The vascular endothelium is richly endowed with caveolae, which are specialized membrane microdomains that facilitate the integration of specific cellular signal transduction processes. We found that the large conductance Ca\(^{2+}\)-activated K\(^+\) (BK) channels are associated with caveolin-1 in bovine aortic endothelial cells (BAECs). OptiPrep gradient cell fractionation demonstrated that BK channels were concentrated in the caveolae-rich fraction in BAECs. Immunofluorescence imaging showed co-localization of caveolin-1 and BK channels in the BAEC membrane. Immunoprecipitation and glutathione S-transferase pull-down assay results indicated that caveolin-1 and BK channels are physically associated. However, whole cell patch clamp recordings could not detect BK (iberiotoxin-sensitive) currents in cultured BAECs under baseline conditions, even though the presence of BK mRNA and protein expression was confirmed by reverse transcription-PCR and Western blots. Cholesterol depletion redistributed the BK channels to non-caveolar fractions of BAECs, resulting in BK channel activation (7.3 ± 1.6 pA/pF, n = 5). BK currents were also activated by isoproterenol (ISO, 1 μM, 6.9 ± 2.4 pA/pF, n = 6). Inclusion of a caveolin-1 scaffolding domain peptide (10 μM) in the pipette solution completely abrogated the effects of ISO on BK channel activation, whereas inclusion of the scrambled control peptide (10 μM) did not inhibit the ISO effects. We have also found that caveolin-1 knockdown by small interference RNA activated BK currents (3.5 ± 1.4 pA/pF, n = 6). We conclude that: 1) BK channels are targeted to caveolae microdomains in vascular endothelial cells; 2) caveolin-1 interacts with BK channels and exerts a negative regulatory effect on channel functions; and 3) BK channels are inactive under control conditions but can be activated by cholesterol depletion, knockdown of caveolin-1 expression, or ISO stimulation. These novel findings may have important implications for the role of BK channels in the regulation of endothelial function.

The vascular endothelium plays a pivotal role in the regulation of vascular function such as blood pressure control (1), vascular remodeling (2), prevention of inappropriate thrombogenesis, and maintenance of vessel wall integrity (3). Caveolae are ubiquitous and prominent features of endothelial cells, comprising 95% of cell surface vesicles and about 15% of endothelial cell volume (4). In vascular endothelium, caveolae regulate nitric oxide (NO)\(^1\) homeostasis and critically control Ca\(^{2+}\) signaling through the microdomain targeting of Ca\(^{2+}\) influx channels and pumps (5). It is now clear that caveolae and its signature protein, caveolin, provide platforms for integrating specific cellular signal transduction processes by harboring important components of cellular signaling cascades, including G proteins, kinases, endothelial nitric-oxide synthase, hormone receptors, neurotransmitters, and growth factors (6, 7). Insight into the role of caveolin-1 on vascular physiology is provided by studies on caveolin-1 knock-out mice (8–11), in which caveolae structures are absent (8). These animals are viable and fertile but exhibit severe pulmonary and vascular abnormalities. In addition, their systemic NO levels are dramatically elevated, and they develop pulmonary hypertension and cardiomyopathy. These findings confirm the regulatory role of caveolae on NO signaling and vascular homeostasis.

The Ca\(^{2+}\)-activated K\(^+\) channels are important ion channels, linking the metabolic state and cellular Ca\(^{2+}\) homeostasis with cellular excitability. All three types of Ca\(^{2+}\)-activated K\(^+\) channels, the small conductance, intermediate conductance, and large conductance Ca\(^{2+}\)-activated K\(^+\) (BK) channels have been found in endothelial cells (12–14). The most important and commonly observed Ca\(^{2+}\)-activated K\(^+\) channels in blood vessels are BK channels, which are ubiquitous and present in virtually all tissues except in myocardium (15–17). However, whether these channels exist in vascular endothelial cells is still controversial (18). BK channels were first reported in cultured bovine aortic endothelial cells (BAECs) (19), and a later report confirmed their presence in a similar preparation (20). BK channels are also present in primary cultured pig coronary artery endothelial cells (21–23) and in human umbilical vein endothelial cells (24, 25). In addition, BK channels have been observed in freshly isolated rabbit aortic endothelial cells (26) but not in freshly isolated bovine coronary endothelial cells (18). The activity of BK channels in vascular

