing region in the Kir2 protein (Romanenko et al., 2002). Later, a combination of protein homology modeling with point mutagenesis identified such a region at the Kir2.1 channel C-terminus (Epshtein et al., 2009). Likewise, differential modulation of $\alpha 4\beta 2$ nicotinic acetylcholine receptors (Paradiso et al., 2000) or gamma-aminobutyric acid (GABA-A) Cl^- channels (Wittmer et al., 1996) by steroid enantiomers predicted the existence of specific steroid-recognition protein sites in each of these ionotropic receptors, these predictions being confirmed by mutagenesis or computational modeling (Barrantes, 2004; Hosie et al., 2007). In contrast, lack of structural specificity for CLR and analogs to modulate VRAC has been interpreted as steroids acting on ion channels *via* changes in bilayer physical properties secondary to CLR insertion (Romanenko et al., 2004b). This hypothesis was successfully proven by micropipette aspiration measurements of membrane stiffness, which showed that CLR modification of bilayer stiffness lead to modification of VRAC function (Byfield et al., 2006).

The CLR molecule contains three clearly distinct regions: 1) a rather rigid, steroid tetra-ring system with a double bond between C5 and C6; 2) a small polar hydroxyl group in β configuration at C3; and 3) a hydrophobic iso-octyl lateral chain attached to C17 (Fig. 3A). The participation of these structural features in CLR action on BK channels has been probed in a SAR study of CLR and closely related monohydroxysterols on cbv1 channel function

after protein reconstitution into POPE:POPS (3:1 w/w) bilayers (Bukiya et al., 2011a). Results indicate that BK channel inhibition by mono-hydroxysterols is favored by the hydrophobic nature of the side chain, which might ensure sufficient partitioning and correct positioning of the CLR molecule within the bilayer. While having lax requirements on the sterol A/B ring fusion, sterol inhibition of BK channels strictly requires the β configuration of the C3 hydroxyl (Fig. 3B). The latter represents a remarkable finding, as stereospecificity of CLR action on ion channels has been consistently interpreted as evidence that a protein surface contributes to the CLR-sensing element (Sooksawate & Simmonds, 2001; Romanenko et al., 2004a). In addition to the strict stereo isomery of the single polar group at C3, the cbv1-mono-hydroxysterol SAR study documents that CLR inhibition of cbv1 channels strictly depends on the optical isomery of the sterol: in contrast to natural CLR, its enantiomer (ent-cholesterol) is ineffective in blunting BK channel activity. Enantioselectivity in CLR modulation of BK channels has also been found in a very recent study conducted with hslol channels reconstituted into POPE:POPS 3:1 (molar ratio) bilayers (Yuan et al., 2011). As optical isomers, CLR and ent-cholesterol share the same relative configuration. Moreover, because these optical isomers affect membrane physical properties similarly, it is widely accepted that enantiospecificity of protein response to CLR presence reflects the existence of a CLR-recognizing protein site (Sooksawate & Simmonds, 2001; Crowder et al., 2001; Romanenko et al., 2002; Westover & Covey, 2004; Alakoskela et al., 2008). Given the simple composition of the reconstitution system (POPE, POPS, cbv1 subunits) and the α subunit homomeric phenotype of the BK current under study, Bukiya et al. (2011a) hypothesized that the CLR-protein recognition surface that leads to channel inhibition is provided by the BK channel-forming α subunit itself.

The actual location and structural elements of the CLR-recognition site involved in sterol inhibition of BK channel activity remain speculation. Cholesterol-sensing regions in ion channel proteins have been mapped to transmembrane segments, e.g., AchNic receptor (Barrantes, 2004), and cytosolic C-tail regions, e.g., the mitochondrial TSPO protein (Li et al., 2001) and 2TM inwardly rectifying K⁺ channels (Epshtein et al., 2009). To begin to identify the cbv1 region(s) that confers CLR sensitivity to BK channels, CLR was probed on *w*tcbv1 vs. the construct trcbv1S6 engineered by truncation immediately after S6, i.e., between Ile 322 and Ile 323. Single channel protein function was evaluated under identical recording conditions after channel reconstitution into POPE:POPS (3:1 w/w) bilayers. In contrast to *w*tcbv1 channels, trcbv1S6 constructs, while keeping some basic characteristics of BK channels, remained insensitive to the presence of CLR (16–33 mol%). This result indicates that the CTD is necessary for CLR inhibition of BK channel activity (Singh et al., 2011). Whether the CTD actually provides CLR-recognition sites remains to be determined by computational dynamics and/or structural data. Notably, the cbv1 CTD contains a number of CLR recognition/interaction amino acid consensus (CRAC) motifs. CRAC motifs often participate of CLR binding by membrane proteins with consequent modification of protein function (Epan, 2006). We are currently investigating the actual involvement of cbv1 CRAC motifs in CLR functional recognition by the BK channel.

