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Membrane cholesterol content modulates activation of BK channels in colonic epithelia

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Abstract

Changes in the level of membrane cholesterol regulate a variety of signaling processes including those mediated by acylated signaling molecules that localize to lipid rafts. Recently several types of ion channels have been shown to have cholesterol-dependent activity and to localize to lipid rafts. In this study, we have investigated the role of cholesterol in the regulation of ion transport in colonic epithelial cells. We observed that methyl-β-cyclodextrin (MβCD), a cholesterol-sequestering molecule, activated transepithelial short circuit current (I_{sc}), but only from the basolateral side. Similar results were obtained with a cholesterol-binding agent, filipin, and with the sphingomyelin-degrading enzyme, sphingomyelinase. Experiments with ΔF508CFTR mutant mice indicated that raft disruption affected CFTR-mediated anion secretion, while pharmacological studies showed that this effect was due to activation of basolateral large conductance Ca^{2+}-activated K^+ (BK) channels. Sucrose density gradient centrifugation studies demonstrated that BK channels were normally present in the high-density fraction containing the detergent-insoluble cytoskeleton, and that following treatment with MβCD, BK channels redistributed into detergent-soluble fractions. Our evidence therefore implicates novel high-density cholesterol-enriched plasma membrane microdomains in the modulation of BK channel activation and anion secretion in colonic epithelia.

Keywords: Anion secretion; Ussing chamber; CFTR; Lipid raft

1. Introduction

The plasma membrane of eukaryotic cells contains a far greater variety of lipid species than is required to form a lipid bilayer, suggesting that lipids play a unique and determining role in membrane organization [1]. In recent years studies on lipid biophysics, protein sorting and detergent solubility have converged to support the concept that lipids are not homogeneously distributed within the lipid bilayer but spontaneously aggregate to form microdomains enriched in cholesterol and sphingolipid [2,3]. The physical characteristics of these microdomains or "lipid rafts" are proposed to preferentially admit and retain proteins modified by unsaturated long chain fatty acids while excluding the majority of transmembrane proteins [2,4]. Thus, by accumulating signaling molecules and selectively admitting transmembrane proteins, lipid rafts have the potential to compartmentalize signals within the plasma membrane [5]. A potential problem with the lipid raft concept is that most of the evidence for the existence of lipid rafts derives from indirect approaches such as resistance to detergent extraction at sub-physiological temperature, and the use of cholesterol-sequestering agents. This has led some investigators to question the existence of lipid rafts and to offer alternative explanations for the effect of modulating cholesterol and sphingolipids on cell function. For example, it was recently suggested that the major role of cholesterol and sphingolipids might be to regulate the permeability of the exofacial leaflet of the bilayer [6].

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Therefore, while there is consensus that lipids modify cellular functionality in reproducible ways, the mechanisms responsible have yet to be fully resolved.

In the last few years several reports have provided evidence that certain ion channels physically associate with the low-density detergent-insoluble microdomains that characterize lipid rafts. For example, both cardiac [7] and epithelial [8] Na^+ channels were recovered within such fractions. Similarly, the voltage-gated K^+ channels, Kv1.5 and Kv2.1 (but not Kv4.2) [9,10], and the large conductance Ca^{2+}-activated K^+ (BK) channels [11] were observed within cholesterol-enriched microdomains. Associations with lipid rafts have been shown to involve the transport of Kir3.1/3.2 channels to the membrane [12], and to control the activities of both Cl^- [13] and Ca^{2+} [14] channels. Thus, although the functional implications of channel associations with plasma membrane microdomains are not well understood, there is growing evidence that these structures play an important role in the modulation of ion channel activity. The localization of ion channels within lipid rafts is an attractive concept, which would integrate ion channels into multi-component signaling complexes capable of generating localized signals in the plasma membrane.

The role of lipid rafts in intestinal epithelial cell function has been explored in several studies. Cholesterol-enriched microdomains were shown to play a role in cholesterol trafficking [15], and in apical exocytotic membrane trafficking in enterocytes [16,17]. However, very little is known about the role of these microdomains in transepithelial anion transport. Chloride secretion in colonic epithelia drives water into the intestinal lumen, providing for the fluidity of intestinal contents [18]. Either over-secretion or under-secretion of chloride can result in significant pathophysiological events, such as secretory diarrhea or cystic fibrosis, respectively. Therefore, significant efforts have been made to study anion secretion in order to understand the underlying mechanisms involved.

