Cholesterol inhibition of slo1 channels is Ca²⁺-dependent and can be mediated by either high-affinity Ca²⁺-sensing site in the slo1 cytosolic tail

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Abbreviations. Ca^{2+} : calcium; BK: Ca^{2+} - and voltage-gated K^+ channel of large conductance; Ca^{2+}_i : Ca^{2+} present at the cytosolic/cis side of natural membranes/artificial lipid bilayers; CLR: cholesterol; CTD: cytosolic tail domain; POPE: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; POPS: 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine; RCK: Regulator of Conductance for K^+ domain

Abstract

Ca^{2+} -voltage-gated K^+ channels of large conductance (BK) are expressed in the cell membranes of all excitable tissues. Currents mediated by BK channel-forming slo1 homotetramers are consistently inhibited by increases in membrane cholesterol (CLR). The molecular mechanisms leading to this CLR action, however, remain unknown. Slo1 channels are activated by increases in Ca^{2+} nearby Ca^{2+} -recognition sites in the slo1 cytosolic tail: one high-affinity and one low-affinity sites locate to the Regulator of Conductance for K^+ (RCK) 1 domain, while another high-affinity site locates within the RCK2 domain. Here we first evaluated the cross-talking between Ca^{2+} and CLR on the function of slo1 (cbv1 isoform) channels reconstituted into planar lipid bilayers. CLR robustly reduced channel open probability while barely decreasing unitary current amplitude, with CLR maximal effects being observed at 10-30 μM internal Ca^{2+} . CLR actions were not only modulated by internal Ca^{2+} levels but also disappeared in absence of this divalent. Moreover, in absence of Ca^{2+} , BK channel-activating concentrations of Mg^{2+} (10 mM) did not support CLR action. Next, we evaluated CLR actions on channels where the different Ca^{2+} -sensing sites present in the slo1 cytosolic domain became nonfunctional *via* mutagenesis. CLR still reduced the activity of low-affinity Ca^{2+} (RCK1:E379A, E404A) mutants. In contrast, CLR became inefficacious when both high-affinity Ca^{2+} sites were mutated (RCK1:D367A,D372A, and RCK2:D899N,D900N,D901N,D902N,D903N), yet still was able to decrease the activity of each high-affinity site mutant. Therefore, BK channel inhibition by CLR selectively requires optimal levels of Ca^{2+} being recognized by either of the slo1 high-affinity Ca^{2+} -sensing sites.

Significance. Results reveal that the widely reported inhibition of BK (slo1) channels by membrane cholesterol requires a physiologically range of internal Ca^{2+} and is selectively linked to the two high-affinity Ca^{2+} -sensing sites located in the cytosolic tail domain of slo1 proteins, which underscores that Ca^{2+} and cholesterol actions are allosterically coupled to the channel gate. Cholesterol modification of BK channel activity likely contributes to disruption of normal physiology by common health conditions that are triggered by disruption of cholesterol homeostasis.

Introduction

Ca^{2+} - and voltage-gated K^{+} channels of large conductance (MaxiK, BK) are widely distributed in animal tissues (Behrens et al., 2000; Orio et al., 2002). Upon activation, these channels generate outward K^{+} currents that control neuronal firing, smooth muscle contractility, endocrine secretion, circadian rhythm, and the processing of sensory information (Behrens et al., 2000; Orio et al., 2002; Pyott et al., 2007; Whitt et al., 2016; Dopico et al., 2018). Functional channels are formed by tetrameric association of channel-forming slo1 subunits (Salkoff et al., 2006). Slo1 monomers are modular proteins that combine pore-, voltage-, and Ca^{2+} -gating domains (Wang et al., 2009; Wu et al., 2010; Yuan et al., 2010; Giraldez and Rothberg, 2017) (**Fig. 1**). The intracellular cytosolic tail domain (CTD) includes two Regulator of Conductance for K^{+} structures (RCK1 and RCK2) (Xia et al., 2002). Each RCK contains a high-affinity Ca^{2+} -binding site that senses nM-low μM Ca^{2+} (Zeng et al., 2005). In addition, RCK1 contains a low-affinity Ca^{2+} -binding site that is sensitive to 0.1-100 mM Ca^{2+} (Zeng et al., 2005). Ca^{2+} binding to the homotetramer expands an elastic gating ring formed by a total of eight RCKs, which is coupled to the voltage-sensing domain through a rigid linker (Tao et al., 2017).

In most native tissues, BK channel-forming tetramers are accompanied by accessory subunits (β and γ -types) (Brenner et al., 2000; Orio et al., 2002; Yan and Aldrich, 2010; Yan and Aldrich, 2012). These small accessory proteins modulate pharmacological and biophysical properties of slo1, but do not generate current themselves (Brenner et al., 2000; Orio et al., 2002; Bukiya et al., 2009; Yan and Aldrich, 2010).

In turn, cholesterol (CLR) is an essential lipid in animals. At the organismal level, cholesterol serves as a precursor of steroid hormones and bile acids (Luu-The, 2013). Cellular effects of CLR include modulation of cell motility and organization of the actin cytoskeleton (Ramprasad et al., 2007), and the regulation of interactions between bacterial or viral (such as HIV) pathogens and host cells (Goluszko and Nowicki, 2005; Schroeder and Cavacini, 2010).

Within plasma membranes of animal cells, CLR plays the role of a structural lipid, providing an ordering effect on membrane phospholipids and modifying several physical properties of the lipid bilayer (Róg et al., 2009). Moreover, CLR has been increasingly recognized as an important regulator of the function of proteins, receptors in particular, including ion channels (Gimpl et al., 1997; Sooksawate and Simmonds, 2001; Addona et al., 2003; Levitan et al., 2010; Dopico et al., 2012).

CLR-induced regulation of BK-mediated ion currents has been widely reported, with both CLR-driven inhibition and activation of BK current being documented (reviewed in Dopico et al., 2012). While the final effect of CLR on BK-mediated currents is influenced by the channel's accessory subunits (Bukiya et al., 2021), it has been widely documented that exposure of homomeric slo1 to CLR levels found in membrane consistently decreases BK current (Crowley et al., 2003; Bukiya et al., 2011a; Singh et al., 2012). The molecular mechanisms leading to BK inhibition of slo1 remain largely unknown.