5. Modification of BK channel membrane expression and/or distribution by manipulation of cell/tissue-cholesterol levels

Several ion channel populations have been shown to localize within specific plasma membrane microdomains enriched in CLR and sphingolipids and known as lipid or membrane “rafts” (Bloch, 1983; Simons & Ikonen, 1997; Brown & London, 1998; Dopico & Tigyi, 2007). Membrane rafts are thought to operate as sorting platforms that bring together molecules for efficient cross-talk and thus control of cellular signaling cascades (Simons & Toomre, 2000; Dopico & Tigyi, 2007). Location of ion channels in rafts maximizes control of channel function by nearby signaling molecules. In addition, channel

function and membrane expression may be regulated by lipid species that prevail in the raft, such as CLR, whether *via* modification of bilayer physical properties conferred to the raft by these lipids (Section 4.3.1) or *via* direct interaction between raft lipids and proteins that cluster in the raft, including the ion channel protein itself (Section 4.3.2).

Several studies have attributed CLR-dependent modulation of BK channel activity in native cellular membranes to BK channel clustering in membrane rafts. Fractionation and sucrose gradient centrifugation of Madin-Darby canine kidney cells and rat ureter myocytes shows that the hsl1 protein preferentially distributes in the detergent resistant, “raft-like” fraction (Bravo-Zehnder et al., 2000; Babiychuk et al., 2004). In IGR39 human melanoma cells, ~50% of functional BK channels are found in membrane rafts (Tajima et al., 2011). The raft-like membrane fraction also concentrates colonic epithelium BK channels. Moreover, CLR depletion with M β CD redistributes the channel to the detergent-soluble fraction (Lam et al., 2004).

Caveolae are flask-shaped membrane invaginations, considered as a subtype of membrane raft (Razani et al., 2002). A structural element usually found in caveolae is caveolin-1, which acts as scaffolding protein (Goetz et al., 2008). BK channels have been found to reside in caveolae of bovine vascular endothelial cells (Wang et al., 2005), human myometrium (Brainard et al., 2005) and ureter (Babiychuk et al., 2004) myocytes. Moreover, immunoprecipitation and glutathione S-transferase pull-down assays demonstrate that caveolin-1 and BK channels can be physically associated (Wang et al., 2005). Slo1 subunits have two potential caveolin-binding motifs in their CTD, and at least one of them can mediate association with caveolin-1 (Alioua et al., 2008). Because the maintenance of caveolae depends on not only caveolin-1 and associated proteins but also on free CLR levels in the membrane (Dreja et al., 2002; Daniel et al., 2004), the modification of BK current by CLR-depleting treatment can be explained by the loss of direct BK channel inhibition by CLR (Sections 4.3.1 and 4.3.2), and/or by alteration of local raft organization with disruption of interactions between BK and raft components, caveolin-1 in particular. Supporting the later mechanism, inclusion of caveolin-1 scaffolding domain peptide in a patch pipette solution completely ablates isoproterenol activation of BK currents in bovine aortic endothelial cells (Wang et al., 2005). This study demonstrates that BK currents are quiescent under control conditions but activated by CLR depletion, knocking-down caveolin-1 expression, or by isoproterenol stimulation. CLR depletion also increases BK currents in coronary artery myocytes, an effect that is paralleled by a decrease in membrane capacitance (Prendergast et al., 2010). The decrease in capacitance is consistent with the removal of caveolae, as previously shown with electron microscopy (Babiychuk et al., 2004; Potocnik et al., 2007) and by internalization of dextran-conjugated fluorescent indicators (Shmygol et al., 2007).

Whether involving caveolin-1 or *via* direct CLR-BK channel interaction, the usual end result of raft disruption by CLR-depleting treatment is a potentiation of BK currents. This has been shown as an increase in overall macroscopic BK current in colonic epithelium (Lam et al., 2004), BK current density in GH3 pituitary tumor cells (Lin et al., 2006), and BK Po in IGR39 human melanoma cells (Tajima et al., 2011). The later study also demonstrates that replenishing the cells with CLR by exposure to M β CD complexed with CLR decreases BK Po. Potentiation of BK current by raft disruption is likely the underlying mechanism leading to inhibition of rat ureter smooth muscle phasic contractions by CLR depletion (Babiychuk et al., 2004).