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1 The abbreviations used are: NO, nitric oxide; BK channel, large conductance Ca\(^{2+}\)-activated K\(^+\) channel; BAEC, bovine aortic endothelial cell; CD, methyl-β-cyclodextrin; GST, glutathione S-transferase; IBTX, iberiotoxin; ISO, isoproterenol; RT, reverse transcription; siRNA, small interfering RNA; AP, antennapedia; PBS, phosphate-buffered saline; pF, picofarads; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/ERK kinase; hSlo, human BK channel α-subunit; hSlo-V5, hSlo with a C-terminal V5 epitope tag.
endothelial cells could control K+ efflux, membrane potential, and affect intracellular Ca2+ concentration (27), but how the endothelial BK channels are regulated is unknown.

In this study, we provide compelling evidence that BK channels are targeted to caveolae in BAECs. Such membrane microdomain targeting involves the interaction between BK channels and caveolin-1, resulting in a negative regulatory effect on BK channel functions. These results suggest a possible new mechanism of ion channel regulation and help to provide new insights in the regulation of endothelial cell function.

MATERIALS AND METHODS

Materials—The following antibodies were used: custom-made polyclonal anti-BK α-subunit antibody raised in rabbits against a peptide (IKTKEAQRKINGSSQADOTLKLKVIDE) of the bovine BK α-subunit (NCBI accession number AA634318), polyclonal anti-caveolin-1 (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-caveolin-1 (BD Transduction Laboratories), monoclonal anti-clathrin (BD Transduction Laboratories), monoclonal anti-Golgi 58 (Sigma), monoclonal anti-V5 (Invitrogen), and monoclonal anti-actin antibodies (Oncogene Research, San Diego, CA). Cavitrin (28), a peptide containing the putative scaffold domain of caveolin-1 (amino acids 101–110, DTGKAKKSTTSTTVYKWFYR), and AP-Cav-X, the scrambled control peptide (WGIDKAFFSMTFVYKWFYR), were synthesized as fusion peptides to the C terminus of the antennapedia (AP) internalization sequence (RQKIKV-FQNRRMKWKK), purified, and analyzed by reversed-phase high-pressure liquid chromatography and mass spectrometry by the Expression Proteomics and Protein Chemistry Facility at the Mayo Clinic. Methylated siRNAs (with 5′-modified 2′-deoxyuridine) of the bovine BK channel (FTKTEAQKINNGSSQADGTLKPVDE) of the bovine BK subunit (5′-GCCGTATTCGCTCGACCAAT-3′ and right primer: 5′-GCCGTATTCGCTCGACCAAT-3′), and C-terminal BKα and monoclonal anti-caveolin-1 antibodies.

Preparation of Caveolin-rich Membrane Fractions and Immunoprecipitation—To prepare the caveolin-rich fractions, 50 μl of recombinant GST-caveolin-1 fusion protein was created by subcloning the human caveolin-1 cDNA (in pcDNA3) into the GST fusion protein vector, pGEX-4T-2 as previously described (32). GST-caveolin-1 and GST constructs were transformed into BL21 (DE3), induced with isopropyl-1-thio-β-D-galactopyranoside (1 mM) for 3 h, and lysed by sonication with lysosome (200 μg/ml) in a buffer containing 20 mM HEPES (pH 7.2), 100 mM KCl, 2 mM MgCl2, 1 mM dithiothreitol, and 0.1% Triton X-100, pH 7.2. The complexes were solubilized in 40 μl of Laemmli sample buffer, resolved in SDS-PAGE, and blotted against anti-BK and monoclonal anti-caveolin-1 antibodies.

Western Blot Analysis—Proteins were separated by 4–15% SDS-PAGE. The proteins were electroblotted onto nitrocellulose membranes, blocked with 5% non-fat dry milk in Tris-HCl, 0.1 mM EDTA, 2 mM EGTA, and 0.1% Tween 20, washed with PBS, and probed with primary antibodies. After washing, detection was performed by horseradish peroxidase-conjugated goat antirabbit antibody (1:1000 dilution), followed by washing, and then detection with SuperSignal West Pico Chemiluminescent Substrate (Pierce) with an X-ray film. Blots were scanned with a high-resolution scanner (HiScan, GE Healthcare). Western blots were analyzed using ImageJ software (NIH).

