Cholesterol depletion alters amplitude and pharmacology of vascular calcium-activated chloride channels

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Aims

Calcium-activated chloride channels (CACCs) share common pharmacological properties with Kcnma1-encoded large conductance K⁺ channels (BKCa or KCa1.1) and it has been suggested that they may co-exist in a macromolecular complex. As KCa1.1 channels are known to localize to cholesterol and caveolin-rich lipid rafts (caveolae), the present study investigated whether Ca²⁺-sensitive Cl⁻ currents in vascular myocytes were affected by the cholesterol depleting agent methyl-β-cyclodextrin (M-βCD).

Methods and results

Calcium-activated chloride and potassium currents were recorded from single murine portal vein myocytes in whole cell voltage clamp. Western blot was undertaken following sucrose gradient ultracentrifugation using protein lysates from whole portal veins. Ca²⁺-activated Cl⁻ currents were augmented by 3 mg mL⁻¹ M-βCD with a rapid time course (t₀.5 = 1.8 min). M-βCD had no effect on the bi-modal response to niflumic acid or anthracene-9-carboxylate but completely removed the inhibitory effects of the KCa1.1 blockers, paxilline and tamoxifen, as well as the stimulatory effect of the KCa1.1 activator NS1619. Discontinuous sucrose density gradients followed by western blot analysis revealed that the position of lipid raft markers caveolin and flotillin-2 was altered by 15 min application of 3 mg mL⁻¹ M-βCD. The position of KCa1.1 and the newly identified candidate for CACCs, TMEM16A, was also affected by M-βCD.

Conclusion

These data reveal that CACC properties are influenced by lipid raft integrity.

Keywords

Calcium-activated chloride channels • Vascular smooth muscle • TMEM16A • KCa1.1 • Lipid raft

1. Introduction

Calcium-activated chloride channels (CACCs) underpin various physiological activities including smooth muscle contraction, secretion, neuronal firing, and cardiomyocyte depolarization. Study of CACCs has always been plagued by the molecular identity being unknown, the lack of truly selective tools and the complicated interaction of so-called chloride channel blockers with native CACCs. Recent work has revealed a remarkable pharmacological overlap between calcium-activated chloride currents (IClCa) and the large conductance, calcium-activated potassium channel (BKCa or KCa1.1). Thus, a wide range of structurally disparate agents considered to be chloride channel blockers, such as niflumic acid (NFA), anthracene-9-carboxylate (A-9-C) and ethacrynic acid, enhance KCa1.1 currents. Furthermore, in vascular myocytes, the structurally different activators of KCa1.1 channels, NS1619 and isopimaric acid, augment IClCa, and the KCa1.1 blockers paxilline, penitrem A, and iberiotoxin inhibit IClCa. Moreover, tamoxifen, which blocks IClCa, either blocks or activates KCa1.1 channels depending on the presence of a β- auxiliary subunit. In contrast, positive and negative modulators of small and intermediate conductance, Ca²⁺-activated K⁺ channels (KCa2.1 and KCa2.3) do not affect IClCa. These observations led to the postulate that CACCs and KCa1.1 contained a common structural motif or co-existed in a sufficiently narrow micro-domain to allow inter-channel activity.

Much evidence is now available for the compartmentalization of protein complexes into highly organized cholesterol- and sphingolipid-enriched structures within the plasma membrane,
2. Methods

For detailed Methods see Supplementary material online, Methods.

2.1 Tissue collection

BALB/c mice (6–8 weeks) were sacrificed by cervical dislocation in accordance with schedule 1 of the UK Animals Act (1986) and conforms with the Guide and Care of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, 1996).

2.2 Cell dissociation

Individual smooth muscle myocytes were isolated from strips of mouse portal vein (PV) by incubation at 37°C for 20 min with 1.5 mg mL−1 collagenase XI followed by 10 min with 1 mg mL−1 protease XIV, followed by gentle trituration with a wide bore fire polished pipette.