Considering the multiplicity of signals and cross-talking among elements that are found within caveolae and rafts, it is not surprising that BK current potentiation by CLR depletion and raft disruption is not a universal finding. CLR depletion completely removes BK current

in mouse portal vein (Sones et al., 2010) and myometrium myocytes, the latter attributed to channel internalization from the surface of the cell membrane (Shmygol et al., 2007). In glioma cells, CLR depletion disrupts the activation of BK channels by Ca^{2+} released *via* inositol 1,4,5-trisphosphate (IP_3) receptors, resulting in strong reduction of BK current (Weaver et al., 2007).

Finally, alteration of BK channel function by manipulation of cell CLR levels may result from disruption of the tightly regulated local ionic milieu that exist in caveolae and rafts. For example, adenosine-5'-triphosphatase (ATPase) activity, which controls the local ion concentration in rafts and thus, BK activity (Tajima et al., 2011), is regulated by CLR (Cuevas et al., 2006). Moreover, the ATPase $\beta 1$ subunit associates with the slo1 protein and modulates BK current (Jha & Dryer, 2009). Interestingly, BK current inhibition, whether directly *by* paxilline or indirectly *via* ATPase inhibition with ouabain, markedly decreases melanoma cell proliferation (Tajima et al., 2011). In synthesis, manipulation of CLR levels and raft disruption may increase or decrease BK current as several mechanisms that control BK function may differentially contribute across species and cell types to the final effect.

6. Modulation of BK channel pharmacology by cholesterol. The alcohol response

Modulation of ion channel pharmacology by membrane CLR has been documented with voltage-gated Na^+ and L-type Ca^{2+} channels, and GABA-A receptors (Rehberg et al., 1995; Sooksawate & Simmonds, 2001; Pouvreau et al., 2004). Studies of CLR modulation of BK channel pharmacology, however, seem to be restricted to the CLR-alcohol (ethyl alcohol, ethanol) interaction on channel function. Interest on this interaction is driven by multiple fields, including: 1) Biophysics: CLR and ethanol partitioning into model or natural membranes leads to modification of a variety of bilayer physical properties, which is sensed by the BK channel (Section 4.3.1; also: Crowley et al., 2003; 2005; Yuan et al., 2004; 2011). Thus, evaluation of BK channel conduction and gating properties in response to ethanol and/or CLR exposure serves as a reader to understand how modification of bilayer properties translates into altered function of membrane-spanning, globular proteins; 2) Cell Physiology and Pharmacology: in native membranes, BK channels associate with CLR-enriched microdomains (Bravo-Zehnder et al., 2000). On the other hand, ethanol exposure leads to changes in membrane CLR content and distribution (Chin et al., 1978; Wood et al., 1990; Omodeo-Salé et al., 1995), which may play a role in cell adaptation to ethanol exposure (Wood et al., 1990). For example, CLR counteracts ethanol's disordering action in mouse synaptosomal membranes and phospholipid bilayers (Chin & Goldstein, 1981). Moreover, ethanol-induced increase in fluidity of the extracellular leaflet of synaptic plasma membranes is larger than that of the cytoplasmic leaflet, a difference attributed to the larger CLR content in the inner leaflet. After chronic ethanol exposure, however, transbilayer differences in fluidity and CLR content are reduced in concert (Wood et al., 1990); 3) Pathophysiology: abnormal tissue CLR content and alcohol intake constitute risk factors for human disease, such as stroke. BK channels are functional targets of CLR (see above) and ethanol (reviewed in Brodie et al., 2007; Mulholland et al., 2009). Addressing molecular targets and mechanisms underlying BK channel involvement in CLR/ethanol-related pathophysiology may render a rationale for novel therapeutic agents that target BK channels.

Ethanol effects on BK channels are complex, resulting from the orchestration of several factors that include channel subunit composition, post-translational modification of slo1 subunit, lipid composition around the BK complex, time of alcohol exposure, and internal concentration of the channel activating ligand, that is, Ca^{2+} (reviewed in Brodie et al., 2007; Mulholland et al., 2009). Acute exposure to ethanol at concentrations reached in blood during intoxication (15–75 mM ethanol) usually evokes increased BK current, provided the

system under study does not contain BK $\beta 1$ subunits and Ca^{2+}_i levels remain at submicromolar to low micromolar (Brodie et al., 2007; Liu et al., 2008c). The increase in current results from enhanced Po with minor, if any, changes in channel conductance, and leads to decreased excitability in isolated neuroendocrine or neuronal elements (Dopico et al., 1999; Brodie et al., 2007; Martin et al., 2008).