Modification of BK current by endogenous ligands or exogenous pharmacological agents is often influenced by activating levels of Ca^{2+} in the vicinity of the channel. Indeed, BK current *potentiation* by lithocholic acid, leukotriene B₄, and ethyl alcohol are all modified by internal Ca^{2+} (Ca^{2+}_i) levels (Bukiya et al., 2007; Liu et al., 2008; Bukiya et al., 2014b). Moreover, ethyl alcohol, whose activation of slo1 is CLR-dependent (Bukiya et al., 2011a), is unable to increase slo1 activity in absence of this activating divalent. Based on these precedents, here we utilized lipid bilayers to test the hypothesis that BK current *inhibition* by CLR is Ca^{2+} -dependent and, if so, determined by specific Ca^{2+} -sensing sites within the slo1 protein. Our results prove this hypothesis by revealing an optimal Ca^{2+}_i level for CLR-induced inhibition of slo1 currents, an effect that involves major reduction in channel steady-state activity and minor reduction in unitary conductance. Moreover, using engineered slo1s that make one or more Ca^{2+} -sensing sites nonfunctional, we demonstrate that as far as Ca^{2+}_i reaches levels to optimally activate the

channel *via* either of the two high-affinity Ca^{2+} sites located in the CTD, CLR inhibits channel activity. This CLR action, however, is lost when the channel is solely activated via its low affinity site Ca^{2+} site also present in the slo1 CTD. Our study reveals the molecular mechanism of CLR-induced slo1 channel inhibition and thus, expands our understanding of CLR effects on ion channels dually gated by ligands and transmembrane voltage.

Materials and Methods

Preparation of slo1 protein. BK channel-forming α subunit cDNA (cbv1; AY330293) cloned from rat cerebral artery myocytes (Jaggar et al., 2005) was inserted into the pcDNA3.1 plasmid vector as described (Singh et al., 2012). Ca^{2+} -sensing site mutants were introduced into cDNAs coding for cbv1 inserted into the pBluescript as described (Liu et al., 2008). The presence of desired nucleotide substitutions and absence of unwanted mutations were verified by automatic sequencing at the Molecular Research Center of the University of Tennessee Health Science Center. Human embryonic kidney (HEK) 293 cells were transiently transfected with slo1-carrying pcDNA3.1 plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Mutated slo1-carrying pBluescript plasmids were transfected into Chinese hamster ovary cell using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfected cell lines were grown to confluence, pelleted, and re-suspended on ice in 10 ml of buffer solution of following composition (mM): 30 KCl, 2 MgCl_2 , 10 HEPES, 5 EGTA; pH 7.2. A membrane preparation was obtained using a sucrose gradient as previously described (Crowley et al., 2003). Aliquots were stored at -80°C .

Recording of ionic current following slo1 reconstitution into planar lipid bilayers. CLR was dissolved in chloroform and then introduced into a lipid mixture of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) 3:1 (w/w), to render a final CLR concentration of 20% w/w (33 mol%). Lipid mixtures, whether containing CLR or not, were dried under N_2 gas and re-suspended in 25 mg/ml of n-decane. Vertical bilayers (80–120 pF) were formed by painting the lipid mix across a 200 μm diameter hole in a delrin cup (Warner Instruments). The membrane preparation of cbv1 protein was added to the *cis* chamber. Fusion between the membrane preparation and the bilayer was promoted by osmosis, with the *cis* chamber recording solution being

hyperosmotic to the *trans* chamber solution. In all recordings, the *cis* and *trans* chambers were set as the intracellular and extracellular compartments, respectively. Solution for the *cis* chamber included (mM): 300 KCl, 10 HEPES; pH 7.2. Solution for the *trans* chamber included (mM): 30 KCl, 10 HEPES; pH 7.2. In all experiments, free Ca^{2+} in *cis* and *trans* chamber solutions was the same, with free Ca^{2+} being adjusted to the desired value by adding CaCl_2 and EGTA into the *cis* and *trans* chamber solutions. For the experiments with $\text{Ca}^{2+} \geq 1 \mu\text{M}$, HEDTA was also added. In Ca^{2+} -free solution, CaCl_2 was omitted. Nominal free Ca^{2+} in solution was calculated using the MaxChelator Sliders program (C. Patton, Stanford University) and validated experimentally using a Ca^{2+} -selective electrode (Cole-Parmer) (Dopico, 2003). The *trans* chamber was connected to ground while the *cis* chamber voltage was clamped at potentials relative to ground. Only cbv1 with its Ca^{2+} -sensors oriented toward the *cis* chamber was used for data acquisition and analysis.

Ionic currents were acquired during 20–30 sec of continuous recording at various voltages using a Warner BC-525D amplifier, low passed-filtered at 1 kHz using the 4-pole Bessel filter built into the amplifier and sampled at 5 kHz with Digidata 1322A/pCLAMP 8 (Molecular Devices). Data from mutated cbv1 were acquired using identical parameters yet sampled using Digidata 1550B/pClamp10. For proper comparisons with previous data obtained by us (Crowley et al., 2003; Bukiya et al., 2008; 2011a; 2011b) and others (Chang et al., 1995; Yuan et al., 2007), studies were conducted at room temperature (20–25 °C). We used NPo as an index of channel steady-state activity, where N=number of functional channels present in the bilayer and Po=individual channel opening probability. At end of the experiment, channels were exposed to high ($>>100 \mu\text{M}$) Ca^{2+} solutions and positive voltage (70 mV) at the *cis* side of the bilayer. Under these conditions, slo1 Po approaches 1 and thus an approximation of N can be obtained from the maximal number of overlapping opening levels, which led us to calculate Po from the computed NPo. NPo and unitary current amplitude were computed using a built-in

routine in Clampfit 10.6 (Molecular Devices, San Jose, CA, USA) using a 50% threshold for event detection. For the calculation of V_{half} , that is, the voltage at which $(N)Po/(N)Po_{max}$ reached 0.5 (half-maximal activity), $(N)Po/(N)Po_{max}$ -V plots were created fitting data to a Boltzmann function using a fitting routine in Origin 8.5.1 (Originlab).

