Cardiovascular Research (2010) **87**, 476–484
doi:10.1093/cvr/cvq057

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

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Received 3 July 2009; revised 22 January 2010; accepted 14 February 2010; online publish-ahead-of-print 18 February 2010

Time for primary review: 23 days

Aims Calcium-activated chloride channels (CACCs) share common pharmacological properties with Kcnma1-encoded large conductance K^+ channels (BK_{Ca} or $K_{Ca}1.1$) and it has been suggested that they may co-exist in a macromolecular complex. As $K_{Ca}1.1$ channels are known to localize to cholesterol and caveolin-rich lipid rafts (caveolae), the present study investigated whether Ca^{2+}-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. Ca^{2+}-activated Cl^- currents were augmented by 3 mg mL^{-1} M-βCD with a rapid time course ($t_{0.5}$ = 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 $K_{Ca}1.1$ blockers, paxilline and tamoxifen, as well as the stimulatory effect of the $K_{Ca}1.1$ 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 $K_{Ca}1.1$ 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 • $K_{Ca}1.1$ • 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,[1] the lack of truly selective tools[2] and the complicated interaction of so called chloride channel blockers with native CACCs.[2–5] Recent work has revealed a remarkable pharmacological overlap between calcium-activated chloride currents (I_{ClCa}) and the large conductance, calcium-activated potassium channel (BK_{Ca} or $K_{Ca}1.1$).[2] 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 $K_{Ca}1.1$ currents.[6–8] Furthermore, in vascular myocytes, the structurally different activators of $K_{Ca}1.1$ channels, NS1619 and isopimaric acid, augment I_{ClCa},[9] and the $K_{Ca}1.1$ blockers paxilline, penitrem A, and iberiotoxin inhibit I_{ClCa}.[10] Moreover, tamoxifen, which blocks I_{ClCa} effectively,[10] either blocks or activates $K_{Ca}1.1$ channels depending on the presence of a β-auxiliary subunit.[11] In contrast, positive and negative modulators of small and intermediate conductance, Ca^{2+}-activated K^+ channels ($K_{Ca}2.1$ and $K_{Ca}2.3$) do not affect I_{ClCa}.[10] These observations led to the postulate that CACCs and $K_{Ca}1.1$ contained a common structural motif or co-existed in a sufficiently narrow micro-domain to allow inter-channel activity.[2]

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

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termed lipid rafts.[12] These moieties selectively include, but also exclude, certain proteins from macromolecular complexes in order to aid efficient signalling and trafficking. The presence of the protein caveolin (isoforms: 1–3) in these specialized lipid-rich domains results in the formation of caveolae, a subtype of lipid raft, that form membrane invaginations of 50–100 nm in diameter that can be visualized by electron microscopy. $K_{Ca}1.1$ channels have been shown to localize to lipid rafts within cell membranes[13,14] and surface expression and conductance of $K_{Ca}1.1$ channels are modulated by interaction with caveolin-1 protein.[15] Treatment of cells with cholesterol depleting agents, such as methyl-β-cyclodextrin (M-βCD), modulates $K_{Ca}1.1$ channel currents.[14,16] The present study aimed to assess whether M-βCD affected the biophysical and pharmacological properties of CACCs. The data presented suggest that CACCs are present in lipid sub-domains. More strikingly, this study reveals that the distinctive pharmacology of CACCs relies upon intact lipid rafts. Overall, these data lend weight to the possibility that native CACCs exist as part of a multi-protein complex.

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 Na_2ATP, 0.2 mM GTP-Na, 10 mM HEPES, 10 mM BAPTA, 1.1 mM $MgCl_2$, and a sustained Cl^- channel activation evoked by a fixed free [Ca^{2+}] of 500 nM obtained through the addition of 7.8 mM $CaCl_2$ as calculated by EqCal (Biosoft, Ferguson, MO, USA).[4,9,10,17–19] The external solution contained 126 mM NaCl, 11 mM glucose, 10 mM HEPES, 10 mM TEA-Cl, 1.2 mM $MgCl_2$, and 1.5 mM $CaCl_2$ (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 glucose, 10 mM HEPES, 1.2 mM $MgCl_2$, and 1.5 mM $CaCl_2$. The pipette solution composed of 126 mM KCl, 10 mM HEPES, 3 mM Na_2ATP, 0.2 mM GTP-Na, 10 mM BAPTA, 1.2 mM $MgCl_2$, and a fixed free [Ca^{2+}] of 250 nM obtained through the addition of 6.4 mM $CaCl_2$. Cells were held at −50 mV and I_K was 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.[20–23] M-βCD pre-bound to cholesterol was used to rule out possible cholesterol independent effects.[20]