The aim of our study was to characterize the role of cholesterol-enriched microdomains in the regulation of transepithelial anion secretion. Using a combination of biophysical, pharmacological and biochemical approaches, we have investigated the role of cholesterol and plasma membrane microdomains in transepithelial anion secretion in colonic epithelia. Our studies show that lowering the cholesterol and sphingomyelin content of the basolateral plasma membrane leads to the activation of BK channels, to an increase in the detergent solubility of BK channels, and to the stimulation of transepithelial anion secretion. Our results therefore identify a novel cholesterol-dependent mechanism of BK channel regulation operative in colonic epithelia. BK channels were not detected within conventional low-density detergent-insoluble microdomains, but were associated with a high-density detergent-insoluble pellet containing the cytoskeleton. Since BK channels and the raft associated protein caveolin both translocated into a high-density detergent-soluble phase on reducing cholesterol, we propose that BK channels may be the first ion channel shown to associate with a cytoskeletally anchored lipid raft.

2. Materials and methods

2.1. Epithelial cells

The colonic epithelia were from three different strains of mice: BALB/c, C57BL/6J and cystic fibrosis (CF) mice. The breeding colony of CF mice (B6.129S6-*Cftr*^{tm1Kth}, Jackson Laboratory, Bar Harbor, ME) was housed in a pathogen-free environment (Health Sciences Laboratory Animal Services, University of Alberta). All experiments were carried out with the approval of the Health Sciences Animal Policy and Welfare Committee, University of Alberta. Pups were weaned at 21 days of age, and genotyped as described previously [19]. Mice were killed by CO_2 narcosis, and 6-cm-long pieces of colon were removed from ~2 cm below the caecum and immediately placed in cold Krebs–Henseleit solution (KHS) containing (mM): 116 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 11.1 glucose, pH 7.4. The colons were opened up, the muscle layers dissected away and four pieces were mounted in Ussing chambers with a 0.2-cm^2 recording area.

2.2. Transepithelial measurements

Ussing chamber experiments were performed as described earlier [19]. For basolateral membrane K^+ current measurements, apical NaCl was replaced by equimolar potassium gluconate, while basolateral NaCl was substituted with sodium gluconate and the Ca^{2+} concentration was increased to 5 mM in both solutions, to compensate for the Ca^{2+}-buffering capacity of gluconate. In addition, 100 μM ouabain was added to the basolateral compartment to inhibit the Na^+/K^+-ATPase. Subsequent permeabilization of the apical membrane with nystatin (90 μg/ml) allowed measurement of K^+ current as these ions moved down their concentration gradient through basolateral K^+ channels.

2.3. Lipid raft protein isolation and immunoblotting

Isolated epithelium was treated with or without 10 mM methyl-β-cyclodextrin (MβCD) for 30 min (in KHS, bubbled with 95% O_2/5% CO_2) and then lysed in 1 ml of cold MES-buffered saline (25 mM MES, 150 mM NaCl, 5 mM $Na_4P_2O_7 \cdot 10H_2O$, 1 mM $NaVO_4$, 2 mM NaF, pH 6.5, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin, leupeptin, pepstatin) for 90 min at 4 °C. The lysate (containing 200 μg of protein) was loaded with an equal volume of 90% sucrose and overlaid with 30% and 5% sucrose. Centrifugation was performed at 165,000 RCF (relative centrifugal force) for 18 h at 4 °C in a Beckman SW60Ti rotor. Starting from the top of the gradient, nine fractions, including the

pellet, were collected and separated by SDS-PAGE (5–20% gradient gel). The proteins were transferred to nitrocellulose membranes incubated with 5% (w/v) non-fat milk powder in phosphate-buffered saline plus Tween 20 (PBST: 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.05% (v/v) Tween 20, pH 7.2), washed and incubated with polyclonal rabbit antibodies against the BK channel α subunit (Chemicon International, Temecula, CA) diluted 1:500, or with polyclonal rabbit antibodies against caveolin-1 (Santa Cruz Biotech., Santa Cruz, CA) diluted 1:200. The membranes were incubated with 1:10,000 peroxidase-conjugated goat anti-rabbit IgG (H+L, Jackson ImmunoResearch Laboratory, West Grove, PA) and then washed in PBST. Detection was carried out using the ECL kit (Amersham Biosciences, Buckinghamshire, England) and Hyperfilm™ (Amersham Biosciences).