Chemicals. CLR, POPS (sodium salt) and POPE were purchased from Avanti Polar Lipids (Alabaster, AL, USA). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Statistical Analysis. Analysis was conducted using InStat 3.0 (GraphPad, San Diego, CA, USA). Groups of data are shown as mean $\pm$ S.D. Normal distribution of data was determined by the Kolmogorov-Smirnov's test. A set of channel recordings under a given experimental condition (e.g., CLR-free) throughout the entire voltage range from a single bilayer was considered a single experimental observation. When number of observations ≥ 10 and data distribution was Gaussian, unpaired Student's t-test (one-tail) was used to determine the statistical significance between individual means. However, when number of observations ≤ 10 or data distribution was not Gaussian, Mann-Whitney test (one-tail) was used. Comparisons of data, whether Po or unitary current amplitude, between CLR-containing vs. CLR-free bilayers were conducted for each specific voltage. V_{half} s and fitting data to Boltzmann functions were determined for each bilayer recorded, with data being acquired throughout the entire range of voltages.

Results

In the absence of Ca^{2+}_i CLR fails to significantly inhibit slo1-mediated currents. We first set to determine whether the presence of the slo1 (cbv1) physiological activating ligand (Ca^{2+}) was critical for CLR to inhibit channel function. The basic BK phenotype of cbv1-mediated currents in this system was established as previously described (Bukiya et al., 2011a,b). The CLR amount used corresponds to a molar fraction of 0.33 (i.e., 33 mol%), which is within the CLR range found in the plasma membrane of animal cells in most tissues (30–50 mol%; Gennis, 1989). The CLR molar fraction used also corresponds to the CLR concentration that reduces the steady state activity (NPo) of human brain hslo1 subunits (Crowley et al., 2003) and cbv1 itself (Bukiya et al., 2008) when reconstituted into binary phospholipid bilayers identical to those used in the current experiments. Following cbv1 incorporation, we perfused both the *cis* and *trans* sides of the bilayer with Ca^{2+} -free solution, i.e., a high K^+ solution that contained only trace amounts of Ca^{2+} (~0.5 nM; Cox and Aldrich, 2000). Channel activity was then recorded at a wide range of transbilayer voltage (-60 to +80 mV). More extreme voltages, whether of positive or negative polarity, resulted in unstable bilayers which, under our experimental conditions, consistently broke within a few seconds after formation.

CLR failed to modify cbv1 Po in the absence of activating Ca^{2+} (**Fig. 2A-B**), a result that was replicated in all bilayers (n=7 for controls; n=6 for CLR-containing bilayers). The extremely low activity of cbv1 in absence of activating Ca^{2+} , as found for other slo1 (Cox and Aldrich, 2000), prevented us from experimentally determining maximal cbv1 activity and thus obtain Po/Po_{max} plots straightforwardly. However, previous reports from our laboratory and others estimated 300-400 mV as the voltage range at which slo1s, including cbv1, reached maximal steady-steady activity (V_{max}) in absence of Ca^{2+} (Cox and Aldrich, 2000; Horrigan and Aldrich, 2002; Kuntamallappanavar and Dopico, 2016). Using these V_{max} values, we conducted a Boltzmann fitting of our current data to estimate cbv1 current V_{half} : 138.28 ± 14.72 and

138.94±26.16 mV in control and CLR-containing bilayers, respectively. The values obtained in control bilayers are similar to those previously reported for slo1 other than cbv1 (Cox and Aldrich, 2000; Horrigan and Aldrich, 2002) and cbv1 itself (Kuntamallappanavar and Dopico, 2016) under these Ca^{2+} -free conditions. Thus, present data demonstrate that CLR fails to significantly reduce cbv1 steady-state activity in the absence of activating Ca^{2+}_i , that is, when slo1 activity is driven by voltage- and/or intrinsic-gating (i.e., closed to open transitions in absence of Ca^{2+}_i and with the voltage sensors largely dwelling in their resting state; Cui et al., 1997; Horrigan and Aldrich, 1999).

In addition to its failure to modify steady-state activity, CLR failed to modify cbv1 unitary current amplitude (i) when evaluated in the absence of Ca^{2+} (Fig. 2C). Total ionic current due to cbv1 expression in a bilayer is determined by N , P_o and i . Given the lack of CLR action on i and NP_o , it is possible to conclude that in the absence of Ca^{2+} CLR fails to significantly modify the total ionic current mediated by cbv1.

Cholesterol-induced inhibition of slo1 currents requires a specific range of Ca^{2+} levels.

As Ca^{2+}_i reaches μM levels, modification of Ca^{2+} -driven gating processes more effectively translates into changes in BK P_o (Meera et al., 1996; Cui et al., 1997; Rothberg and Magleby, 2000). At these levels, Ca^{2+} begins to be sensed by the two high-affinity Ca^{2+} -recognition sites present in the slo1 CTD, opening the octameric gating ring and leading to increased P_o (Hite et al., 2017). At 1 μM Ca^{2+}_i , however, slo1 P_o and unitary current amplitudes in CLR-containing bilayers still remained basically unmodified from their counterparts in CLR-free bilayers (Fig. 2D-F). V_{half} s reached 139.05±38.74 and 112.28±16.93 mV in CLR-containing vs. control bilayers, respectively. Remarkably, in Ca^{2+} -free systems, exposure of slo1 channels to 10 mM Mg^{2+}_i , i.e., a Mg^{2+}_i concentration that activates BK channels on its own (i.e, independently of Ca^{2+}_i) failed to endow the slo1 channel with sensitivity to CLR inhibitory effect (Supp. Fig. 2).

Data indicate that the dependence of CLR inhibition of slo1 channels on activating Ca^{2+}_i does not generalize to include another physiological divalent, and suggest the existence of a rather selective coupling between CLR- and Ca^{2+} -sensing elements in the slo1 protein.