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 (Invitrogen, 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-caveolin[24] 1:10 000; BD Biosciences) and flotillin-2[25] (1:20 000; BD Biosciences), the non-lipid raft protein marker β-adaptin[26] (1:1500; Santa Cruz), $K_{Ca}1.1$ (1:200; Alomone), and TMEM16A (ab53213; Abcam; a 1:5 dilution of a prediluted 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 Ca^{2+} 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.[9,10,17–19] 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 τ_{open} of 199 ± 13 ms ($n = 10$). Repolarization to −80 mV evoked an immediate inward current of -13.8 ± 1.9 pA pF^{-1} ($I_{-80\ mV}$) which decayed to -1.9 ± 0.3 pA pF^{-1} with a τ_{close} of 54 ± 3 ms.

3.1 Effect of M-βCD on native Ca^{2+}-activated Cl^- currents in mPV myocytes

Application of M-βCD (3 mg mL^{-1}) rapidly augmented I_{ClCa} (*Figure 1A*) with half maximal enhancement ($t_{0.5}$) occurring after 1.8 min (*Figure 1D*). *Figure 1B* shows that augmentation of I_{ClCa} was

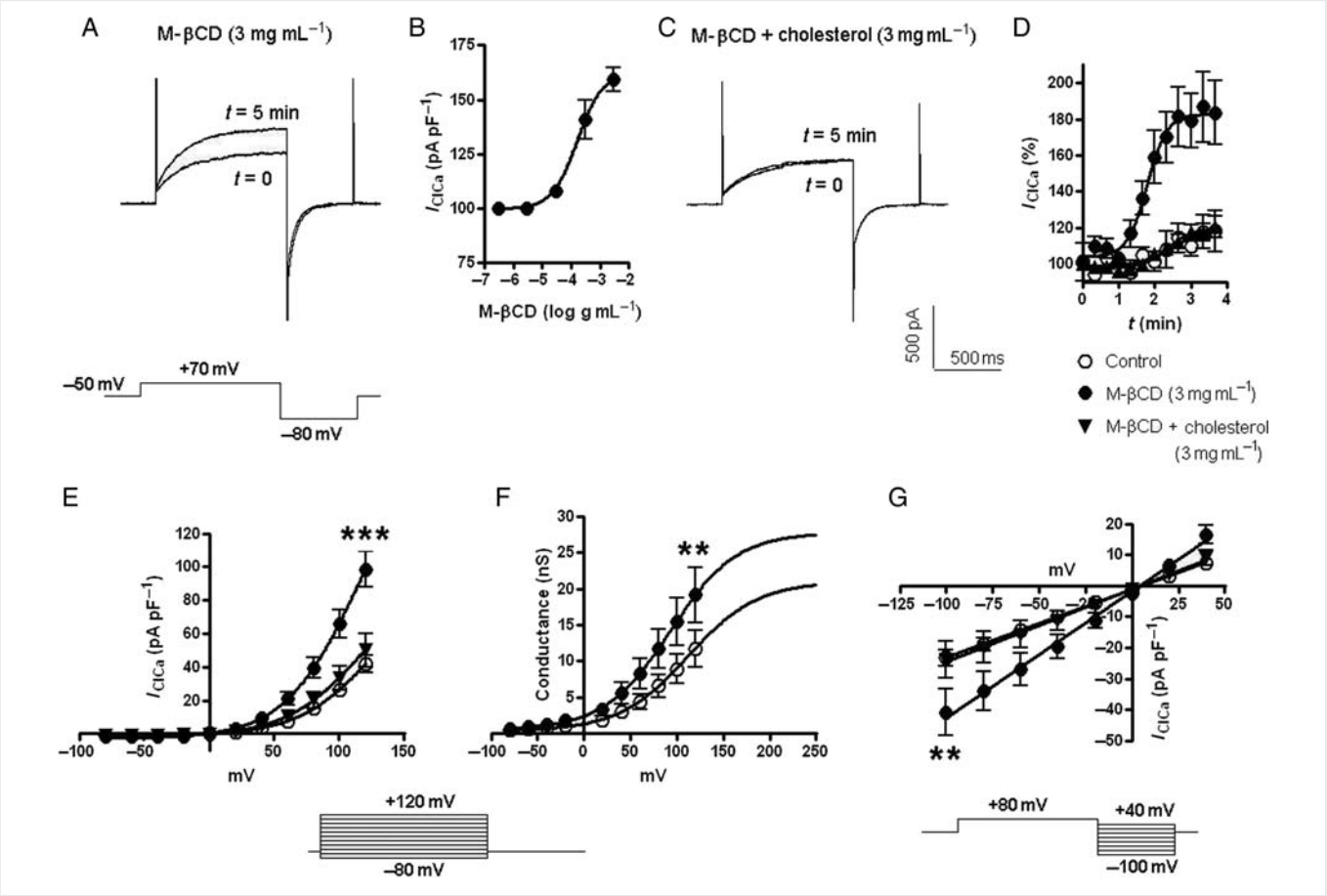

Figure 1 Effects of M-βCD on I_{ClCa}. Examples of I_{ClCa} recorded in the absence and after 5 min application of 3 mg mL^{-1} M-βCD (*A*). I_{ClCa} was evoked by step depolarization to +70 mV followed by repolarization to −80 mV. (*B*) Concentration dependence of the increase in I_{ClCa} at +70 mV produced by M-βCD (mean of 3–9 cells). (*C*) Lack of effect of 3 mg mL^{-1} M-βCD when bound to cholesterol (M-βCD + cholesterol). (*D*) Time taken for a change in I_{ClCa} (expressed as a percentage of the maximum current at +70 mV prior to application of M-βCD). (*E*) *I–V* relationship of I_{ClCa} in the absence (open circle), or presence of M-βCD (filled circle), or M-βCD bound to cholesterol (filled inverted triangle). (*F*) Voltage-dependence of activation in the presence and absence of M-βCD. (*G*) Reversal potential of the evoked current was not affected by either M-βCD or cholesterol bound M-βCD. All protocols are shown as inserts. Each point comprises data from at least five cells with error bars depicting SEM $^{**}P < 0.01$ and $^{***}P < 0.001$ for paired Student's *t*-test comparisons between data acquired prior to and post-application of M-βCD at the indicated voltage step.