2.3 Electrophysiology

Macroscopic I_{ClCa} were recorded from a total of 85 cells from 46 different mice using pipette solutions containing 106 mM CsCl, 20 mM TEA, 3 mM Na2ATP, 0.2 mM GTP-Na, 10 mM HEPES, 10 mM BAPTA, 1.1 mM MgCl2, and a sustained Ca2+ channel activation evoked by a fixed free [Ca2+] of 500 nM obtained through the addition of 7.8 mM CaCl2 as calculated by EqCaI (Biosoft, Ferguson, MO, USA). The external solution contained 126 mM NaCl, 11 mM glucose, 10 mM HEPES, 10 mM TEA-Cl, 1.2 mM MgCl2, and 1.5 mM CaCl2 (pH was adjusted to 7.2 with NaOH). I_{ClCa} were recorded at different potentials using protocols described in Figure 1. Potassium currents (I_k) were recorded in cells bathed in a solution containing 136 mM NaCl, 5 mM KCl, 11 mM CaCl2. The pipette solution consisted of 126 mM KCl, 10 mM HEPES, 3 mM Na2ATP, 0.2 mM GTP-Na, 10 mM BAPTA, 1.2 mM MgCl2, and a fixed free [Ca2+] of 250 nM obtained through the addition of 6.4 mM CaCl2. Cells were held at −50 mV and I_k were recorded at different potentials by application of voltage ramps (200 mV s−1) from −100 to +100 mV every 15 s. All experiments were performed at room temperature.

2.4 Cholesterol modifying agents

Once I_{ClCa} had stabilized the role of lipid rafts was determined by application of M-βCD (0.03–3 mg mL−1), a cholesterol chelating agent which enhances aqueous solubility of cholesterol, as described previously. M-βCD pre-bound to cholesterol was used to rule out possible cholesterol independent effects.

2.5 Lipid raft isolation

Portal veins (PV) from 30 mice were crushed in liquid nitrogen and homogenized in 1 ml lysis buffer (Sigma-Aldrich; Poole, UK) containing 1% Triton X-100. One half of the total lysate was incubated with 3 mg mL−1 M-βCD for 15 min, whereas the other half was incubated with the appropriate vehicle. Both lysates were fractionated using the Caveolae/Raft Isolation Kit (Sigma-Aldrich). A discontinuous sucrose gradient (0–40%) was constructed using different volumes of OptiPrep and centrifuged at 200 000 g for 4 h at 4 °C. After centrifugation, 12 fractions of 1 mL were collected from the top (fraction 1) to the bottom (fraction 12) of the tube and stored at −20°C until required for western blot analysis.

2.6 Western blots

Protein samples were denatured at 95°C for 5 min in the presence of reducing agent (Inviron, UK), loaded onto a pre-cast sodium dodecyl sulphate-polyacrylamide gel (4–12% Bis-Tris, Invitrogen, UK), subjected to electrophoresis and then transferred onto PVDF membranes (Amersham Biosciences). The membranes were then probed for the lipid raft marker proteins caveolin (pan-caveolin24 1:10 000; BD Biosciences) and flotillin-21 2:12 000; BD Biosciences), the non-lipid raft protein marker β-adaptin25 (1:1500; Santa Cruz), K Ca1.1 (1:200; Alomone), and TMEM16A (ab53213; Abcam; a 1:5 dilution of a preludited form). Protein bands were visualized using ECL (Thermo Scientific) and hyperfilm (Amersham Bioscience). All antibodies had been tested to determine effective concentrations and non-specific effects on samples of whole heart and whole PV in previous experiments (data not shown). Owing to low levels of protein, SignalBoost Immunoreaction Enhancer (Calbiochem; Nottingham, UK) was used with the anti-flotillin-2 antibody; it was not suitable/required for use with the other antibodies used in this investigation.

2.7 Statistical analysis

All data are means ± SEM taken from at least three animals. Statistical comparison was performed between the stable response observed prior to exposure to modulators (t = 0) and that obtained in the presence of modulators using either paired Student’s t-test or ANOVA. All drugs were purchased from Sigma-Aldrich unless otherwise indicated.

3. Results

I_{ClCa} evoked by pipette solutions containing 500 nM free Ca2+ exhibited a rapid rundown immediately after membrane rupture and then remained constant for the duration of the experiment. At —50 mV, the mean inward current was —1.6 ± 0.2 pA pF−1 (n = 7) and depolarization to +70 mV yielded currents with distinctive outward kinetics (Figure 1A) that are characteristic of I_{ClCa} recorded by this technique. The current at +70 mV increased from a mean level of 2.4 ± 0.2 pA pF−1 immediately after depolarization to 10.6 ± 1.5 pA pF−1 after 750 ms with a t_{rise} of 199 ± 13 ms (n = 10). Repolarization to —80 mV evoked an immediate inward current of —13.8 ± 1.9 pA pF−1 (t —80 ms) which decayed to —1.9 ± 0.3 pA pF−1 with a t_{close} of 54 ± 3 ms.