As Ca^{2+}_i was further increased to 10 μM , the effects of CLR on slo1 function became clearly evident: CLR was able to significantly ($P < 0.05$) decrease cbv1 activity within a window of transmembrane voltages from -20 to +30 mV (**Fig. 3A-B**). CLR-induced cbv1 inhibition at 10 μM Ca^{2+}_i translated into a significant increase in V_{half} from -3.88 ± 22.95 to 22.57 ± 35.90 mV (**Fig. 3C-D**). Unitary current amplitude, however, remained basically unchanged by CLR (**Fig. 3E**), consistent with previous findings that have reported minor, if any, decrease in unitary conductance of native or recombinant BK channels in response to CLR molar fractions that drastically decrease steady-state activity (reviewed in Dopico et al., 2012). CLR-induced slo1 inhibition became even more significant as Ca^{2+}_i was increased to 30 μM (**Fig. 3F**). At this Ca^{2+}_i , the CLR-induced decrease in cbv1 Po became more robust reaching significance across a wider voltage-range (from -50 to +30 mV) (**Fig. 3G**). Thus, CLR evoked a parallel, rightward shift in $\text{Po}/\text{Po}_{\text{max}}$ -V curve along the voltage axis (**Fig. 3H**) which translated into a significant increase in V_{half} from -27.10 ± 24.91 to 3.54 ± 10.72 mV (**Fig. 3I**). While CLR effect on unitary current amplitude at 30 μM free Ca^{2+} did not reach significance, p values of comparison between CLR-free and CLR-containing bilayers were consistently between 0.08-0.05. Thus, had the sample size been larger, the CLR-evoked minor decrease in unitary current amplitude under these conditions would have likely reached significance (**Fig. 3J**). Moreover, titration of CLR in the bilayer led to modification of slo1 channel function in a concentration-dependent manner: both unitary current amplitude and Po were barely affected by 10 or 20 mol% CLR, significantly reduced by 33 mol% (see above), and further reduced by 45 mol% (**Supp. Fig. 1**).

It is noteworthy that the robust inhibition of slo1 channel activity evoked in the presence of 30 μM Ca^{2+}_i was identical in the absence and presence of activating Mg^{2+}_i (10 mM Mg^{2+}_i ;

Supp. Fig. 3), underscoring a lack of synergism between the two physiological divalents on CLR inhibition of slo1 channels, and the selectivity in the coupling of CLR-sensing to Ca^{2+} -driven gating.

We next evaluated the CLR response of cbv1 at even higher Ca^{2+} levels ($\geq 100 \mu\text{M}$). These levels are above those needed to fully activate the slo1's two high-affinity Ca^{2+} -sensing sites while able to begin to activate these channels through a low affinity Ca^{2+} -sensing site (Zeng et al., 2005). At $100 \mu\text{M} \text{Ca}^{2+}_i$, CLR somewhat decreased cbv1 Po, yet this effect was significant within a voltage range (from -20 to +20 mV) (**Fig. 4A-B**) narrower than that observed at $10\text{-}30 \mu\text{M} \text{Ca}^{2+}_i$. As CLR did not evoke a substantial shift in the $\text{Po}/\text{Po}_{\text{max}}\text{-V}$ relationship along the x-axis (**Fig. 4C**) V_{half} the difference in V_{half} between CLR-free and CLR-containing was not significant, reaching 42.04 ± 4.94 and 34.57 ± 12.38 mV, respectively (**Fig. 4D**). In turn, a mild decrease in unitary current amplitude in CLR-containing bilayers was detected (**Fig. 4E**).

Finally, increasing Ca^{2+} levels to $850 \mu\text{M}$ sustained the CLR-driven decrease in slo1 Po, only between -40 and 0 mV (**Fig. 4F-G**). At this high level of Ca^{2+} CLR action did not result in a significant shift of the $\text{Po}/\text{Po}_{\text{max}}\text{-V}$ curve (**Fig. 4H**); averaged V_{half} values were very close, reaching -46.34 ± 12.61 and -39.85 ± 24.43 mV in CLR-free and CLR-containing bilayers, respectively (**Fig. 4I**). In addition, the CLR-induced decrease in unitary current amplitude that was observed within $1\text{-}100 \mu\text{M} \text{Ca}^{2+}$ was lost (**Fig. 4E versus J**). Collectively, our evaluation of CLR action on cbv1 function within a wide Ca^{2+} range ($0\text{-}850 \mu\text{M}$) shows that under the extreme points of this range CLR-induced reduction of cbv1 Po (robust) and i (mild) are lost whereas CLR action is maximal at the Ca^{2+}_i range of $10\text{-}100 \mu\text{M}$ (**Fig. 5**).

CLR-induced cbv1 inhibition is mediated by the high-affinity Ca^{2+} -sensing sites located in the channel subunit cytosolic tail domain. To begin to understand the structural basis of Ca^{2+} -CLR interactions on slo1 activity, we evaluated the effects of CLR on the activity of cbv1s

in which their Ca^{2+} -sensing site(s) were made nonfunctional by point mutagenesis and in the presence of Ca^{2+}_i levels that fully activated/saturated the site under analysis. Thus, CLR significantly decreased the NPo of the low-affinity RCK1 (E379A, E404A) mutant when evaluated at 30 μM Ca^{2+}_i (**Fig. 6**). In contrast, CLR-driven decrease in Po was lost in a construct with both high-affinity sites for Ca^{2+}_i being nonfunctional (RCK1:D367A, D372A, and RCK2:D899N, D900N, D901N, D902N, D903N) when recorded at 10 mM Ca^{2+}_i , a concentration fully able to activate slo1 through their low-affinity RCK1 site ([Zeng et al., 2005](#)). There was no significant CLR-induced decrease in unitary current amplitude detected in either the low- or high-affinity site cbv1 mutants. Notably, CLR was still able to decrease the NPo and unitary current amplitude of each individual high-affinity site mutant, i.e., RCK1:D367A, D372A, and RCK2:D899N, D900N, D901N, D902N, D903N when evaluated at 30 μM Ca^{2+}_i (**Fig. 7**). Collectively, evaluation of CLR action on these constructs indicates that CLR inhibition of cbv1 is coupled to activating levels of Ca^{2+}_i being recognized by either of the two high-affinity Ca^{2+}_i -sensing sites present in the cbv1 CTD.