also observed with 0.3 mg mL^{-1} M-βCD but not lower concentrations (0.03–0.1 mg mL^{-1}, $n \geq 3$). In contrast, application of 3 mg mL^{-1} M-βCD pre-bound with an equivalent concentration of cholesterol did not produce any changes in I_{ClCa} 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 $V_{0.5}$ 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 ($n = 3$). The augmentation of I_{ClCa} was not due to the *de novo* recruitment of a new ionic conductance as neither the reversal potential (*Figure 1G*) nor the kinetics at +70 or −80 mV were affected by M-βCD. These data are the first to show that Ca^{2+}-activated Cl^- currents are affected by cholesterol depletion.

3.2 Effect of M-βCD on native Ca^{2+}-activated K^+ currents in mPV myocytes

Experiments were performed to see if M-βCD also modulated large conductance $K_{Ca}1.1$ currents in mPV myocytes similar to previous findings in uterine smooth muscles cells and gliomas.[14,16] As *Figure 2A* shows application of a depolarizing voltage ramp with pipette solutions containing [Ca^{2+}] fixed at 250 nM evoked an outwardly rectifying I_K superimposed by considerable current fluctuations at positive potentials that was inhibited by the $K_{Ca}1.1$ channel blocker 1 μM paxilline[27,28] by 80 ± 2% ($P < 0.05$; $n = 4$). Application of M-βCD (3 mg mL^{-1}) reduced I_K (*Figure 2A* and *B*) with a time course similar to the effect on I_{ClCa} ($t_{0.5} = 2.7$ min, *Figure 2C*), which was not manifest in cells pre-treated with paxilline (*Figure 2B*). The effect of M-βCD on I_K was also maintained after washout of the cyclodextrin ($n = 3$). Thus, $K_{Ca}1.1$ channels in vascular myocytes are inhibited by cholesterol depletion.