3.1 Effect of M-βCD on native Ca2+-activated Cl− currents in mPV myocytes

Application of M-βCD (3 mg mL−1) rapidly augmented I_{ClCa} (Figure 1A) with half maximal enhancement (I_0.5) occurring after 1.8 min (Figure 1D). Figure 1B shows that augmentation of I_{ClCa} was
also observed with 0.3 mg mL⁻¹ M-βCD but not lower concentrations (0.03–0.1 mg mL⁻¹, n ≥ 3). In contrast, application of 3 mg mL⁻¹ M-βCD pre-bound with an equivalent concentration of cholesterol did not produce any changes in ICaCl that were significant from vehicle effects (n = 6; Figure 1D and E). Application of 3 mg mL⁻¹ M-βCD increased the current at +50 mV from 1.6 ± 0.2 pA pF⁻¹ to 1.9 ± 0.3 pA pF⁻¹ (n = 7; P < 0.05) and at all test potentials (Figure 1E, significance only at +120 mV shown for clarity). Maximal extrapolated whole-cell conductance increased from 21.1 to 27.8 nS, whereas Vₐ₅ decreased from 113 to 93 mV (Figure 1F). The stimulatory effects of M-βCD were not reversed upon removal from the bath solution for up to 15 min (n = 3). Thus, KCa1.1 channels in vascular myocytes are inhibited by cholesterol depletion.

### 3.2 Effect of M-βCD on native Ca²⁺-activated K⁺ currents in mPV myocytes

Experiments were performed to see if M-βCD also modulated large conductance KCa1.1 currents in mPV myocytes similar to previous findings in uterine smooth muscles cells and gliomas. As Figure 2A shows application of a depolarizing voltage ramp with pipette solutions containing [Ca²⁺] fixed at 250 nM evoked an outwardly rectifying Iₓ superimposed by considerable current fluctuations at positive potentials that was inhibited by the KCa1.1 channel blocker 1 µM paxilline by 80 ± 2% (P < 0.05; n = 4). Application of M-βCD (3 mg mL⁻¹) reduced Iₓ (Figure 2A and B) with a time course similar to the effect on ICaCl (t₀.₅ = 2.7 min, Figure 2C), which was not manifest in cells pre-treated with paxilline (Figure 2B). The effect of M-βCD on Iₓ was also maintained after washout of the cyclodextrin (n = 3). Thus, KCa1.1 channels in vascular myocytes are inhibited by cholesterol depletion.
3.3 Effects of Cl\textsuperscript{−} channel blockers on \(I_{\text{ClCa}}\) in the presence of M-\(\beta\)CD

Experiments were undertaken with the Cl\textsuperscript{−} channel blockers NFA and A-9-C to assess whether disruption of cholesterol levels affected the ability of these chloride channel blockers to modify \(I_{\text{ClCa}}\). In vascular myocytes, these agents produce complex effects on sustained \(I_{\text{ClCa}}\), which is manifest as a significant but not complete inhibition at positive potentials and a paradoxical stimulation of \(I_{\text{ClCa}}\) at negative potentials.\textsuperscript{3–5,9} Figure 3 shows that NFA (100 \(\mu\)M) produced effects, comparable to those observed previously in mPV myocytes by increasing holding current at \(-50\) mV, inhibiting late outward current and increasing inward current upon repolarization to \(-80\) mV (\(n = 4\); Figure 3Ai). Similar effects were observed in the presence of M-\(\beta\)CD (Figure 3Aii). In the absence of M-\(\beta\)CD, NFA evoked a decrease in the outward time-dependent current of 2.6 ± 1.3 pA pF\textsuperscript{−1}, whereas in the presence of M-\(\beta\)CD, a decrease of 3.9 ± 0.8 pA pF\textsuperscript{−1} was observed. In addition, the inward current at \(-80\) mV in the presence of NFA was larger in M-\(\beta\)CD (Figure 3Aiii). Both observations reflect a heightened activation of the underlying CACCs. Application of A-9-C (500 \(\mu\)M) produced effects similar to those previously reported in the rabbit pulmonary artery\textsuperscript{4} that were manifest as a reduction in current at \(+70\) mV and a marked augmentation of the current recorded upon repolarization to \(-80\) mV (\(n = 3\); Figure 3Bi), which were also apparent after application of 3 mg mL\textsuperscript{−1} M-\(\beta\)CD (\(n = 4\); Figure 3Bii). However, the enhancement of the current at \(-80\) mV produced by A-9-C increased from 6.1 ± 1.2 to 8.8 ± 3.8 pA pF\textsuperscript{−1} in M-\(\beta\)CD (\(n = 4\); Figure 3Biii) again consistent with an increase in the availability of the underlying CACC. These data show that the distinctive bimodal effects of NFA and A-9-C on sustained \(I_{\text{ClCa}}\) were maintained after incubation with M-\(\beta\)CD.