Remarkably, ethanol-induced increase in BK Po is maintained when the drug is evaluated on hslol channels reconstituted into POPE:POPS (3:1 w/w) bilayers (Crowley et al., 2003). This outcome suggests that, as found for CLR (see section 4.3.2), a minimum set of targets (two species of phospholipids and slo1 subunits) suffice for ethanol action. Increasing CLR level in this bilayer type antagonizes ethanol-induced activation of hslol channels (Crowley et al., 2003). CLR-ethanol antagonism on protein activity may result, at least partially, from CLR-induced decrease in ethanol partition into phospholipid bilayers (Trandum et al., 2000), as found for other small compounds that target the CNS (Colley & Metcalfe, 1972; Miller & Yu, 1977; Lechleiter et al., 1986). Cholesterol-induced reduction in ethanol partitioning has been linked to the fact that CLR presence in the bilayer leads to the disappearance of the gel-liquid crystalline interface where ethanol preferentially resides (Trandum et al., 1999; 2000; Crowley et al., 2003).

It is noteworthy that bilayer CLR antagonism on ethanol-induced potentiation of hslol channel activity is concentration-dependent and paralleled by CLR-induced inhibition of hslol channel basal activity, that is, in absence of ethanol. Thus, alternatively or supplementary to a CLR-induced reduction of EtOH partitioning into lipid membranes, CLR antagonism of ethanol effects on BK current may result from CLR antagonism of ethanol *action* on its target(s). Remarkably, CLR-induced inhibition and ethanol-induced activation of BK currents share striking similarities: 1) effect on current is totally due to changes in Po , with undetectable modification of ion conductance; 2) change in Po results from agent-induced modification of both open and closed time distributions; 3) final effect on Po usually results from major changes in channel closed times with minor changes in open times. It has been speculated that if CLR and ethanol acted through a single, common mechanism, we might expect these agents to exert reciprocal actions on common dwell states of the channel (Crowley et al., 2003). However, derivation of mechanistic interpretations from BK dwell-times distributions is highly speculative, as cause-effect cannot be established and coincidental modifications cannot be rule out. Even at the phenomenological level, CLR and ethanol actions on BK channels differ in two basic aspects: 1) ethanol action on is critically modulated by accessory $\beta 1$ subunits whereas CLR action is not (Feinberg-Zadek & Treisman, 2007; Bukiya et al., 2009a), and 2) ethanol action on slo1 channel activity requires, and is a function of, activating Ca^{2+}_i (Liu et al., 2008c) whereas CLR inhibition of slo1 activity does not (Singh et al., 2011). Thus, while ethanol and CLR may affect Po by a common mechanism(s), each agent must also alter Po *via* separate gating pathways.

In a recent study, CLR modulation of ethanol action on slo1 channels has been evaluated after channel protein reconstitution into a DOPE:SPM (3:2 molar ratio) bilayer (Yuan et al., 2011). This bilayer is of particular interest because CLR and SPM form lipid microdomains that have been used to model lipid raft behavior in native membranes (London, 2002; 2005; Yuan et al., 2002; Simons and Vaz, 2004; Johnston, 2007). In contrast to data obtained in POPE:POPS (3:1 w/w) bilayers where inhibition of ethanol potentiation of hslol channel activity is a monotonic function of bilayer CLR (Crowley et al., 2003), CLR presence in the DOPE:SPM bilayer introduces qualitative changes in the channel response to 50 mM ethanol: in the CLR-free bilayer, ethanol reduces hslol Po , which is switched to channel activation by 20% (molar ratio) CLR, and back to ethanol-induced reduction of Po when CLR molar fraction 30%. A thorough discussion of the differential ethanol effects on BK

channels in SPM-free vs. SPM-containing bilayers relates the channel response to the bilayer thickness (Yuan et al., 2008).