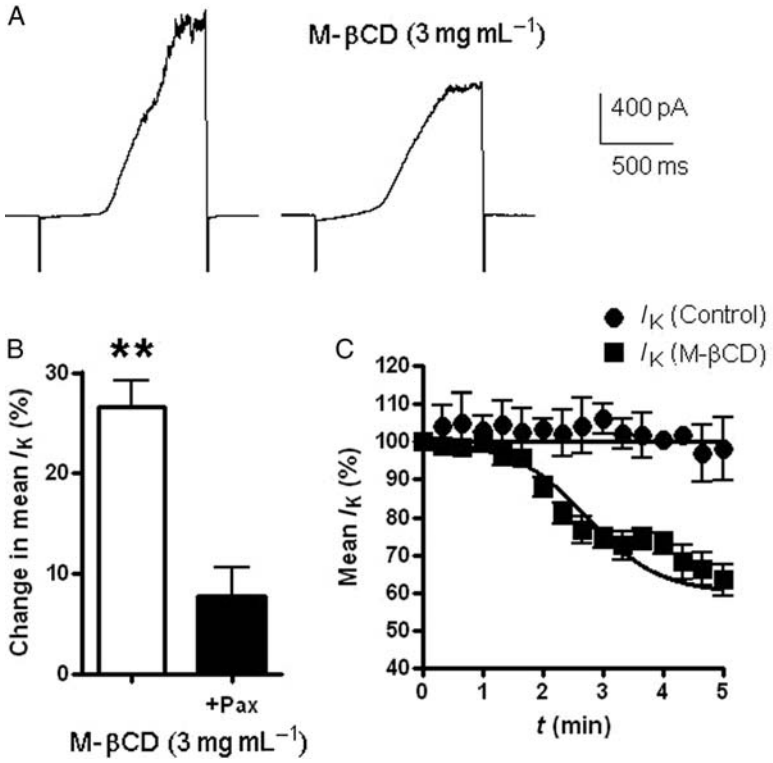

Figure 2 M-βCD and I_K. (*A*) Collection of traces elicited by a ramp protocol [−100 mV to +100 mV (at 200 mV s^{-1}) taken from a cell prior to and after 5 min incubation in M-βCD (3 mg mL^{-1})]. (B) Change in mean I_K amplitude evoked by M-βCD in the absence and presence of paxilline (1 μM). (*C*) Time course of M-βCD modulation of I_K at +100 mV compared with control currents recorded over a similar time course. Each point comprises data from four cells with error bars depicting SEM. $^{**}P < 0.01$ for paired Student's *t*-test comparisons between data acquired prior to and post-application of M-βCD.

3.3 Effects of Cl^- channel blockers on I_{ClCa} in the presence of M-βCD

Experiments were undertaken with the Cl^- 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_{ClCa}. In vascular myocytes, these agents produce complex effects on sustained I_{ClCa}, which is manifest as a significant but not complete inhibition at positive potentials and a paradoxical stimulation of I_{ClCa} at negative potentials.[3–5,9] *Figure 3* shows that NFA (100 μM) 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 3*Ai). Similar effects were observed in the presence of M-βCD (*Figure 3*Aii). In the absence of M-βCD, NFA evoked a decrease in the outward time-dependent current of 2.6 ± 1.3 pA pF^{-1}, whereas in the presence of M-βCD, a decrease of 3.9 ± 0.8 pA pF^{-1} was observed. In addition, the inward current at −80 mV in the presence of NFA was larger in M-βCD (*Figure 3*Aiii). Both observations reflect a heightened activation of the underlying CACCs. Application of A-9-C (500 μM) produced effects similar to those previously reported in the rabbit pulmonary artery[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 3*Bi), which were also apparent after application of 3 mg mL^{-1} M-βCD ($n = 4$; *Figure 3*Bii). 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^{-1} in M-βCD ($n = 4$, *Figure 3*Biii) 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_{ClCa} were maintained after incubation with M-βCD.

3.4 Effects of $K_{Ca}1.1$ modulators on I_{ClCa} in the presence of M-βCD

We have recently shown that the $K_{Ca}1.1$ inhibitor paxilline and the $K_{Ca}1.1$ activator NS1619 modulate I_{ClCa}.[9,10] The observation, in the present study, that I_{ClCa}, as well as $K_{Ca}1.1$ current, are affected by M-βCD suggests that the pharmacological cross over could stem from a close physical interaction between the Cl^- channel and $K_{Ca}1.1$ in the same microdomain. Consequently, disruption of the local environment by M-βCD might alter the ability of $K_{Ca}1.1$ channel modulators to affect I_{ClCa}. Similar to our previous report,[10] 1 μM paxilline reduced I_{ClCa} markedly at all potentials but did not affect the reversal potential of the evoked current ($n = 4$, *Figure 4*Ai–Aiii). In stark contrast, paxilline had no effect on I_{ClCa} in the presence of M-βCD (3 mg mL^{-1}; $n = 4$; *Figure 4*Bi–Biii). Moreover, as *Figure 4C* shows addition of M-βCD (3 mg mL^{-1}) reversed completely the inhibition of I_{ClCa} produced by paxilline with a time course similar to the stimulatory effect of M-βCD alone ($\tau = 2.1$ min, *Figure 4*Civ, $n = 4$). M-βCD did not reverse the inhibitory