3.4 Effects of \(K_{\text{Ca1.1}}\) modulators on \(I_{\text{ClCa}}\) in the presence of M-\(\beta\)CD

We have recently shown that the \(K_{\text{Ca1.1}}\) inhibitor paxilline and the \(K_{\text{Ca1.1}}\) activator NS1619 modulate \(I_{\text{ClCa}}\).\textsuperscript{9,10} The observation, in the present study, that \(I_{\text{ClCa}}\), as well as \(K_{\text{Ca1.1}}\) current, are affected by M-\(\beta\)CD suggests that the pharmacological cross over could stem from a close physical interaction between the Cl\textsuperscript{−} channel and \(K_{\text{Ca1.1}}\) in the same microdomain. Consequently, disruption of the local environment by M-\(\beta\)CD might alter the ability of \(K_{\text{Ca1.1}}\) channel modulators to affect \(I_{\text{ClCa}}\). Similar to our previous report,
\textsuperscript{10} 1 \(\mu\)M paxilline reduced \(I_{\text{ClCa}}\) markedly at all potentials but did not affect the reversal potential of the evoked current (\(n = 4\); Figure 4Al–Aii). In stark contrast, paxilline had no effect on \(I_{\text{ClCa}}\) in the presence of M-\(\beta\)CD (3 mg mL\textsuperscript{−1}; \(n = 4\); Figure 4Bi–Bii). Moreover, as Figure 4C shows addition of M-\(\beta\)CD (3 mg mL\textsuperscript{−1}) reversed completely the inhibition of \(I_{\text{ClCa}}\) produced by paxilline with a time course similar to the stimulatory effect of M-\(\beta\)CD alone (\(\tau = 2.1\) min, Figure 4Civ, \(n = 4\)). M-\(\beta\)CD did not reverse the inhibitory
Figure 3  Effect of NFA and A-9-C in the presence of M-βCD. (A) Effect of 100 μM NFA on I_{ClCa} in the absence and presence of 3 mg mL^{-1} M-βCD. (Ai and Aii) Representative traces. (Aiii) Effect of NFA on I_{ClCa} amplitude recorded at different potentials, using the reversal protocol in Figure 1G, in the absence and presence of 3 mg mL^{-1} M-βCD (see insert). *P < 0.05 for paired Student’s t-test comparisons between data acquired prior to and post-application of NFA at the indicated step. (Bi and Bii) Representative traces showing effects of 500 μM A-9-C in the absence and presence of 3 mg mL^{-1} M-βCD, respectively. (Biii) Mean effect of A-9-C on I_{ClCa} amplitude recorded at different potential, using the reversal protocol in Figure 1G, in the absence and presence of 3 mg mL^{-1} M-βCD (see insert). Each point comprises data from at least four cells with error bars depicting SEM.

Figure 4  M-βCD modulates the effect of paxilline on I_{ClCa}. (A) Effect of 10 μM paxilline (Pax) on I_{ClCa} (Ai) Representative currents recorded in the absence and presence of paxilline (5 min). (Aii and Aiii) I–V relationships and reversal potential of I_{ClCa} in the absence (open circle) and presence of paxilline (filled circle). (B) Effect of 10 μM paxilline on I_{ClCa} in the absence (open square) and presence of 3 mg mL^{-1} M-βCD (filled square). (C) Reversal of paxilline modulation by M-βCD. (Ci) Representative traces, (Cii) I–V relationships, (Ciii) Reversal potentials of I_{ClCa} and (Civ) Time course for the effect of M-βCD in the continued presence of 10 μM paxilline. In each graph, control currents are (open circle) + paxilline (open triangle) and paxilline plus M-βCD (filled triangle). Each point comprises data from at least four cells from different animals with error bars depicting SEM.
Figure 5 Effect of tamoxifen and NS1619 in the presence of M-βCD. (A) Representative currents and the I–V relationship recorded in the absence (open circle) and presence of tamoxifen (5 min; filled circle) in the absence of M-βCD. (B) Recordings performed in the absence (open square) and presence of tamoxifen (filled square) in cells pre-incubated in 3 mg mL⁻¹ M-βCD. (C) Representative traces and the I–V relationship recorded prior to (open triangle) and post-exposure to NS1619 (filled triangle) min the absence of M-βCD. (D) In the presence of 3 mg mL⁻¹ M-βCD, traces and the I–V relationship recorded before (inverted triangle) and after exposure to NS1619 (filled inverted triangle). Each point comprises data from at least three cells with error bars depicting SEM. *P < 0.05 and **P < 0.01 for paired Student’s t-test comparisons between data acquired prior to and post-application at the indicated step.