2.4. Chemicals

Amiloride (10 mM), $BaCl_2$ (500 mM), ouabain (10 mM) and tetrapentylammonium chloride (TPeA, 100 mM) were dissolved in H_2O, MβCD and mannitol (80 mM) in KHS, diphenylamine-2-carboxylate (DPC, 1 M) and pimaric acid (10 mM, a generous gift from J. Clay, Helix Biotech, New Westminster, BC) in dimethyl sulfoxide (DMSO). Furosemide (100 mM) was prepared in H_2O with a drop of 1 N NaOH, charybdotoxin (10 μM) in KHS containing 0.1% BSA. Bumetanide, ceramide, clotrimazole, and tolbutamide were made as at least 1000-fold stock solutions in ethanol. Filipin (10 mg/ml in methanol) and nystatin (90 mg/ml in DMSO) were prepared fresh before each experiment. XE991 (10 mM, a generous gift from Dr. B.S. Brown, DuPont, Wilmington, DE) was dissolved in 0.1 N HCl. $BaCl_2$ and mannitol were from Fisher Scientific (Fair Lawn, NJ). All other chemicals were from Sigma-Aldrich.

2.5. Statistical analysis

Data are expressed as means±S.E. with the number of preparations used (n). Statistical difference was determined by Student's t-test or one-way ANOVA. Values of $P<0.05$ were considered statistically significant.

3. Results

3.1. Basolateral lipid rafts affect anion secretion in colonic epithelia

The cyclic oligosaccharide MβCD is a membrane impermeable molecule that selectively and rapidly extracts cholesterol from the plasma membrane. We applied MβCD to either the apical or basolateral side of epithelial cell sheets from the colon and measured the effect on transepithelial short circuit current (I_{sc}). Fig. 1A shows that in KHS, apical MβCD (10 mM, n=11) had no significant effect on I_{sc} for at least 30 min ($P>0.05$, Student's t-test). In contrast, basolateral MβCD (10 mM, n=38) produced a biphasic I_{sc} response, with an initial peak followed by an elevated plateau. When MβCD was added unilaterally, mannitol (10 mM) was added to the opposite side to compensate for changes in osmotic pressure. Mannitol by itself had no effect on I_{sc} (n=9, $P>0.05$). Bilateral MβCD produced a change in I_{sc} that was identical to that caused by basolateral MβCD (n=8). MβCD had no effect on transepithelial resistance for at least 90 min ($P>0.05$, paired Student's t-test, n=12), indicating that this treatment does not affect the paracellular pathway. Similarly, it did not affect cell viability, since the relative I_{sc} activated by cAMP- or Ca^{2+}-dependent secretagogues (10 μM forskolin and 100 μM carbachol, respectively) was not altered by MβCD treatment (10 mM, basolateral, 90 min; Table 1). Moreover, tetrodotoxin (5 μM, data not shown) did not affect the response to basolateral MβCD, ruling out the contribution of residual neural activity. The effect of MβCD was also not affected by the presence of the epithelial Na^+ channel (ENaC) blocker, amiloride (10 μM, n=12, $P>0.05$). Therefore, all subsequent experiments were performed with amiloride in the apical compartment to inhibit ENaC-mediated Na^+ absorption.

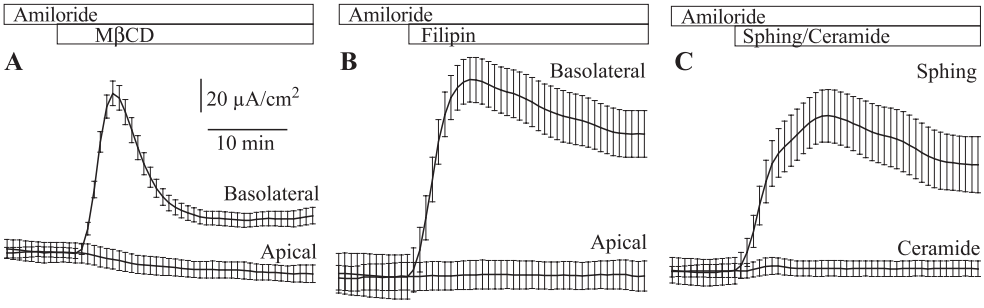

Fig. 1. The effects of MβCD, filipin and sphingomyelinase on I_{sc}. (A) Basolateral MβCD (10 mM, n=38) produced a biphasic I_{sc} response, with an initial increase followed by a plateau that was attained after ~20 min. Apical MβCD (n=11) had no significant effect on I_{sc} for at least 30 min. Both filipin (B, 10μg/ml, n=8) and neutral sphingomyelinase (C, 1.6 U/ml, n=6) activated I_{sc} from the basolateral side only. The product of sphingomyelinase metabolism, ceramide (10 μg/ml, both sides), had no effect on I_{sc} (n=6, $P>0.05$, Student's t-test). In all experiments, 10 μM amiloride was present in the apical compartment to inhibit Na^+ channels.