It is noteworthy that CLR modulation of cbv1 channel pharmacology seems to be somewhat selective: in the same bilayer preparation, ethanol action on Po is regulated by membrane CLR while paxilline action is not (Bukiya et al., 2011c). This conserved sensitivity to paxilline contrasts with data from mouse portal vein myocytes, in which M β CD treatment drastically alters the channel pharmacology, including its response to paxilline (Sones et al., 2010). The molecular basis for the ethanol-paxilline dichotomy in bilayer studies remains elusive. However, effective BK channel block by paxilline is associated with elements of the slo1 protein S5-P loop-S6 domains (Gly311 in particular) (Zhou et al., 2010). On the other hand, ethanol action on BK channels does not interfere with ion conduction and voltage-gating (Dopico et al., 1996; 1998) but requires activating Ca²⁺ interactions with defined areas in the slo1 CTD (Liu et al., 2008). Conceivably, CLR influence on BK channel pharmacology is largely limited to gating modifiers (i.e., ethanol) that alter channel activity *via* CTD-driven gating mechanisms.

The presence of BK channels with heterooligomeric subunit composition and protracted ethanol exposure, conditions that are more relevant to understand CLR-ethanol actions on tissue function, bring additional complexities to the CLR-ethanol interaction. Application of 50 mM ethanol to vascular smooth muscle (VSM) BK channels for 3–10 min results in significant decrease in BK NPo in membrane patches excised from freshly isolated cerebral artery myocytes of rat and mice (Liu et al., 2004; Bukiya et al., 2011c), these channels consisting of cbv1 and β 1 subunits (see below). It is noteworthy that vascular smooth muscle BK channel inhibition by ethanol translates into cerebral artery constriction, as shown with rat and mouse cerebral arteries pressurized *in vitro* (Liu et al., 2004; Bukiya et al., 2011c). In these systems, CLR depletion from the arterial wall by preincubating the arteries with M β CD results in complete loss of ethanol-induced vasoconstriction. This outcome is identical in intact and endothelium-free arteries, advancing the idea that CLR in the smooth muscle itself (and independently of circulating, paracrine or endothelial factors) plays a key role in regulating ethanol action on BK channels and vascular pathophysiology (Bukiya et al., 2011c).

Consistent with the evaluation of CLR-ethanol interaction on myogenic tone and the key role of BK channels in mediating ethanol-induced cerebral artery constriction (Liu et al., 2004), electrophysiology on membrane patches excised from rat and mouse cerebral artery myocytes reveals that ethanol-induced BK channel inhibition is lost after CLR-depleting treatment. Moreover, cbv1 $\pm$ β 1 channels reconstituted into POPE:POPS (3:1 w/w) bilayers show are inhibited and unresponsive to 50 mM ethanol in CLR-containing (33 mol%) and CLR-free bilayers, respectively (Bukiya et al., 2011c). Data from excised membrane patches and artificial bilayers underscore that the CLR-ethanol interaction on BK channel activity requires neither cytosolic signaling nor the complex organization of cell membranes. Remarkably, CLR not only enables ethanol-induced inhibition of VSM BK channels but amplifies it (Bukiya et al., 2011c). Thus, while regulation of BK channel gating by CLR and ethanol is differentially modulated by BK auxiliary subunits and activating Ca²⁺, there must be a common mechanism(s) that leads to CLR amplification of ethanol inhibition of BK channels and cerebral artery constriction. In synthesis, the molecular bases of CLR-ethanol interactions on BK channels are complex, and further electrophysiological studies complemented with imaging, biochemical, structural and computational approaches are necessary to unveil mechanisms and structural requirements.

7. Modification of BK channel function during hypercholesterolemia

Hypercholesterolemia is defined by elevated CLR levels (>240 mg/dL) in serum. In humans, and also replicated in numerous animal models, hypercholesterolemia has been linked to a variety of abnormal conditions, including atherosclerosis, arterial hypertension, stroke and cognitive dysfunction (Hu et al., 2008; Bui et al., 2009; Granger et al., 2010; Miller et al., 2010; Gorelik et al., 2011). Like most human diseases, these conditions are better understood at the organismal and organ levels, yet effective intervention often requires identification of underlying subcellular mechanisms and molecular targets that respond to increased CLR levels.