effect of paxilline on Iₖ (n = 3). Similarly tamoxifen (10 μM), another KCa1.1 modulator, which inhibits IClCa (n = 4), induced no change in IClCa after pre-incubation with M-βCD (3 mg mL⁻¹; n = 4; Figure 5A and B). The presence of M-βCD also limited the stimulatory effect of the KCa1.1 channel activator NS1619 on IClCa (Figure 5C and D). Figure 5C shows that similar to Saleh et al. Thirty micromolar NS1619 increased IClCa at all potentials with the current at the end of a 750 ms step to +70 mV increasing from 7.7 ± 2.1 to 16.9 ± 2.9 pA pF⁻¹ (P < 0.05, n = 4). In contrast, IClCa was unaffected by the application of 30 μM NS1619 when the myocytes had been previously treated with M-βCD (3 mg mL⁻¹; n = 4; Figure 5D). These data provide evidence that the effects of KCa1.1 modulators on IClCa are reliant upon functional lipid microdomains.

3.5 Lipid fractionation studies

In the present study, electrophysiological effects were observed with a relatively mild treatment with M-βCD compared with previous studies where higher concentrations (5–10 mg mL⁻¹) and longer application times (1–3 h) have been used.22,23,25,26 Thus, a series of experiments were undertaken to assess whether the M-βCD treatment used in the electrophysiology studies could affect the distribution of lipid raft markers. Figure 6A shows representative western blot analysis following discontinuous sucrose density ultracentrifugation of mPV tissue. Immunodetection with antibodies directed against β-adaptin and caveolin produced a localization profile similar to previous work in rat aorta.23,26 The localization pattern for flotillin-2 was also consistent with earlier work when the same concentration of Triton-X (1%) was used, indicating that flotillin-2-enriched lipid rafts are susceptible to glycerophospholipid depletion.25 Treatment of the protein lysate for 15 min incubation with M-βCD (3 mg mL⁻¹) produced an obvious reduction in density of the bands for caveolin and flotillin-2 at lower fractions and the appearance of bands in later fractions (Figure 6A). The non-raft marker β-adaptin was affected considerably less by incubation with M-βCD (Figure 6A). These data show that the electrophysiological effects of M-βCD are associated with changes in the buoyancy of lipid raft markers.

The molecular identity of CACCs is unknown but recently the gene TMEM16A has been proposed as a strong candidate for this channel.29–31 Consequently, we ascertained whether TMEM16A could be detected in mPV lysates and whether it existed in the same lipid fractions as KCa1.1. Figure 6B shows that TMEM16A and KCa1.1 immunoreactivity was detected in PV lysates. KCa1.1 staining appeared most abundant in fractions that overlapped with caveolin and not with β-adaptin, suggesting that this ion channel may be located in the caveolae fraction of lipid rafts similar to other smooth muscles. TMEM16A staining was apparent in some of the same fractions as KCa1.1 but was generally present in less buoyant fractions (Figure 6B). Treatment with M-βCD caused a small, but obvious shift in the appearance of TMEM16A and KCa1.1 to less buoyant fractions.

4. Discussion

The work of the present study shows that the amplitude and pharmacology of CACCs in vascular smooth muscle cells is drastically altered by short application of M-βCD, an agent shown to deplete cholesterol levels. Western blot analysis after sucrose gradient
ultracentrifugation showed that lipid raft markers caveolin and flotillin-2 migrated to less buoyant fractions upon treatment with M-βCD. This agent had no effect on the non-raft marker β-adaptin but had a subtle effect on the staining pattern for K<sub>Ca</sub>1.1 and the recently identified molecular candidate for CACCs, TMEM16A. This study, by analogy with past work, suggests that CACCs exist in localized lipid microdomains or rafts where an interaction with K<sub>Ca</sub>1.1 may dictate biophysical and pharmacological properties.