Table 1
I_{sc} activated by forskolin and carbachol under control and MβCD-pretreated conditions

	Control ΔI_{sc} ($\mu A/cm^2$)	N	MβCD (10 mM) ΔI_{sc} ($\mu A/cm^2$)	N
Forskolin (10 μM, bilateral)	322.4±9.6	13	326.8±16.0	7
Carbachol (100 μM, basolateral)	183.0±10.6	15	182.4±16.7	10

N—number of experiments.

Filipin is a cholesterol-binding reagent that is frequently used in lipid raft studies. We found that apical filipin (10 μg/ml, n=4) had no effect on the baseline I_{sc} (Fig. 1B). However, basolateral filipin (n=8) increased I_{sc} similarly to MβCD, although the peak was broader and the plateau level was attained at higher I_{sc} (Fig. 1B).

Lipid raft integrity depends on the presence of both cholesterol and sphingomyelin. We reasoned that treating cells with sphingomyelinase would alter raft structure or composition and affect ion flux mediated by these domains, if they were involved in this process. Apical sphingomyelinase (1.6 U/ml) had no effect on the baseline I_{sc} (n=4), but basolateral sphingomyelinase produced a change in I_{sc} that was qualitatively similar to the effects of MβCD and filipin (Fig. 1C). One of the products of sphingomyelinase action is ceramide. Therefore, we applied ceramide (10 μg/ml, both sides) to investigate if the effect of sphingomyelinase could be related to ceramide production. Fig. 1C shows that ceramide had no effect on I_{sc} (n=6, P>0.05, Student's t-test). The fact that three dissimilar treatments that affect lipid raft integrity produced qualitatively similar effects on I_{sc} suggests that these microdomains are involved in the regulation of ion transport in epithelial cells.

3.2. Lipid raft disruption affects CFTR-mediated anion secretion

In order to identify the current affected by cholesterol depletion, we used colonic epithelium from ΔF508CFTR mice. Fig. 2A shows that MβCD has no significant effect on I_{sc} in CF mice (n=6). Similarly, the CFTR Cl^- channel blocker, DPC (0.5 mM, apical, n=8), significantly inhibited I_{sc} activation by MβCD. These experiments indicate that disruption of lipid rafts affects CFTR-mediated anion secretion.

Anion secretion in epithelial cells is coordinated by a network of ion channels, transporters, and energy-dependent pumps that are selectively expressed in the apical or basolateral aspects of the epithelium. Since MβCD, filipin and sphingomyelinase affected I_{sc} from the basolateral side only, we focused on this side to identify the target(s) that were affected by these reagents.

3.3. Basolateral BK channels are associated with lipid rafts

An inhibitor of the basolateral Na^+-K^+-$2Cl^-$ cotransporter, furosemide (1 mM), had no effect on the baseline current (ΔI_{sc}=−1.6±2.1 $\mu A/cm^2$, n=4, P>0.05 Student's t-test), and in its presence MβCD increased I_{sc} by 92.8±13.6 $\mu A/cm^2$ (n=4), which is not significantly different from control conditions. Similar results were obtained with another inhibitor of the basolateral Na^+-K^+-$2Cl^-$ cotransporter, bumetanide (10 μM, n=6). These results indicate that cholesterol depletion does not affect Na^+-K^+-$2Cl^-$ cotransporter function in colonic epithelia.

The effect of MβCD on Na^+/K^+-ATPase activity was investigated with the use of the ionophore nystatin in order to bypass the apical membrane (Fig. 2B). In regular Krebs–Henseleit solution, nystatin (90 μg/ml, apical) activated similar currents in the presence or absence of MβCD (10 mM, n=4 in both sets, P>0.05, Student's t-test), indicating that removal of cholesterol from the basolateral side does not affect Na^+/K^+-ATPase function in colonic epithelia. The effect of nystatin was inhibited by basolateral ouabain (100 μM, n=3), confirming that the nystatin-induced current is generated by the Na^+/K^+-ATPase.