Confluent Immunofluorescence Microscopy—BAECs were plated onto Lab-Tek chamber slides (Nunc, Naperville, IL), fixed with ice-cold methanol (100% for 15 min), and then permeabilized with 0.1% Triton X-100 in PBS for 2 min. After incubation with 10% normal goat serum in PBS for 30 min, cells were incubated with a monoclonal anti-caveolin-1 antibody (1:200 dilution) plus a polyclonal anti-BK α-subunit antibody (1:100 dilution). The controls were incubated with normal goat serum or pre-immune rabbit serum for 1 h. The primary antibodies were detected with fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (1:1000 dilution) or with Texas Red-conjugated goat-anti-rabbit secondary antibody (1:500 dilution) and mounted in antifade medium (ProLong Gold). Cells were washed with PBS after both the primary and the secondary antibody incubations. Cells nuclei were stained with Hoechst 33258. Cells were visualized using a confocal laser microscope (LSM 510, Zeiss, Germany) with a 100× oil immersion lens.

Caveolin-1 Knockdown by Small Interfering RNA—A caveolin-1 siRNA duplex corresponding to bovine caveolin-1 mRNA targeting against the open reading frame, 223–241 bases (5′-CCA GAA GGA ACA DAA GC-3′ and 5′-CCG GAT AGG CAC CAG-3′), was synthesized and purchased from Dharmacon (Thermo Fisher Scientific). siRNA duplexes were transfected into BAECs using the DharmaFECT delivery reagent according to the manufacturer’s instructions. Results were analyzed by Western blotting, fluorescence microscopy, and flow cytometry.

Preparation of Caveolin-rich Membrane Fractions and Immunoprecipitation—To prepare the caveolin-rich fractions, 50 × 10^6 BAECs were harvested, homogenized in cold buffer A (0.25 mM sucrose, 1 mM EDTA, and 20 mM Tricine, pH 7.8), layered onto 30% Percoll, and centrifuged at 84,000 × g for 30 min (28, 30). The plasma membrane fraction was collected and brought to a volume of 2 ml with buffer A. The crude membrane fraction was then sonicated three times for 5 s each, resuspended in a 23% solution of OptiPrep, and then placed in a centrifuge tube. A linear 20% to 10% OptiPrep gradient was layered on top and centrifuged at 52,000 × g for 90 min. Eight fractions (1.5 ml each, the lightest in fraction 1 and the heaviest in fraction 8) were collected for further analysis. Cholesterol depletion of BAECs was achieved by incubation with 10 mM of the cholesterol binding drug, CD, in serum-free medium for 1 h, and the cells were fractionated by density gradient centrifugation as in control cells. Immunoprecipitation of caveolin-associated proteins was performed as previously described with modifications (31). Briefly, the caveolin-enriched fraction (fraction 2) of the density gradient was diluted with lysis buffer (50 mM NaCl, 50 mM NaF, 50 mM sodium pyrophosphate, 5 mM EDTA, 5 mM EGTA, 0.1 mM NaVO4, 1% Triton X-100, 10 mM HEPES, pH 7.4) containing protease inhibitors (1.04 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 15 μM pepstatin A, 14 μM E-64, 40 μM bestatin, 20 μM leupeptin, and 0.8 μM aprotinin), and procelerated by incubation for 2 h with protein-G-agarose beads (30 μl). Precleared supernatants were incubated with 2 μg of polyclonal anti-caveolin-1 antibody at 4 °C overnight with gentle mixing, followed by the addition of 25 μl of recombinant protein-G-agarose and continued incubation for another 2 h. The immune complexes were collected by centrifugation at 2000 × g for 5 min, washed two times with 0.7 ml of buffer 1 (500 mM KCl, 10 mM Tris, 1 mM dithiothreitol, and 0.1% Triton X-100, pH 7.6) and two times with buffer 2 (20 mM HEPES, 2 mM EGTA, 10 mM MgCl2, 1 mM dithiothreitol, and 0.1% Triton X-100, pH 7.2). The complexes were solubilized in 40 μl of Laemmli sample buffer, resolved in SDS-PAGE, and blotted against anti-BK and monoclonal anti-caveolin-1 antibodies.