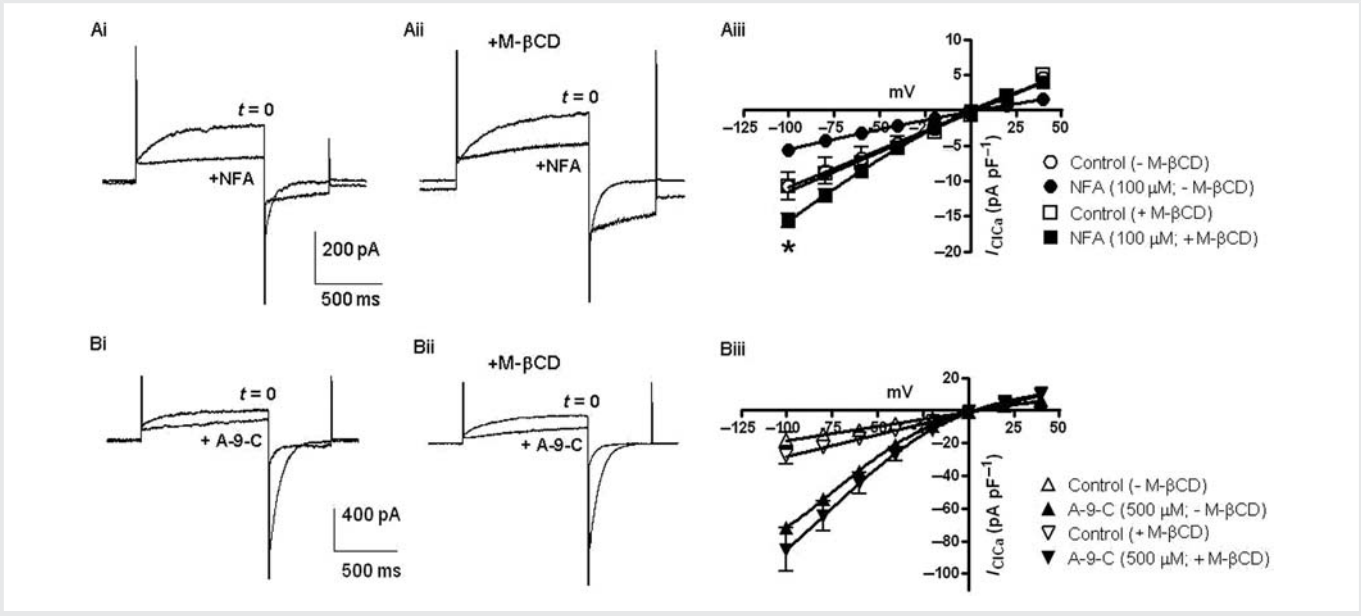

Figure 3 Effect of NFA and A-9-C in the presence of M-βCD. (*A*) Effect of 100 μM NFA on I_{ClCa} in the absence and presence of 3 mg mL^{-1} M-βCD. (*Ai* and *Aii*) Representative traces. (*Aiii*) Effect of NFA on I_{ClCa} amplitude recorded at different potentials, using the reversal protocol in *Figure 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.