The effect of MβCD on basolateral K^+ channels was assessed in nystatin-permeabilized epithelia in the presence of ouabain (100 μM), to inhibit the Na^+/K^+-ATPase, and an apical-to-basolateral directed K^+ gradient (Fig. 3A). Nysta-

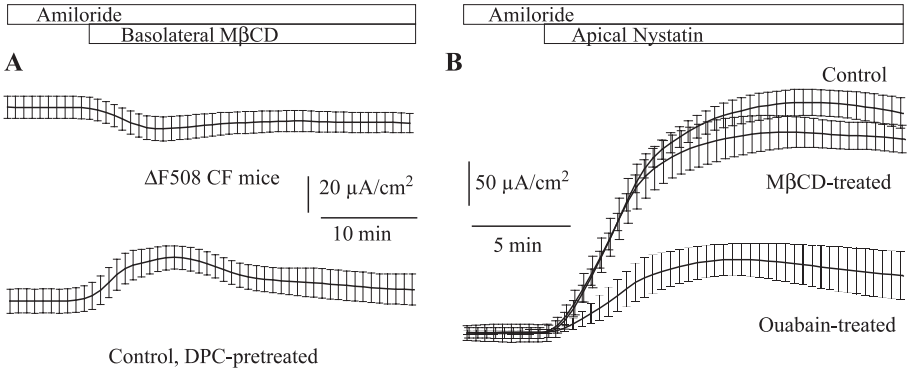

Fig. 2. MβCD treatment affects CFTR-mediated anion secretion. (A) MβCD did not activate I_{sc} in ΔF508 CF colonic epithelia (n=6), and DPC (0.5 mM, apical) blocked the effect of MβCD (10 mM, n=8) on I_{sc} in wild-type mice. (B) MβCD treatment has no effect on I_{sc} generated by the Na^+/K^+-ATPase in nystatin-permeabilized epithelia. The data are means±S.E. from three to four recordings.

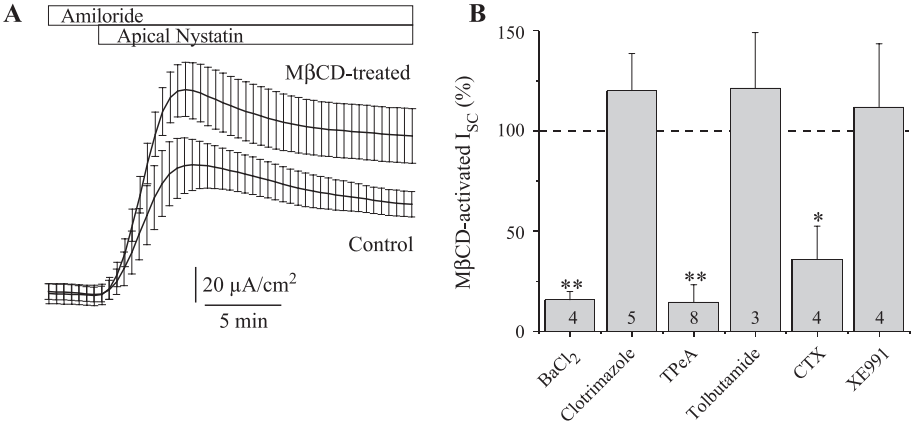

Fig. 3. Activation of basolateral K^+ channels by MβCD treatment. Epithelia pre-incubated with ouabain (100 μM, basolateral) were subjected to an apical-to-basolateral K^+ gradient and nystatin (90 μg/ml, apical) was added at the time indicated (A). The data are shown as means±S.E. from four control and seven MβCD-treated I_{sc} measurements. (B) The effect of K^+ channel blockers on I_{sc} activation by MβCD. Epithelia were pre-treated from the basolateral side with $BaCl_2$ (5 mM), clotrimazole (50 μM), TPeA (100 μM), tolbutamide (100 μM), charybdotoxin (CTX, 100 nM) or XE991 (30 μM), before MβCD treatment (10 mM, basolateral). Values are expressed as percentages of MβCD-activated I_{sc} in the absence of K^+ channel blockers. The number of experiments is shown in the histogram. *P<0.05, **P<0.01.

tin (90 μg/ml, apical) increased I_{sc} from 72.6±19.8 μA/cm^2 (n=4) under control conditions to 125.6±21.3 μA/cm^2 (n=4, P<0.05 Student's t-test) in MβCD-treated epithelia (Fig. 3A), indicating that removal of cholesterol from the basolateral membrane activated K^+ channels in colonic epithelia. Further studies were designed to identify the K^+ channels activated by MβCD.