Several animal models have been used to document that plasmalemma BK channels are sensitive to hypercholesterolemia (reviewed by Sobey, 2001). Driven by epidemiological and clinical links between hypercholesterolemia and cardiovascular disease, and the key role of BK channels in regulating vascular tone (Section 2.2), most of the evidence comes from the cardiovascular system. Endothelium-dependent and independent *in vitro* relaxations have been evaluated in hypercholesterolemic rabbits that were fed with a 0.5% CLR diet for 12 weeks. Determinations of isometric tension in intact *vs.* de-endothelized carotid artery rings reveal that artery dilation in response to NO, sodium nitroprusside and 8-bromoguanosine 3',5'-cyclic monophosphate (8-Brc-GMP) remain similar in control and hypercholesterolemic rabbits (Najibi & Cohen, 1995). However, NO-mediated responses are significantly attenuated by selective BK block in hypercholesterolemic animals. From these studies, it has been suggested that hypercholesterolemia induces an over-compensatory response of BK channels, presumably in the arterial smooth muscle layer itself (Najibi et al., 1994; Najibi & Cohen, 1995). It has also been suggested that an enhanced role of vascular BK channels under hypercholesterolemic conditions could be associated with lower basal channel activity, in which case the availability of BK channels to acetylcholine stimulation should be increased (Sobey, 2001). However, increased BK P_o has been actually reported after cell-attached recordings of smooth muscle from the atherosclerotic plaque, when compared to arterial media segments (Wiecha et al., 1997). This difference is lost when BK channel function is evaluated in cell-free, inside-out membrane patches (Wiecha et al., 1997), pointing to the involvement of intracellular organelles and/or freely diffusible cytosolic signals in the increased smooth muscle BK channel activity that is associated with the atherosclerotic plaque.

In contrast to the studies mentioned in the previous paragraph, *in vivo* studies on rabbits show a reduced vasodilation (i.e., hindlimb vascular conductance) in response to acetylcholine and bradykinin following a high CLR diet (0.5% CLR for 16 weeks). The different vascular dilation in control *vs.* hypercholesterolemic groups persists after treatment with the NO-synthase antagonist N-nitro-L-arginine methyl ester (L-NAME). The remaining, NO-independent, component of acetylcholine- and bradykinin-induced vasodilation is almost totally abolished by block with tetraethylammonium or charybdotoxin +apamin. Thus, authors have concluded that hypercholesterolemia impairs BK-mediated *in vivo* vasodilation (Jeremy & McCarron, 2000).

Findings from an *in vitro* study on rat cerebral arteries from our laboratory are consistent with the conclusions driven from the rabbit hindlimb *in vivo* dilation results. Middle cerebral arteries of rats on control *vs.* high CLR diet (2% CLR for 10 weeks) were de-endothelized and then pressurized at 60 mm Hg. While the responses to a depolarizing solution, containing 60 mM KCl are similar in control and hypercholesterolemic arteries, paxilline-induced vasoconstriction in arteries from rats on high CLR diet is more than $\times 2$ smaller than that from rat littermates on isocaloric chaw. Notably, BK channel function is altered rather selectively: arterial diameter responses to 4-aminopyridine, which blocks

voltage-gated K⁺ channels other than BK, do not differ in control vs. hypercholesterolemic animals. These results demonstrate that while the fundamental contractile machinery of the artery (tested with high KCl) remains unaltered by high CLR diet, endothelium-independent, smooth muscle BK channel-mediated dilation is ablated in cerebral vessels from hypercholesterolemic rats (Bukiya & Dopico, 2008a).

The reduction in paxilline-induced cerebral artery constriction by hypercholesterolemia described in the previous paragraph may result from a decreased number of BK channels in arterial smooth muscle and/or alterations in individual channel function. In particular, hypercholesterolemia may down-regulate accessory, smooth muscle type β subunit ($\beta 1$). Remarkably, cerebral arteries of *KCNMB1* K/O mice are insensitive to selective BK channel block by peptide blocker iberiotoxin (Brenner et al., 2000b). The molecular basis for hypercholesterolemia-driven attenuation of BK channel function in cerebral arteries remains unknown and is under current investigation in our laboratory.

Hypercholesterolemia-induced changes in BK $\beta 1$ subunit level have been studied in the circular smooth muscle strips from the sphincter of Oddi (Du et al., 2006).

Immunohistochemical staining and Western blotting using polyclonal antibody against BK $\beta 1$ subunits show higher levels of $\beta 1$ protein in the in control group of rabbits compared to animals on high CLR diet (1.0 g of CLR/day for 4 weeks). These results led authors of the study to conclude that hypercholesterolemia down-regulates BK $\beta 1$ subunit expression in this smooth muscle specimen (Du et al., 2006).

In synthesis, several studies demonstrate that hypercholesterolemia affects BK channel function in different organs and tissues, smooth muscle in particular. Among many others, it remains a possibility that hypercholesterolemia modulates BK function secondary to actual partition of CLR molecules into cell membranes where the BK channel resides, with modulation of channel function resulting from CLR-mediated of bilayer physical properties (Section 4.3.1) and/or direct sterol-protein interactions (Section 4.3.2).