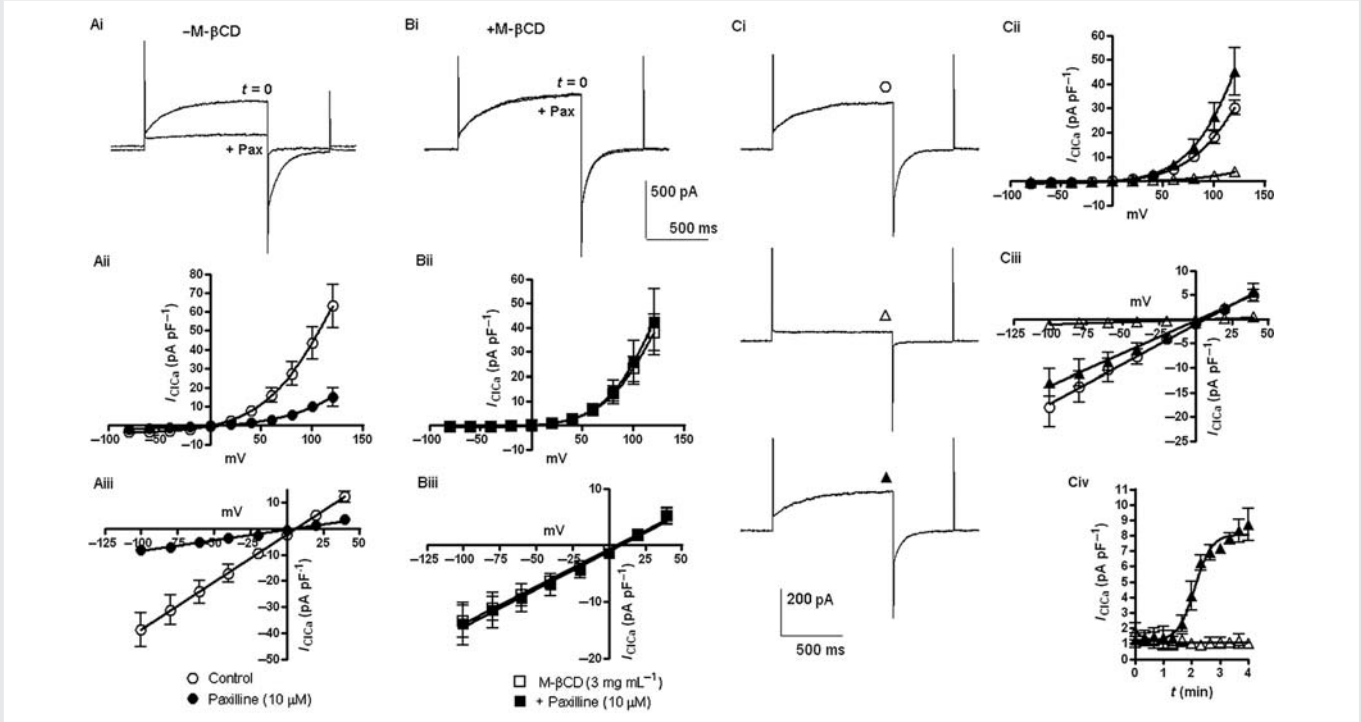

Figure 4 M-βCD modulates the effect of paxilline on I_{ClCa}. (*A*) Effect of 10 μM paxilline (Pax) on I_{ClCa}. (*Ai*) Representative currents recorded in the absence and presence of paxilline (5 min). (*Aii* and *Aiii*) *I*–*V* relationships and reversal potential of I_{ClCa} in the absence (open circle) 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.