At least four biophysically and pharmacologically distinct types of K^+ channels contribute to the basolateral K^+ conductance in mammalian colon: a cAMP-activated K^+ channel (KCNQ1) [20], an intermediate conductance Ca^{2+}-activated K^+ channel (IK-1) [21], a large conductance Ca^{2+}-activated K^+ channel (BK) [20], and ATP-dependent K^+ channels (K_{ATP}) [19]. The KCNQ1 channel can be specifically blocked by the cognitive enhancer XE991, the IK-1 channel-by an antifungal antibiotic, clotrimazole, the BK channel-by Ba^{2+}, charybdotoxin or TPeA, and the K_{ATP} channel-by tolbutamide [22]. We used the abovementioned blockers to identify the K^+ channels activated by MβCD. We found that XE991 (30 μM, n=4), clotrimazole (50 μM, n=5) and tolbutamide (100 μM, n=3) did not affect I_{sc} activation by MβCD (Fig. 3B). However, in the presence of TPeA (100 μM, n=4) I_{sc} activation by MβCD was reduced by 85.5±8.7% (n=4, P<0.01, paired Student's t-test). Similar results were obtained using charybdotoxin and $BaCl_2$ (Fig. 3B), suggesting that BK channels were activated by cholesterol removal in colonic epithelia. This conclusion has been further supported by experiments with the specific BK channel opener, pimaric acid [23]. Fig. 4 shows that I_{sc} activation by pimaric acid (90 μM, n=8) was significantly attenuated after pretreatment of the epithelium with MβCD. The I_{sc} peak response to pimaric acid was reduced from 65 μA/cm^2 under control conditions to 12.5 μA/cm^2 after MβCD treatment.

In subsequent studies we used rabbit polyclonal antibodies against the BK channel α subunit to investigate BK channel expression in colonic epithelia. Western blot experiments have shown that this antibody recognized a ~125-kDa protein band that disappeared after pre-absorption of the primary antibody with a blocking peptide supplied by the manufacturer (data not shown). This antibody was used to detect BK channels in fractions obtained after sucrose density gradient centrifugation. Fig. 5 shows that the BK channel is present mainly in the detergent-insoluble pellet that contains the actin cytoskeleton. The gradient fractions were also blotted for the caveolae marker, caveolin-1, which was found not only in the low buoyant density raft fractions 4–5, but also in higher density fractions including the pellet. Interestingly, after MβCD treatment the majority of BK channels translocated from the pellet to lighter density

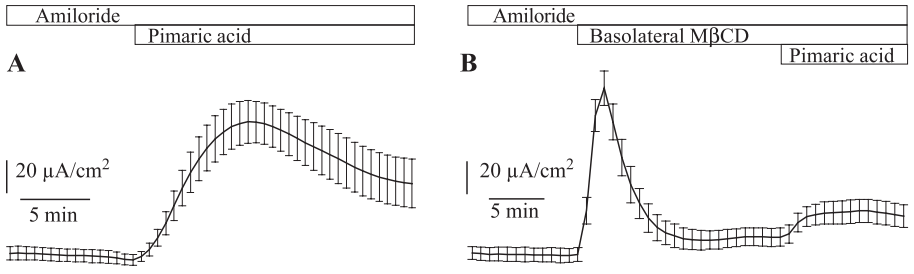

Fig. 4. Activation of I_{sc} by pimaric acid (90 μM, both sides) in control (A, n=6) and MβCD-treated (B, 10 mM, n=8) epithelia.