Discussion

Present data demonstrate that CLR inhibition of cbv1 is selectively coupled to Ca^{2+}_i -driven gating; when cbv1 activity is driven by intrinsic/voltage-gating CLR becomes ineffective. Moreover, there is a narrow range of Ca^{2+}_i (10-30 μM) at which (a) this divalent fully activates slo1 through its Ca^{2+} -sensing high-affinity sites (Bao et al., 2002; 2004) and (b) CLR action is more robust and reaches significance across a wider voltage range. Robustness in CLR inhibition of slo 1 channels within this narrow Ca^{2+}_i range likely explains the previously reported high efficacy of 33 mol% CLR to reduce the Po of hslo1 (cloned from human brain; Crowley et al., 2003) and cbv1 themselves (Bukiya et al., 2011a) when expressed in a similar binary bilayer, as data were obtained at 10 and 50 μM Ca^{2+}_i . Current data also show that CLR modulation of gating is lost when Ca^{2+}_i gets close to 1 mM (850 μM), i.e., levels at which the high-affinity sites are saturated but cbv1 activation by Ca^{2+}_i can still be evoked by the low-affinity site present in the CTD (Zhang et al., 2001). Consistent with the lack of involvement of this site in CLR action, 10 mM Mg^{2+}_i neither endows slo1 channels with CLR sensitivity in the absence of Ca^{2+}_i (Supp. Fig. 2) nor modifies CLR action in the presence of optimal levels of Ca^{2+}_i (30 μM) (Supp. Fig. 3). Moreover, data from engineered slo1 reveal that CLR action, as reported for ethanol (Liu et al., 2008), involves *either* of the high-affinity Ca^{2+} -sensing sites being optimally activated by Ca^{2+}_i . Of note, while the RCK1 high-affinity site is much closer to the pore-gate region than RCK2, the logarithmic relationships between V_{half} and activating Ca^{2+}_i suggest that the electrical distances between the binding sites (and thus their functional coupling to the gate) are equivalent (Cui et al., 1997). Remarkably, both high-affinity sites are surrounded by hydrophobic residues on which CLR can potentially dock. In particular, Tyr450, one of the many CRAC domain central tyrosines that participate in CLR-sensing by slo1 (Singh et al., 2012) is located within the flexible interface between RCK1 and RCK2 (see below).

The requirement for an optimal Ca^{2+} level to enable CLR-induced cbv1 inhibition is open to several interpretations. One is that Ca^{2+} binding promotes a cbv1 conformation that enables access and accommodation of CLR into a CLR binding site(s). This role for Ca^{2+} , i.e., enabling accommodation of a modulatory agent into a “newly available” recognition site in the slo1 CTD has been demonstrated for ethanol and related n-alkanols. These alcohols dock onto a water-accessible site within a flexible interface positioned between the Ca^{2+} -sensors and the gate. When Ca^{2+} is bound, the ethanol site between Lys361 and Arg514 is revealed. In the Ca^{2+} -unbound state, however, spatial reorientation repositions Met909, which now prevents the ethanol molecule from accessing Lys361 for hydrogen bonding and eventual alcohol-induced increase in Po (Bukiya et al., 2014a). This ethanol-CLR analogy is particularly appealing as modulation of slo1 by these ligands shares many features: 1-minor, if any, effects on ion conduction at ligand concentrations that drastically change Po; 2-need of Ca^{2+} presence for ligand action; 3-coupling of ligand action to Ca^{2+} interaction with any of the two high-affinity, but not the low-affinity, Ca^{2+} -sensing sites in the CTD (current data and Liu et al., 2008).

In a second scenario, CLR could still bind to slo1 in absence of Ca^{2+} , yet the expansion of the gating ring driven by Ca^{2+} -binding that eventually leads to channel opening (Yuan et al., 2011; Hite et al., 2017; Jia et al., 2018) should introduce a conformational change that allows CLR binding to negatively feedback on Ca^{2+} binding itself and/or its effect on the gating ring. The precise location of CLR binding to cbv1 remains speculation. Although we did identify CLR-sensing areas (seven CRAC domains, with CRAC4 including Tyr450) in the slo1 CTD that contribute to CLR-induced cbv1 inhibition (Singh et al., 2012), it remains to be determined whether any of these CRACs actually provides CLR binding or, rather, participates in the CLR sensitivity of cbv1 as an allosteric modulatory site. The role of CRAC domains as CLR binding sites in proteins, including voltage-gated inwardly-rectifying K^+ channels, has been put into question (Rosenhouse-Dantsker et al., 2013; Bukiya and Dopico, 2017), and we cannot rule out

the existence of CLR recognition sites in the cbv1 CTD (e.g., Asn590-Phe1060) rather than the identified CRACs (Bukiya and Dopico, 2017). To complicate structural interpretations further, partial structural elements within different CRACs (or within their reverse sequence CARS; Fantini et al., 2019) with or without involvement of residues outside these motifs may work individually to conform CLR binding sites of low-affinity, which are usually of regulatory nature (Jian & Levitan, *in press*). At any rate, in either of these two scenarios, Ca^{2+} -recognition sites and the putative CLR-recognition sites in the CTD are different (current **Fig. 1**, and Yuan et al., 2011; Singh et al., 2012; Hite et al., 2017). Thus, activating physiological Ca^{2+}_i , inhibitory CLR and activatory n-alkanols should all be considered heterotropic ligands of slo1s.

CLR-induced slo1 inhibition, however, differs from that of other BK inhibitors; paxilline effect is more evident at low levels of Ca^{2+}_i and at negative transmembrane voltages (Sanchez and McManus, 1996; Zhou and Lingle, 2014). Likewise, lolitrem B inhibits hslo1 currents at $\text{Ca}^{2+}_i < 50$ nM (Imlach et al., 2011), which is Ca^{2+}_i too low to gate slo1 on its own. Current data show the opposite: CLR-induced inhibition is more robust as Ca^{2+} reaches activating (≥ 1 μM) concentrations for slo1. Moreover, at a given Ca^{2+} level, more depolarizing voltages, which confer higher P_o , enable a more robust inhibition of cbv1 by CLR (**Figs. 3B-G, 4B-G**). Thus, CLR decreases cbv1 P_o *via* a significant interaction with the channel open state(s). This hypothesis finds support from re-analysis of our earlier data documenting that CLR 33 mol% robustly decreases mslo1 P_o due to not only a major increase in mean closed time (x16 times) but also a *major decrease in mean open time* (34% of control) (Crowley et al., 2003). Likewise, 10-30% w/w CLR decreases the mean open time of rat brain BK channels reconstituted into POPE/POPS (55/45 w/w) to less than 50% of control (Chang et al., 1995).