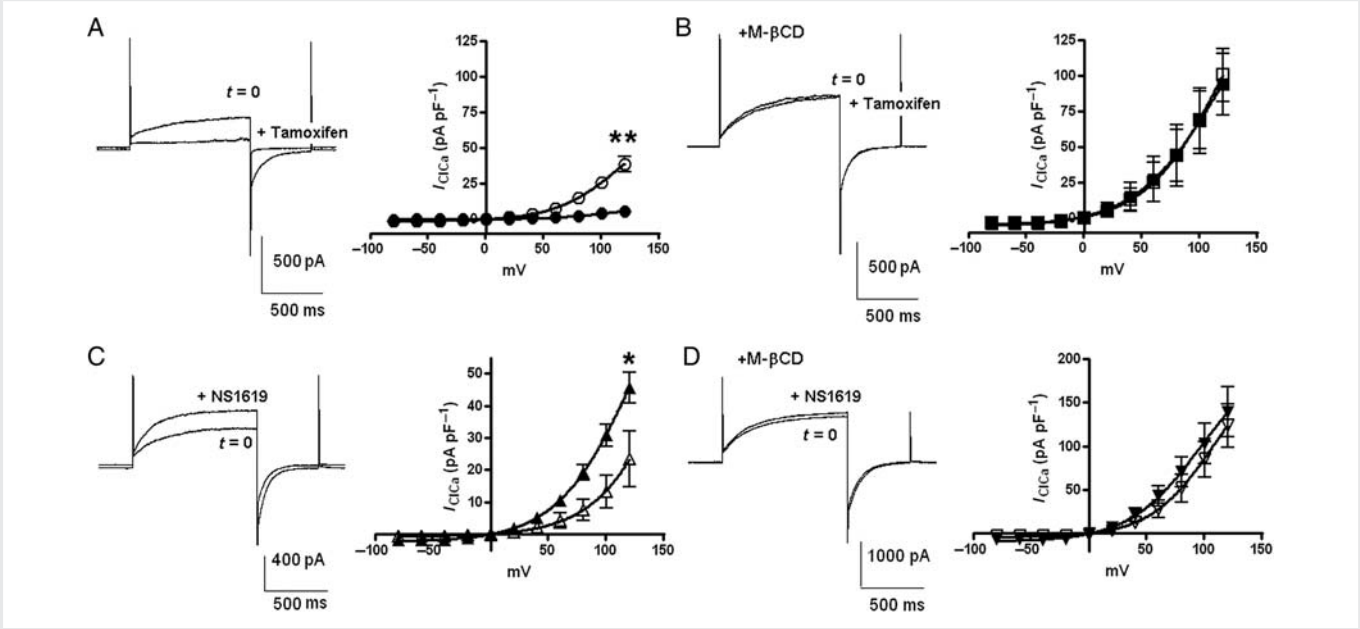

Figure 5 Effect of tamoxifen and NS1619 in the presence of M-βCD. (*A*) Representative currents and the *I–V* relationship recorded in the absence (open circle) and presence of tamoxifen (5 min; filled circle) in the absence of M-βCD. (*B*) Recordings performed in the absence (open square) and presence 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 I_K ($n = 3$). Similarly tamoxifen (10 μM), another $K_{Ca}1.1$ modulator, which inhibits I_{ClCa}[10] ($n = 4$), induced no change in I_{ClCa} 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 $K_{Ca}1.1$ channel activator NS1619 on I_{ClCa} (*Figure 5C* and *D*). *Figure 5C* shows that similar to Saleh *et al.*[9] Thirty micromolar NS1619 increased I_{ClCa} 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, I_{ClCa} 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 $K_{Ca}1.1$ modulators on I_{ClCa} 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 $K_{Ca}1.1$. *Figure 6B* shows that TMEM16A and $K_{Ca}1.1$ immunoreactivity was detected in PV lysates. $K_{Ca}1.1$ 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 $K_{Ca}1.1$ 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 $K_{Ca}1.1$ 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