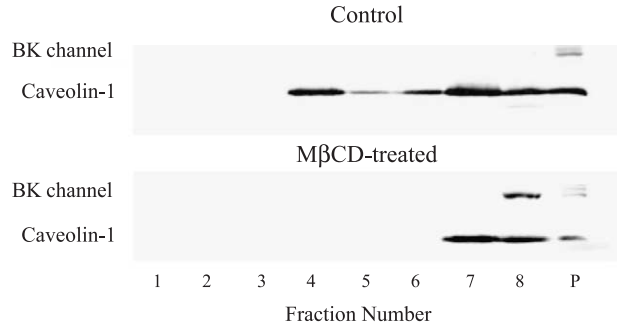

Fig. 5. BK channels partition in the pellet fraction of sucrose density gradients and do not co-localize with caveolin in colonic epithelial cells. Cells were lysed in MES-buffered saline+1% Triton X-100 and fractionated in sucrose gradients. Fractions were collected from the gradient top (1) to bottom (8), where P is the pellet, and analyzed by immunoblotting with the indicated antibodies. For MβCD treatment, cells were incubated for 30 min with 10 mM MβCD before lysis. The BK channel antibody recognizes the C-terminal region of the α subunit, and the expected size is 125 kDa. The caveolin-1 antibody recognizes a 24-kDa protein.

fraction 8 which contains detergent-soluble proteins (Fig. 5). The translocation of BK channels from the pellet was accompanied by a similar shift in the buoyant density of caveolin, which also accumulated in fractions 7 and 8. Since MβCD treatment displaced caveolin from the low buoyant density fractions, it was effective in disrupting caveolin-containing lipid rafts. Thus, our evidence indicates BK channels are selectively associated with the cytoskeleton and not within the low-density lipid rafts, but are nevertheless dependent upon cholesterol for anchorage to the cytoskeleton. This suggests that BK channels are associated with a subset of lipid rafts that are selectively anchored to the cytoskeleton.

4. Discussion

The main finding of our study is that BK channels present in the basolateral membrane of colonic epithelial cells control transepithelial anion secretion through a cholesterol-dependent mechanism. The convergent effects of cholesterol depletion by MβCD, filipin and of sphingolipid depletion by sphingomyelinase provide strong evidence that lipid microdomains in the plasma membrane play an important role in the regulation of anion secretion.

Lipid rafts in intestinal epithelial cells have been extensively characterized [15,17,24]. The apical (brush border) membrane contains at least two different types of lipid raft microdomains that are different from rafts present in the basolateral membrane or on the surface of other cell types [24]. In particular, apical rafts are stable rather than transient, and their core components include glycolipids as well as the divalent lectin galectin-4 [24]. Although apical rafts contain cholesterol, it is not essential for raft stability [17]. The results of our studies show that the removal of cholesterol from the apical membrane or treatment with the sphingomyelin-degrading enzyme, sphingomyelinase, has no effect on transepithelial anion secretion. Since the majority of apical anion conductance in colonic epithelia is mediated by CFTR [20], this suggests that raft integrity is not essential for CFTR function.

Anion secretion in epithelial cells depends on the coordinated activity of apical Cl^- channels as well as basolateral Na^+/K^+-ATPases, Na^+-K^+-$2Cl^-$ cotransporters and K^+ channels. The role of rafts in the regulation of Na^+/K^+-ATPase is controversial. Several studies have shown that caveolae do not contain Na^+/K^+-ATPase [16,25], whereas others have suggested that Na^+/K^+-ATPase is present in lipid rafts [26]. These contradictory conclusions were based on the biochemical analysis of proteins found in caveolae, and could be related to differences in the experimental methods used to isolate cell membrane microdomains (e.g., in the presence or absence of Triton X-100). Our results indicate that disruption of basolateral rafts has no effect on Na^+/K^+-ATPase activity in colonic epithelia. In addition, pharmacological studies showed that the Na^+-K^+-$2Cl^-$ cotransporter does not play a role in the response to basolateral lipid raft disruption.

A number of distinct K^+ channels have been identified on the basolateral membrane of mammalian colon [18]. In our earlier studies we have shown that K_{ATP}, but not BK channels, were tonically active and controlled baseline anion secretion in murine colonic epithelia [19]. The results of this study further expand those observations by showing that lipid rafts control the activity of basolateral BK channels. Under baseline conditions these channels are found mainly in the high-density pellet fraction, and are inactive, since charybdotoxin has no effect on the baseline I_{sc}. Following MβCD treatment, they translocate out of the pellet fraction and become active, resulting in cell hyperpolarization and stimulation of anion secretion. The fact that pimaric acid-induced BK channel activation is inhibited after MβCD treatment suggests that BK channels may either be fully activated by cholesterol removal or that pimaric acid activates only those BK channels that are associated with lipid rafts.