8. Pathophysiological implications and concluding remarks

Considering the ubiquitous distribution of CLR and BK channels, their common location in specific membrane domains and their participation in regulating a long list of physiological processes, it is not surprising that most authors that evaluated CLR-BK channel interactions attempted to infer how such interactions impact cell physiology and/or participate in pathological developments. Connections between CLR-BK channel interaction and tissue pathophysiology have been largely advanced from data on excitable cells. In neurons, CLR modulation of BK channel activity has been linked to plastic changes that occur in the neuronal membrane in response to protracted or repeated alcohol exposure (Crowley et al., 2003; Yuan et al., 2007). Change in firing properties by CLR modulation of BK currents in GH3 cells is likely to alter the cell excito-contraction coupling and thus, neuropeptide release (Lin et al., 2006). In afferently-innervated hair cells, CLR modulation of BK currents could reshape electrical potentials, alter tuning and temporal coding and, by changing the membrane time constant, the capability of the cell to accurately respond to repetitive stimuli (Purcell et al., 2011). Interestingly, these authors point to a correlation between hearing loss and dyslipidemia (Campbell et al., 1996).

In vascular smooth muscle, hypercholesterolemia leads to impairment of BK-mediated vasodilation, as reported with hindlimb (Jeremy & McCarron, 2000) and cerebral arteries (Bukiya & Dopico, 2008a). Such mechanism could contribute to vascular pathology associated with systemic hypertension, vascular peripheral disease, cerebrovascular constriction and stroke. In particular, the synergism between alcohol and CLR in reducing BK current in cerebral artery smooth muscle could contribute to the fact that

hypercholesterolemia and moderate-heavy alcohol intake constitute risk factors for cerebrovascular disease (Bukiya et al. 2011c). BK channels do not seem to play a significant role in the coronary hyperemia reactive to ischemia that occurs in swine fed with a hypercaloric diet supplemented with 2% CLR (Borhouse et al., 2010). However, this treatment does reduce coronary vasodilation in response to BK channel activation (Borhouse et al., 2009). Consistently, CLR depletion leads to increased BK currents in rat coronary arteries (Prendergast et al., 2010). It has been speculated that CLR influence on BK channel function contributes to the coronary dysfunction described in obese subjects with metabolic syndrome (Knudson et al., 2007; Borhouse et al., 2009).

Contribution of CLR-BK channel interaction to smooth muscle pathophysiology is not limited to vascular smooth muscle. In both rat (Shmygol et al., 2007) and human (Zhang et al., 2007) uterine smooth muscle CLR depletion leads to disruption of caveolae and BK function and, thus, increase in both spontaneous and oxytocin-evoked contractility. On the other hand, authors speculate that BK channel targeting by increased CLR in obese pregnant women could contribute to poor uterine contractility with increased rate of C-sections found in this group (Crane et al., 1997; Shmygol et al., 2007; Zhang et al., 2007).

Regarding non-excitabile tissues, CLR modulation of BK channels in colonic epithelium will likely impact anionic secretion in the colon (Lam et al., 2004). Cholesterol-BK channel associations could also control K^+ gradients that are necessary for glioma cell migration (Weaver et al., 2007) and IGR39 melanoma cell proliferation (Tajima et al., 2011).

Collectively, the data from excitable and nonexcitable cells described above suggest that disruption of BK channel-membrane CLR interaction is associated with a variety of pathophysiological processes. However, a causative role for such interaction remains to be established in most cases. This shortcoming is particularly applicable to studies documenting alteration of BK currents with hypercholesterolemia, as this *in vivo* condition could alter BK channel activity by triggering genetic, epigenetic or posttranslational mechanisms that may have nothing to do with a direct membrane CLR-BK channel interaction. Uncertainties in our knowledge are also present from studies at the cellular level: while there is little doubt that BK channel is preferentially associated with CLR-enriched domains in natural membranes, the physiological impact of such association remains to be fully deciphered. Finally, a variety of approaches combining structural biology, mutagenesis, single channel electrophysiology and computational dynamics will be necessary to determine the relative role of lipid and protein interfaces in CLR recognition by the BK channel. Identification of a slo1 protein site for CLR could lead to identification of polymorphisms in human populations that would be more/less susceptible to CLR modulation of BK channel function. It may also open new avenues in Medicinal Chemistry for designing pharmacological agents that regulate tissue function and/or control disease by targeting CLR-BK channel interactions. The current uncertainties at the organismal, cellular and molecular levels should be seen as an optimal opportunity for biomedical researchers to contribute to the field of CLR-BK channel interactions.