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CA GAG U-tTdT-3') and a negative control siRNA (5'-GGG CGC UUU GUA GGA UUC G-tTdT-3') were selected for caveolin-1 knockdown (33). siRNA duplex oligonucleotides were purchased from Dharmacon (Lafayette, CO). BAECs at 70% confluence were used for the transfection of siRNA. Different concentrations (20, 30, and 60 nM) of siRNAs were tested in the transfections using Lipofectamine 2000 (Invitrogen). Fresh medium was added 5 h after transfection, and the cells were analyzed 48 h after transfection.

Electrophysiology Studies—Whole cell current recordings were performed as previously described (34). BAECs seeded on coverslips were placed in a 1-mL chamber on the stage of an inverted microscope. The averaged cellular capacitance of BAECs was 11.9 ± 0.6 pF (n = 28). Bath solution (in mM: NaCl 145, KCl 4, MgCl2 1.0, CaCl2 1.0, HEPES 10, glucose 10, pH 7.4) was superfused through the chamber at 1–2 ml/min. Borosilicate glass capillary patch pipettes (Corning 7056, Warner Instrument, Grand Hamden, CT) were fire-polished. The electrical resistance when filled with the pipette solution (in mM: KCl 140, MgCl2 0.5, Na2ATP 5.0, Na2GTP 0.5, HEPES 10, EGTA 1.0, pH 7.2, and CaCl2 was added to provide 1.0 μM free Ca2+) as calculated using Chelator software) was usually 1–5 MΩ, and the typical seal resistance was >10 GΩ. Whole cell BK currents were recorded with an Axopatch 200B integrating amplifier (Axon Instruments, Foster City, CA), and the output of the amplifier was filtered through an 8-pole low pass Bessel filter at 1 kHz and digitized at 12-bit resolution (10-kHz sampling rate, Digidata 1200, Axon). Subsequently, the data were acquired using pClamp 8.0 software (Axon) with a personal computer for further offline analysis. The effect of drugs on BK currents was measured by pClamp 8.0 software (Axon) with a personal computer for further offline analysis. The averaged cellular capacitance of BAECs was 11.9 ± 0.6 pF (n = 28). Bath solution (in mM: NaCl 145, KCl 4, MgCl2 1.0, CaCl2 1.0, HEPES 10, glucose 10, pH 7.4) was superfused through the chamber at 1–2 ml/min. Borosilicate glass capillary patch pipettes (Corning 7056, Warner Instrument, Grand Hamden, CT) were fire-polished. The electrical resistance when filled with the pipette solution (in mM: KCl 140, MgCl2 0.5, Na2ATP 5.0, Na2GTP 0.5, HEPES 10, EGTA 1.0, pH 7.2, and CaCl2 was added to provide 1.0 μM free Ca2+) as calculated using Chelator software) was usually 1–5 MΩ, and the typical seal resistance was >10 GΩ. Whole cell BK currents were recorded with an Axopatch 200B integrating amplifier (Axon Instruments, Foster City, CA), and the output of the amplifier was filtered through an 8-pole low pass Bessel filter at 1 kHz and digitized at 12-bit resolution (10-kHz sampling rate, Digidata 1200, Axon). Subsequently, the data were acquired using pClamp 8.0 software (Axon) with a personal computer for further offline analysis. The effect of drugs on BK currents was measured by pClamp 8.0 software (Axon) with a personal computer for further offline analysis.

RESULTS

BK Channels Are Present in BAECs—Because the existence of BK channels in vascular endothelial cells is controversial (18–22, 24), we first sought to determine whether BK channels are present in BAECs by measuring BK channel mRNA in these cells. Total RNA was isolated from BAECs and treated with DNase I. The cDNAs obtained by RT were amplified using primers specific to a C-terminal sequence of the bovine BK α-subunit as described under “Experimental Procedures.” No template and no RT negative controls were used to rule out possible reagent and genomic DNA contamination. Expression of BK channel mRNA in BAECs was confirmed as shown in Fig. 1A.