Considering that CLR inhibition of slo1 involves a major destabilization of the channel open state(s) and increases as Ca^{2+}_i activates these channels, it is remarkable that CLR-action begins to attenuate as Ca^{2+}_i reaches 100 μM and, furthermore, vanishes in the presence of sub-

mM Ca^{2+}_i (i.e., 850 μM) (**Fig. 4F-J**). Ca^{2+}_i at 10-100 μM activates and saturates both high-affinity Ca^{2+} -sensing sites while Ca^{2+} exceeding ~ 1 mM starts occupying the low-affinity site with eventual channel activation (Xia et al., 2002; Bao et al., 2004; Zeng et al., 2005). Thus, the affinity limits of the high-affinity Ca^{2+} -sensing sites match the Ca^{2+}_i -dependence of CLR action on *wt cbv1* (**Fig. 3**). As Ca^{2+} occupancy of the low-affinity sites starts to take place ($[\text{Ca}^{2+}]_{\text{free}} > \sim 1$ mM), the ability of CLR to decrease P_o and i tend to vanish. A simple explanation for the reduction of CLR action as Ca^{2+}_i goes from 10 to 850 μM is that Ca^{2+}_i binding to the *cbv1* low-affinity Ca^{2+} site increases *cbv1* activity and thus, counteracts the CLR inhibitory action that is coupled to Ca^{2+}_i binding to the *cbv1* high-affinity Ca^{2+}_i sites. However, data demonstrate that (a) CLR does not activate the channel when the two high-affinity Ca^{2+}_i sites are nonfunctional (**Fig. 6D**) and (b) CLR inhibitory action is not amplified in the low-affinity Ca^{2+}_i site mutant (**Fig. 6A-C versus Fig. 3F-J**). At $\text{Ca}^{2+}_i > 10$ μM , however, both recombinant (*mslo1*) and native BK channels enter a low P_o mode, equivalent to a desensitized state(s) (Rothberg et al., 1996; Liu et al., 2008). The lack of CLR action at hundredths of μM Ca^{2+}_i thus could reflect the insensitivity of this channel state(s) to CLR. If so, CLR and ethanol actions on *slo1* channel gating drastically differ: ethanol inhibition of *mslo1* at $\text{Ca}^{2+}_i > 10$ μM is driven by favoring channel entry into this Ca^{2+} -desensitized state(s) (Liu et al., 2008).

We should point out that, as observed for data obtained at 850 μM Ca^{2+} , CLR-induced *slo1* inhibition was not significant at maximal voltages (> 40 mV) when Ca^{2+} levels were optimal to facilitate CLR action (10-30 μM Ca^{2+}_i). At these Ca^{2+}_i levels and voltages *cbv1* activity reaches maximal levels (**Figs. 3B-G, 4B-G**). Thus, there seems to be an optimal level of *cbv1* activity and/or allosteric coupling between Ca^{2+}_i - and voltage-gating that favors CLR-inhibition of *cbv1*. According to the “lipid-dependent gating” theory, CLR, as a type II lipid, would be expected to disrupt *slo1* voltage-sensor activation, as reported for its relative the KvAP channel (Jiang, 2019). The individual parameters that underlie the Ca^{2+} -dependence of CLR on *slo1* can

be obtained through the Horrigan-Aldrich allosteric gating model (Horrigan and Aldrich, 2002) which requires varied voltage-protocols, some of which not sustainable in a bilayer system. Based on present data, a simplistic model for the CLR- Ca^{2+}_i functional interaction on slo1 channels is given in **Fig. 8**.

The CLR- Ca^{2+}_i -slo1 interaction reported here occurs at (a) CLR molar fractions found in native membranes (van Meer et al., 2008; Slayden et al., 2020; Jian and Levitan, in press) and to modulate BK (Dopico et al., 2012; Bukiya and Dopico, 2019), and (b) at physiological Ca^{2+}_i that gates BK channels: in cerebrovascular myocytes, the activity of sarcoplasmic ryanodine receptors generates local Ca^{2+} sparks which, given the close vicinity of membrane BK channels and sarcoplasmic ryanodine receptors in these cells, raises Ca^{2+} to 4-30 μM *in the vicinity of the BK channel's Ca^{2+} -sensors* (Pérez et al., 2001). The final effect of CLR on BK channel function, however, is drastically modified by the presence of regulatory β_1 subunits, which are highly abundant in cerebrovascular smooth muscle (Kuntamallappanavar et al., 2017; North et al., 2018): CLR enrichment of middle cerebral arteries leads to a trafficking-dependent increase in membrane levels of BK β_1 , a process that overrides CLR direct inhibition of slo1 channels and leads to an actual increased in BK currents (Bukiya et al., 2021). The gating underpinnings of CLR- β_1 antagonism on slo1 channel function remain to be determined, although it is worthy to mention that β_1 regulation of slo1 channel activity is significantly linked to Ca^{2+} -driven gating (Meera et al., 1996; Cox and Aldrich, 2000; Orio et al., 2002; Morrow et al., 2006). Present findings, however, make it clear that CLR inhibition of BK channels will be particularly relevant in tissues that poorly express β_1 , whether naturally or under pathological conditions. Regarding vascular smooth muscle, it is noteworthy that prevalent pathologies wherein abnormal myogenic tone and vasodilation have been reported, such as arterial hypertension and diabetes, are characterized by a decreased expression of BK β_1 (Table I in Dopico et al., 2018) and increased CLR tissue levels.

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Authorship Contributions:

Participated in research design: North, Bukiya, Dopico,

Conducted experiments: North, Zhang, Singh, Zaytseva, Slayden

Contributed new reagents or analytic tools: Bukiya, Dopico

Performed data analysis: North, Zhang, Singh, Zaytseva, Bukiya

Wrote or contributed to the writing of the manuscript: North, Zhang, Slayden, Bukiya, Dopico

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Figure Legends.

Figure 1. Schematic lateral view of the plasmalemma including four BK channel-forming slo1 proteins. Each slo1 (also known as BK channel α subunit) contains a voltage sensor domain (VSD), a central pore–gate domain (PGD) and a long C-terminus of intracellular location termed cytosolic tail domain (CTD). Each CTD contains two Regulators of Conductance for K^+ (RCK) domains. RCK1 (in blue) includes binding sites for Ca^{2+} : a low-affinity Ca^{2+} -sensing site (pink amino acid structures; highlighted by pink star) and a high-affinity Ca^{2+} -sensing site (red amino acid structures; highlighted by red stars). The RCK2 domain (in orange) includes another high-affinity Ca^{2+} -sensing site (green amino acid structures; highlighted by green star). VSD and CTD are connected to the PGD by linkers and through domain-domain interface contacts. The mutations conducted are presented according to the hslo1 amino acid sequence ([Yuan et al., 2010](#)) and color coded to the high- and low-affinity sites. Thus, slo1 mutations are: RCK1 high-affinity: D362A, D367A; RCK1 low-affinity: E374A, E399A; RCK2 high-affinity: D897A, D898A, D899A, D900A, D901A.