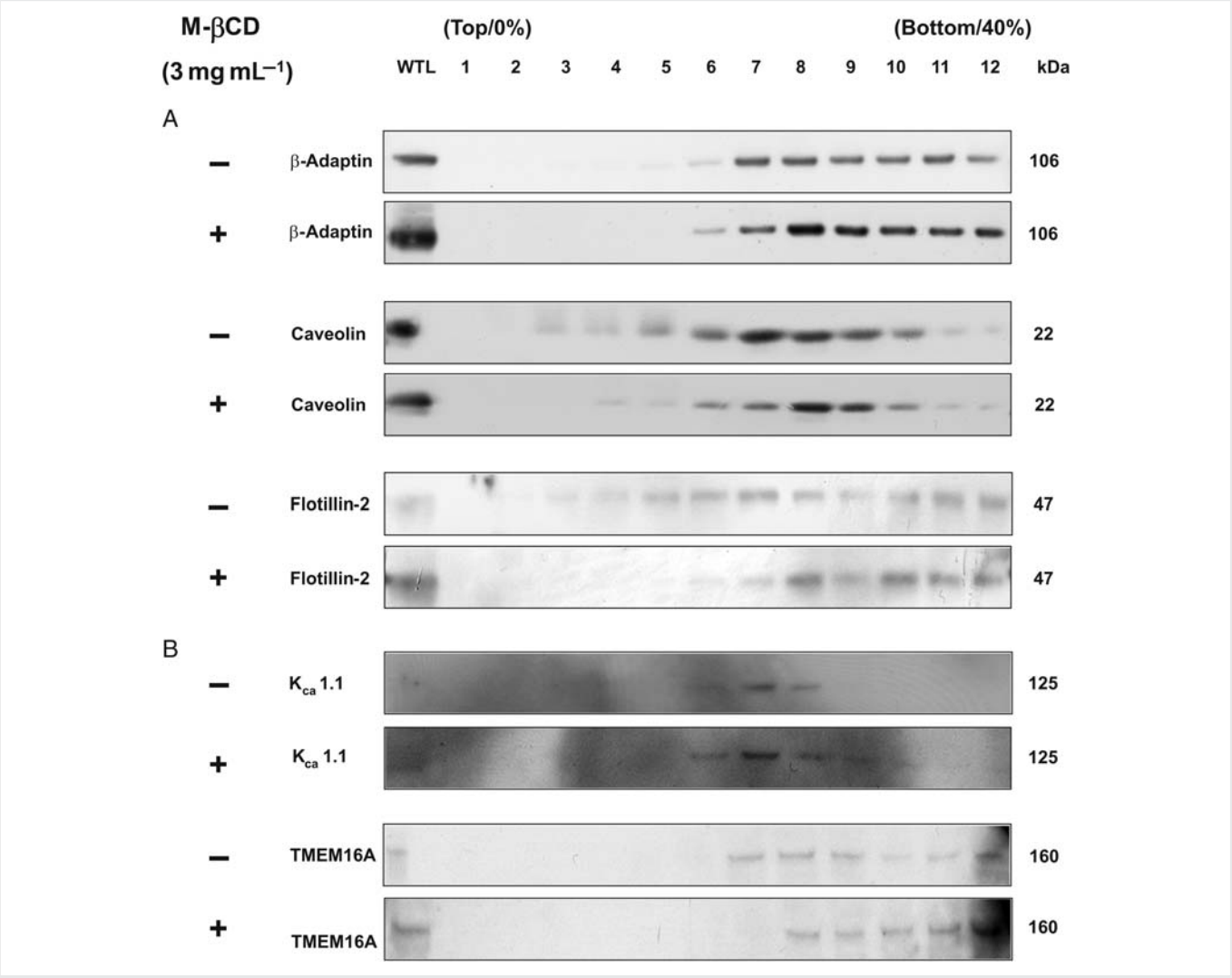

Figure 6 Effect of M-βCD on lipid raft-enriched fractions prepared from murine portal vein. (*A*) Western blot analysis of membrane proteins separated by a discontinuous sucrose density gradient (0, 20, 25, 30, 35, 40% sucrose) in the absence (−) and presence of 3 mg mL^{-1} M-βCD for 15 min (+). (*B*) A representative western blot for fractionated proteins probed with antibodies against K_{Ca}1.1 and TMEM16A ± M-βCD for 15 min.

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_{Ca}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_{Ca}1.1 may dictate biophysical and pharmacological properties.

K_{Ca}1.1 channels have been identified in caveolin-enriched lipid microdomains in endothelial cells,[32,33] glioma cells[14] as well as ureter and uterine smooth muscle.[16,26,34] Physical interaction with caveolin-1 protein in these microdomains acts as a tether and a regulator.[15,33] Disruption of cholesterol-rich lipid domains by cyclodextrins reduces K_{Ca}1.1 activity in glioma and uterine smooth muscle cells,[14,16] which may be due to dismemberment from IP_3-mediated Ca^{2+} release sites[14] as caveolin and the sarcoplasmic reticulum make physical nanocontacts.[35] The present study shows that a brief application of M-βCD, at relatively low concentrations (3 mg mL^{-1}) compared with previous studies,[22–26] also reduced macroscopic paxilline-sensitive K^+ currents in PV myocytes. Importantly, the present study also shows that the same low concentration of M-βCD increased the amplitude of I_{ClCa} with the same, rapid time course as the effect on K^+ currents and caused translocation of the lipid raft markers caveolin and flotillin-2 to less buoyant fractions. No stimulation of I_{ClCa} was observed when M-βCD had been pre-incubated with cholesterol showing that the effect on I_{ClCa} 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_K and I_{ClCa} 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_{Ca}1.1 channels on I_{ClCa}. Hence, the selective K_{Ca}1.1 blocker paxilline, which abolishes I_{ClCa} at all voltages in mPV myocytes[10] and in the present study, was totally ineffective in the presence of M-βCD.

Similarly, tamoxifen, which activates or inhibits $K_{Ca}1.1$ depending on the presence of an auxiliary subunit,[11] abolished I_{ClCa} in the absence but not in the presence of M-βCD[10] and in the present study. Moreover, the $K_{Ca}1.1$ opener NS1619, shown to enhance I_{ClCa} in vascular myocytes,[9] had no effect on I_{ClCa} in the presence of M-βCD. This inability to augment I_{ClCa} was not due to M-βCD increasing the current to saturating levels, because Saleh *et al.*[9] showed that NS1619 augmented I_{ClCa} generated by a higher activating $[Ca^{2+}]$ (1 μM vs. 500 nM). In contrast, the bimodal inhibition and stimulation of I_{ClCa} reported for the so-called Cl^- channel blockers NFA and A-9-C[3–5] 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 Ca^{2+}-activated Cl^- channels shown recently.[36] Overall, the present data provide support for $K_{Ca}1.1$ and Ca^{2+}-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 Ca^{2+}-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, $K_{Ca}1.1$ and various signalling moieties such as CaMKII, calcineurin, and PP1 known to regulate vascular I_{ClCa}.[1,18,19,37] However, the sucrose gradient work of the present study suggests that TMEM16A is distributed 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 $K_{Ca}1.1$ 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.

Acknowledgements

Thanks to Mr Thomas Jepps for technical support and the advice of Profs Johnstone and Whitely at St George's, University of London. The advice of Drs C. Dart (University of Liverpool) and C. Vial (University of Leicester) is acknowledged.

Conflict of interest: none declared.

Funding

W.R.S. and A.J.D. were funded by British Heart Foundation grants to I.A.G. (PG/05/038 and PG/07/127/24235). This study was supported by a grant to N.L. from the National Institutes of Health (Grant 5 RO1 HL 075477) and from the National Center for Research Resources (NCRR 5 P20 RR15581).

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