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Abbreviations

AP action potential

BK	large conductance, calcium- and voltage-gated potassium
cbv1	BK pore-forming subunit encoded by gene cloned from rat cerebral artery smooth muscle
Ca_v	voltage-gated calcium
CLR	cholesterol
CRAC	cholesterol recognition/interaction amino acid consensus
CTD	cytosolic tail domain
DOPE	1,2-dioleoyl-3-phosphatidylethanolamine
hslol	BK pore-forming subunit encoded by gene cloned from human brain
K/O	knock-out
KCNMA1	gene coding for BK α subunit
KCNMB	gene coding for BK β subunit
Kir	inwardly rectifying K ⁺
MβCD	methyl- β -cyclodextrin
nAChR	nicotinic acetylcholine receptor
NO	nitric oxide
PKA	protein kinase A
Po	channel open probability
POPE	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine
POPS	1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-l-serine
RCK	regulatory of conductance for K ⁺
SAR	structure-activity relationship
SPM	sphingomyelin
TM	transmembrane (domain/region)
STOC	spontaneous transient outward current
VRAC	volume-regulated anion channel

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Figure 1. Schematic structure of a BK channel heterodimer made of channel-forming (α) and accessory (β) subunits

BK channel-forming α (slo, slo1) subunit belongs to the superfamily of 6TM (S1–S6 segments, shown in blue) voltage-gated ion channels. Four α subunits render a functional BK channel. S4 includes Arg213, which has been shown to control the channel voltage sensitivity. Regions in S5 and S6 assemble to form the pore domain. In addition to the 6TM core, each BK α subunit contains a TM segment (S0) and a large cytosolic tail domain (CTD). These two features seem unique to BK channels and are shown in red. CTD contains two Regulatory of Conductance for K^+ domains (RCK1,2). The two RCKs are thought to provide the structural basis for Ca^{2+} sensitivity and form an octameric gating ring to promote channel opening in response to activating Ca^{2+} . Ca^{2+} -driven force is transmitted to the channel pore domain *via* the S6-RCK1 linker (spring). In most mammalian tissues, BK channels are tightly associated with 2TM β subunits (violet). Expression of these subunits is highly tissue-specific. BK β subunits greatly determine the BK current phenotype, including its pharmacological profile.

Figure 2. Cholesterol may reduce BK channel activity via three types of interactions

The top left panel shows a dimer of BK channel-forming α subunits (in blue and red) with the gate open, allowing outward flow of K^+ through the pore. Cholesterol molecules are shown in orange. The bottom left panel depicts CLR-lipid interactions as mediators of CLR inhibition of BK channels. CLR presence in a lipid membrane is associated with formation of CLR-rich domains characterized by increased order of phospholipid hydrocarbon chains (dark grey). As a result, the bilayer physical properties within such domains change (e.g., increased in lateral stress), favoring closure of the BK channel. The top right panel depicts a channel protein-CLR direct interaction mediating CLR inhibition of BK channels; the channel protein provides an actual CLR-docking surface that senses the presence of CLR rather selectively. Upon CLR recognition by this site, channel closure is favored. Lipid- and protein-CLR interactions are not mutually exclusive (see main text). Finally, the bottom right panel shows a CLR molecule interacting at a lipid-protein interface. In this case, the CLR-docking area is contributed by both protein and lipid surfaces.

Figure 3. Cholesterol and related analogs used in structure-activity relationship studies of sterol-sensing by BK channels

A. Cholesterol molecule (top) possesses three distinct features. First, a single polar hydroxyl group at carbon 3 (C3, shown in red), which is in β configuration. Second, CLR is characterized by a set of hydrophobic A–D steroid rings with the A/B ring junction containing an unsaturated bond between C5 and C6 (shown in blue). Lastly, CLR contains a hydrophobic iso-octyl tail (C17–C25). Part of this tail (shown in pink) was substituted by a carboxyl group in the SAR study. Enantiomeric cholesterol (ent-cholesterol, shown at the bottom) is a mirror image of natural cholesterol (i.e., with all chiral centers inverted). A horizontal dashed grey line represents the plane of the mirror (M). **B.** CLR and its saturated

analogs cholestanol and coprostanol (left column) with differential geometry of A/B ring junction are all active in reducing BK channel activity. In contrast, all α epimers (epicholesterol, epicholestanol, and epicoprostanol) are inactive on BK channel activity.