Although lipid rafts are by definition recovered within low buoyant density fractions, subsets of lipid rafts may associate with the cytoskeleton. For example, multivalent ligation of the Fc receptor in neutrophils results in translocation into high buoyant density lipid rafts [27,28]. Other studies have shown that a subset of plasma membrane skeleton proteins co-isolate with cholesterol-rich, detergent-resistant membrane fragments that exhibit a high buoyant density in sucrose [29]. Similarly, clustering the hyaluronan receptor CD44 promotes association with lipid rafts and the redirection of actin bundles into the raft [30]. Mass spectrometric analysis of lipid raft proteomes, including our own studies, identifies the structural components of the intermediate filament and actin cytoskeleton as frequent components of low-density detergent-insoluble fractions [29,31–35]. Actin is a common component of such fractions and clustering raft-based glycolipids or GPI-linked proteins

promotes local organization of F-actin [36]. Similarly, cortical F-actin spikes originate within the caveolae of adipocytes [37]. Thus, although their exact roles remain to be determined, we have speculated that lipid rafts regulate cytoskeletal assembly and, conversely, that cytoskeletal assemblies with lipid rafts might provide submembranous scaffolds for the assembly and function of signaling molecules [34]. According to this view, the functions of lipid rafts and the assembly of cortical cytoskeletal assemblies are reciprocally linked. If lipid rafts are closely associated with F-actin assembly, then those rafts, which are most tightly associated with the cytoskeleton, might be expected to segregate with the cytoskeletal pellet following detergent extraction. In the current study failure to identify BK channels within the low buoyant density fractions demonstrates that BK channels are not associated with conventional lipid rafts. However, translocation of BK channels from the cytoskeleton to the detergent-soluble fraction on lowering cholesterol would be compatible with the disruption of cytoskeletally associated lipid rafts, and with the redistribution of caveolin from both low- and high-density fractions to those of intermediate density. The absence of BK channels from low-density domains indicates that if BK channels associate with lipid rafts they do so only as a very late event and may therefore be components of a subset of lipid rafts that associate exclusively with the cytoskeleton.

Many studies of lipid rafts have been conducted in transformed cell lines that are non-polarized. In polarized epithelia the cytoskeleton is most elaborated on the baso-lateral margin. It therefore seems likely that apically located lipid rafts would be recovered within the low buoyant density fractions whereas basolaterally located rafts would associate with the cytoskeleton. The cytoskeleton can provide a scaffold for the assembly, juxtaposition and three-dimensional orientation of signaling complexes. The mechanism of BK channel activation by MβCD is presently unknown. However, the fact that under control conditions BK channels are found in the pellet fraction suggests that they are constitutively present in large macromolecular complexes that maintain the channel in the closed state. Cholesterol removal could cause the breakup of these structures leading to BK channel translocation into a lighter fraction and simultaneous removal of inhibitory interactions that hold the channel in the closed state. A similar mechanism has been recently proposed for adenylyl cyclase activation, based on the observation that treatment with MβCD augmented the enzyme activity [38]. Lipid rafts are also known to contain proteins that are involved in Ca^{2+} homeostasis [14,26], and changes in $[Ca^{2+}]_i$ due to lipid raft disruption could lead to BK channel activation. However, a recent study has shown that basal levels of $[Ca^{2+}]_i$ in MβCD-treated and untreated cells were identical [39].

The physiological significance of BK channel regulation by lipid rafts, in particular cholesterol, is presently unknown. However, in vivo studies show that feeding mice with *n*-3 polyunsaturated fatty acids may reduce cell membrane cholesterol content in colonic epithelia by 46%, without altering total cellular levels [40]. The results of this study indicate that such a change would have a major effect on transepithelial anion secretion in the colon. Similarly, changes in membrane cholesterol concentration could be related to reverse cholesterol transport, the process whereby cholesterol is removed from peripheral tissues and is delivered to the liver for subsequent excretion into bile [41]. The ability of peripheral cells to participate in reverse cholesterol transport has been proposed to be essential in establishing the proper cholesterol distribution in cells. Measurements of the cholesterol efflux from epithelial cells have shown that cholesterol efflux occurs less readily from the apical than the basolateral membrane [42], a result consistent with the observed effect of cholesterol removal on I_{sc} reported in this study. In summary, we have shown that lipid raft disruption stimulates transepithelial anion secretion by a mechanism compatible with the activation of basolateral BK channels in colonic epithelial cells. These channels are of particular interest because they transform intracellular signals into changes in membrane conductance, and are thought to be loci where the regulation of anion secretion is accomplished.

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