K<sub>Ca</sub>1.1 channels have been identified in caveolin-enriched lipid microdomains in endothelial cells, glioma cells as well as ureter and uterine smooth muscle. Physical interaction with caveolin-1 protein in these microdomains acts as a tether and a regulator. Disruption of cholesterol-rich lipid domains by cyclodextrins reduces K<sub>Ca</sub>1.1 activity in glioma and uterine smooth muscle cells, which may be due to dismemberment from IP<sub>3</sub>-mediated Ca<sup>2+</sup> release sites as caveolin and the sarcoplasmic reticulum make physical nanocontacts. The present study shows that a brief application of M-βCD, at relatively low concentrations (3 mg mL<sup>−1</sup>) compared with previous studies, also reduced macroscopic paxilline-sensitive K<sup>+</sup> currents in PV myocytes. Importantly, the present study also shows that the same low concentration of M-βCD increased the amplitude of I<sub>ClCa</sub> with the same, rapid time course as the effect on K<sup>+</sup> currents and caused translocation of the lipid raft markers caveolin and flotillin-2 to less buoyant fractions. No stimulation of I<sub>ClCa</sub> was observed when M-βCD had been preincubated with cholesterol showing that the effect on I<sub>ClCa</sub> was due to cholesterol depletion rather than an effect of the cyclodextrin itself. The similar time course of the effect of M-βCD on I<sub>K</sub> and I<sub>ClCa</sub> suggests a common biochemical event is involved, although this may represent a subtle alteration in the macromolecular complex rather than complete disruption of the lipid raft.

An even more striking observation was that cholesterol depletion by M-βCD abolished the effect of agents known to modulate K<sub>Ca</sub>1.1 channels on I<sub>ClCa</sub>. Hence, the selective K<sub>Ca</sub>1.1 blocker paxilline, which abolishes I<sub>ClCa</sub> at all voltages in mPV myocytes and in the present study, was totally ineffective in the presence of M-βCD.

**Figure 6** Effect of M-βCD on lipid raft-enriched fractions prepared from murine portal vein. (A) Western blot analysis of membrane proteins separated by a discontinuous sucrose density gradient (0, 20, 25, 30, 35, 40% sucrose) in the absence (−) and presence of 3 mg mL<sup>−1</sup> M-βCD for 15 min (+). (B) A representative western blot for fractionated proteins probed with antibodies against K<sub>Ca</sub>1.1 and TMEM16A ± M-βCD for 15 min.
Similarly, tamoxifen, which activates or inhibits KCa1.1 depending on the presence of an auxiliary subunit, abolished lI(Ca) in the absence but not in the presence of M-βCD and in the present study. Moreover, the KCa1.1 opener NS1619, shown to enhance lI(Ca) in vascular myocytes, had no effect on lI(Ca) in the presence of M-βCD. This inability to augment lI(Ca) was not due to M-βCD increasing the current to saturating levels, because Saleh et al.9 showed that NS1619 augmented lI(Ca) generated by a higher activating [Ca2+]i (1 μM vs. 500 nM). In contrast, the bimodal inhibition and stimulation of lI(Ca) reported for the so-called Cl− channel blockers NFA and A-9-C1−3 were generally unaffected by M-βCD, although the stimulatory effect of these agents was greater in M-βCD. This reflects a greater availability of the underlying CACCs and may be related to the impact of phosphorylation on the pharmacology of Cl−-activated Cl− channels shown recently.36 Overall, the present data provide support for KCa1.1 and Ca2+-activated Cl− channel proteins existing in a restricted space to allow pharmacological overlap. Disruption of cholesterol by M-βCD uncouples these two proteins and causes the loss of the pharmacological overlap.

It is tempting to speculate that the native Ca2+-activated Cl− channel exists as a multimeric assemblage consisting of a pore forming subunit, possibly TMEM16A after recent publications,29–31 and the expression data of the present study, KCa1.1 and various signalling moieties such as CaMKII, calcineurin, and PP1 known to regulate vascular lI(Ca).18,19,37 However, the sucrose gradient work of the CD uncouples these two proteins existing more evenly in the plasma membrane rather than just localized in rafts, even though some overlap with caveolin was observed and the pattern of TMEM16A staining was altered by mild treatment with M-βCD. These data suggest that if TMEM16A constitutes the native CACCs in smooth muscle then the nature of any molecular interaction with KCa1.1 is more subtle or complex than simple consolidation in a discrete microdomain. Ongoing experiments are aimed at ascertaining whether TMEM16A expression products contribute to native CACCs in smooth muscle.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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