Figure 2. CLR fails to inhibit slo1 channel in absence of Ca^{2+}_i or at low micromolar Ca^{2+}_i .

A. Representative channel recordings obtained after incorporation of slo1 into CLR-free (left column) versus CLR-containing (right column) POPE/POPS (3:1 wt/wt) bilayers bathed by Ca^{2+} -free bilayer recording solutions. Channel openings are shown as upward deflections; arrows indicate the baseline level (all channels closed). B. Averaged data showing open probability (P_o) as a function of transmembrane voltage in CLR-free versus CLR-containing bilayers; data are shown as mean $\pm$ standard deviation. C. Scattered data showing unitary current amplitude values in CLR-free versus CLR-containing bilayers at varying transmembrane voltages; data are shown as mean $\pm$ standard deviation. D. Representative channel recordings obtained after

incorporation of slo1 into CLR-free (left column) versus CLR-containing (right column) POPE/POPS (3:1 wt/wt) bilayers bathed by bilayer recording solution containing 1 μM free Ca^{2+} . Channel openings are shown as upward deflections; arrows indicate the baseline level (channels closed). E. Averaged data showing P_o as a function of transmembrane voltage in CLR-free versus CLR-containing bilayers; data are shown as mean $\pm$ standard deviation. F. Scattered data showing unitary current amplitude values in CLR-free versus CLR-containing bilayers at varying transmembrane voltages; data are shown as mean $\pm$ standard deviation.

Figure 3. CLR-induced slo1 channel inhibition in presence of 10 and 30 μM Ca^{2+} . A. Representative channel recordings obtained after incorporation of slo1 into CLR-free (left column, two channels are incorporated) versus CLR-containing (right column, one channel is incorporated) POPE/POPS (3:1 wt/wt) bilayers. Bilayers are bathed by recording solutions with 10 μM free Ca^{2+} . Channel openings are shown as upward deflections; arrows indicate the baseline level (channels closed). B. Averaged data showing P_o as a function of transmembrane voltage in CLR-free versus CLR-containing bilayers; data are shown as mean $\pm$ standard deviation. *Statistically significant difference from CLR-free bilayers, $p < 0.05$ by a one-tail Mann-Whitney test. C. Averaged data showing $P_o/P_{o_{\text{max}}}$ as a function of transmembrane voltage in CLR-free versus CLR-containing bilayers. D. Scattered data showing V_{half} of individual bilayers; data are shown as mean $\pm$ standard deviation. *Statistically significant difference from CLR-free bilayers, $p = 0.0465$ by a one-tail t-test. E. Scattered data showing unitary current amplitude values in CLR-free versus CLR-containing bilayers at varying transmembrane voltages at 10 μM free Ca^{2+} ; data are shown as mean $\pm$ standard deviation. F. Representative channel recordings obtained after incorporation of slo1 into CLR-free (left column, two channels are incorporated) versus CLR-containing (right column, three channels are incorporated) POPE/POPS (3:1 wt/wt) bilayers bathed by bilayer recording solutions with 30 μM free Ca^{2+} .

Channel openings are shown as upward deflections; arrows indicate the baseline level (channels closed). G. Averaged data showing P_o as a function of transmembrane voltage in CLR-free versus CLR-containing bilayers; data are shown as mean $\pm$ standard deviation. *Statistically significant difference from CLR-free bilayers, $0.010 < p < 0.050$; #Statistically significant difference from CLR-free bilayers, $0.003 < p < 0.010$ (one-tail Mann-Whitney test). H. Averaged data showing $P_o/P_{o_{max}}$ as a function of transmembrane voltage in CLR-free versus CLR-containing bilayers; data are shown as mean $\pm$ standard deviation. I. Scattered data showing V_{half} s of individual bilayers; data are shown as mean $\pm$ standard deviation. #Statistically significant difference from CLR-free bilayers, $p=0.0055$ by a one-tail Mann-Whitney test. J. Scattered data showing unitary current amplitude values in cholesterol-free vs. cholesterol-containing bilayers at varying transmembrane voltages; data are shown as mean $\pm$ standard deviation.

Figure 4. CLR-induced slo1 channel inhibition in presence of 100 and 850 μ M Ca^{2+} . A. Representative channel recordings obtained after incorporation of cbv1 into CLR-free (left column, two channels are incorporated) versus CLR-containing (right column, one channel is incorporated) POPE/POPS (3:1 wt/wt) bilayers bathed by bilayer recording solutions with 100 μ M free Ca^{2+} . Channel openings are shown as upward deflections; arrows indicate the baseline level (channels closed). B. Averaged data showing P_o as a function of transmembrane voltage in CLR-free versus CLR-containing bilayers at 100 μ M free Ca^{2+} ; data are shown as mean $\pm$ standard deviation. *Statistically significant difference from CLR-free bilayers, $p=0.036$; #Statistically significant difference from CLR-free bilayers, $0.0003 < p < 0.010$ (one-tail Mann-Whitney test). C. Averaged data showing $P_o/P_{o_{max}}$ as a function of transmembrane voltage in CLR-free versus CLR-containing bilayers at 100 μ M free Ca^{2+} ; data are shown as mean $\pm$ standard deviation. D. Scattered data showing V_{half} s of individual bilayers at 100 μ M free

Ca²⁺. E. Scattered unitary current amplitude values in CLR-free versus CLR-containing bilayers at varying transmembrane voltages at 100 μ M free Ca²⁺; data are shown as mean $\pm$ standard deviation. *Statistically significant difference from CLR-free bilayers, 0.010<p<0.050; #Statistically significant difference from CLR-free bilayers, 0.007<p<0.010 (one-tail Mann-Whitney test). F. Representative channel recordings obtained after incorporation of slo1 into CLR-free (left column, three channels are incorporated) versus CLR-containing (right column, one channel is incorporated) POPE/POPS (3:1 wt/wt) bilayers bathed by bilayer recording solutions with 850 μ M free Ca²⁺. Channel openings are shown as upward deflections; arrows indicate the baseline level (channels closed). G. Averaged data showing Po as a function of transmembrane voltage in CLR-free versus CLR-containing bilayers; data are shown as mean $\pm$ standard deviation. *Statistically significant difference from CLR-free bilayers, 0.015<p<0.050; #Statistically significant difference from CLR-free bilayers, 0.008<p<0.010 (one-tail Mann-Whitney test). H. Averaged data showing Po/Po_{max} as a function of transmembrane voltage in CLR-free versus CLR-containing bilayers; data are shown as mean $\pm$ standard deviation. I. Scattered data showing V_{half}s of individual bilayers; data are shown as mean $\pm$ standard deviation. J. Scattered unitary current amplitude values in CLR-free versus CLR-containing bilayers at varying transmembrane voltages; data are shown as mean $\pm$ standard deviation.

Figure 5. CLR-induced inhibition of BK channels requires specific Ca²⁺ levels. Averaged data showing Ca²⁺ dependency of CLR-driven increase in V_{half}; data are shown as mean $\pm$ standard deviation. *Statistically significant difference from CLR-free bilayers, p=0.0465 by a one-tail t-test; #Statistically significant difference from CLR-free bilayers, p=0.0055 by a one-tail Mann-Whitney test.

Figure 6. A. Representative channel recordings obtained after incorporation of the low-affinity Ca^{2+} mutant, (RCK1: E379A, E404A) into CLR-free (left column, three channels are incorporated) versus CLR-containing (right column, two channels are incorporated) POPE/POPS (3:1 wt/wt) bilayers bathed by bilayer recording solutions with 30 μM free Ca^{2+} . Channel openings are shown as upward deflections; arrows indicate the baseline level (channels closed). B. Averaged data showing P_o as a function of transmembrane voltage in CLR-free versus CLR-containing bilayers at 30 μM free Ca^{2+} ; data are shown as mean $\pm$ standard deviation. *Statistically significant difference from CLR-free bilayers, $p=0.04$; $p=0.039$ (one-tail Mann-Whitney test). C. Scattered unitary current amplitude values in CLR-free versus CLR-containing bilayers at varying transmembrane voltages at 30 μM free Ca^{2+} ; data are shown as mean $\pm$ standard deviation. D. Representative channel recordings obtained after incorporation of both high-affinity Ca^{2+} site mutant (RCK1:D367A, D372A, and RCK2:D899N, D900N, D901N, D902N, D903N) into CLR-free (left column, two channels are incorporated) versus CLR-containing (right column, two channels are incorporated) POPE/POPS (3:1 wt/wt) bilayers bathed by bilayer recording solutions with 10 mM free Ca^{2+} . Channel openings are shown as upward deflections; arrows indicate the baseline level (channels closed). E. Averaged data showing P_o as a function of transmembrane voltage in CLR-free versus CLR-containing bilayers; data are shown as mean $\pm$ standard deviation. F. Scattered unitary current amplitude values in CLR-free versus CLR-containing bilayers at varying transmembrane voltages; data are shown as mean $\pm$ standard deviation.

Figure 7. A. Representative channel recordings obtained after incorporation of the RCK1 high-affinity Ca^{2+} site mutant (RCK1: D367A, D372A) into CLR-free (left column, two channels are incorporated) versus CLR-containing (right column, two channels are incorporated) POPE/POPS (3:1 wt/wt) bilayers bathed by bilayer recording solutions with 30 μM free Ca^{2+} .

Channel openings are shown as upward deflections; arrows indicate the baseline level (channels closed). B. Averaged data showing P_o as a function of transmembrane voltage in CLR-free versus CLR-containing bilayers at 30 μM free Ca^{2+} ; data are shown as mean $\pm$ standard deviation. *Statistically significant difference from CLR-free bilayers, $p=0.045$; $p=0.030$; $p=0.024$; $p=0.019$ (one-tail Mann-Whitney test). C. Scattered unitary current amplitude values in CLR-free versus CLR-containing bilayers at varying transmembrane voltages at 30 μM free Ca^{2+} ; data are shown as mean $\pm$ standard deviation. *Statistically significant difference from CLR-free bilayers, $p=0.003$, $p=0.011$, $p=0.021$, $p=0.027$, $p=0.044$. D. Representative channel recordings obtained after incorporation the RCK2 high-affinity Ca^{2+} site mutant (D899N, D900N, D901N, D902N, D903N) into CLR-free (left column, one channel is incorporated) versus CLR-containing (right column, one channel is incorporated) POPE/POPS (3:1 wt/wt) bilayers bathed by bilayer recording solutions with 30 μM free Ca^{2+} . Channel openings are shown as upward deflections; arrows indicate the baseline level (channels closed). E. Averaged data showing P_o as a function of transmembrane voltage in CLR-free versus CLR-containing bilayers; data are shown as mean $\pm$ standard deviation. *Statistically significant difference from CLR-free bilayers, $p=0.029$, $p=0.038$. F. Scattered unitary current amplitude values in CLR-free versus CLR-containing bilayers at varying transmembrane voltages; data are shown as mean $\pm$ standard deviation. *Statistically significant difference from CLR-free bilayers, $p=0.02$, $p=0.015$.

Figure 8. Cartoon illustrating the authors' interpretation of CLR modulation of slo1 channel activity. A. A lateral view of the channel is shown as a dimer of slo1 subunits, with their relevant domains shown only in one monomer (right): the "core" (conducting and voltage-sensing domains) is shown in blue; the RCK1 domain is in pink; the Ca^{2+} bowl associated with the RCK2 is in yellow. At lower Ca^{2+}_i , the equilibria from low-conducting via intrinsic gating (A) to high

conducting states due to Ca^{2+} -sensing by the Ca^{2+} -bowl (B) or by the RCK1 high-affinity site (C) are regulated by CLR leading to decreased P_o (black arrow pointing down). The RCK1 high-affinity site sensing of higher $[\text{Ca}^{2+}_i]$ makes the channel dwell in a low-activity mode (D), this state being largely refractory to CLR modulation, leading to no change in overall P_o by this lipid. In addition, CLR does not modify gating involving the RCK1 low-affinity site, which is involved in sensing high μM - mM divalents, whether Ca^{2+} or Mg^{2+} (E). Dark blue arrows: outward K^+ flow; plus symbols: voltage-sensor; circular “pockets”: RCK1 low-affinity Ca^{2+} site; triangular notch: high-affinity Ca^{2+} sites in RCK1 or “bowl”; brown circles: Mg^{2+} ; red drops: Ca^{2+} .

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Figure 1

Figure 2

10 μM $[\text{Ca}^{2+}]$

30 μM $[\text{Ca}^{2+}]$

Figure 3

Figure 4

A

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Figure 5

Figure 6

Figure 7

Figure 8