To determine whether BK channel protein is expressed in BAECs, we used a rabbit polyclonal anti-BK α-subunit antibody that we developed against the peptide KTKEAQQKR-INNSGGAQDVGLKPVDE. This sequence shares a 100% identity with the human, rat, and bovine BK channel α-subunit sequences. The specificity of the antibody was verified by immunoblotting with rat coronary artery lysates showing a single band at about 130 kDa (Fig. 1B), and incubation of the antibody with the above peptide would completely block the binding of the antibody to the BK bands (Fig. 1B, lower panel). Fig. 1C shows the Western blot analysis of BK channel expression in BAEC lysates. The results demonstrated that the BK α-subunit is present in BAECs.

To further determine that functional BK channels are present in BAECs, we performed whole cell patch clamp recordings in BAECs. Under baseline conditions, there were no discernible BK (IBTX-sensitive) currents in cultured BAECs (Fig. 1D, left panel). This is consistent with the previous observation that BK channel densities were very low in cultured endothelial cells under baseline conditions and were observed in 4% of the patches (19). However, BK currents in BAECs could be activated by 1 μM isoproterenol (ISO) (Fig. 1D, right panel). At a holding potential of −60 mV and a testing potential of +100 mV, whole cell K+ currents increased from a baseline of 8.7 ± 1.7 pA/Pf to 21.7 ± 5.4 pA/Pf with ISO (n = 6, p = 0.03 versus baseline), and reduced to 14.6 ± 3.4 pA/Pf with ISO plus 100 nM IBTX (n = 6, p = 0.04 versus ISO), suggesting that more than half of the ISO effects was from BK channel activation. Fig. 1D shows the I-V relationships of whole cell K+ currents at baseline, with 1 μM ISO, and with 1 μM ISO plus 100 nM IBTX. These results suggested that BK channels are expressed and detectable at the mRNA, protein, and functional levels in BAECs. It is interesting that these channels are inactive under baseline static culture conditions but could be activated upon stimulation of the β-adrenergic receptor.

BK Channels Are Targeted to Caveolae in BAECs—Caveolae-rich membrane fractions were prepared using a detergent-free sucrose density gradient method as previously described (29, 30). The cell lysates were separated into eight 1.5-ml fractions (numbered 1 through 8, with sample 1 being the lightest (top) fraction). Immunoblot analysis of the fractions against anti-BK α-subunit antibody (Fig. 2A, upper panel) and anti-caveolin-1 antibody (Fig. 2A, lower panel) demonstrated that BK channels were present in the caveolae-rich fraction (fraction 2). BK channel proteins were also observed in a heavier fraction (fraction 6), which contained Golgi and clathrin-associated membranes as indicated in Fig. 2B. These results suggested that BK channels are targeted to the cholesterol-rich, low buoyant density caveolae-rich fraction of the BAEC membrane.

To confirm that the caveolae-rich fraction is selectively enriched with caveolae, we performed Western blot analysis on the density gradient cell fractions examining for the presence of the clathrin heavy chain, a non-lipid raft membrane marker, and for Golgi 58, a subcellular organelle marker. Clathrin appeared predominantly in the heavier fractions of the gradient (fractions 6–8) and was absent in fraction 2, suggesting the exclusion of clathrin-coated pits as well as clathrin-associated membranes from our caveolae-rich fraction (Fig. 2B, upper panel). The Golgi apparatus was similarly distributed as clathrin in the density fractions, suggesting that the low buoyant density caveolae-rich fraction was relatively free of contamination by intracellular organelles (Fig. 2B, lower panel). These results suggested that the targeting of BK channels to caveolae microdomains is specific.

To further demonstrate that BK channels are localized in cholesterol-enriched membrane microdomains, we treated BAECs with CD. This treatment depletes cholesterol from the plasma membrane and causes the loss of compartmentalization of caveolae-associated molecules (35–37). Our results showed that treatment with CD disrupted the targeting of BK channels, along with caveolin-1, to the low buoyant density membrane fraction. Fraction 2 was depleted in caveolin-1 and devoid of BK channels, which were found only in the heavy fractions (Fig. 2C), in contrast to the distribution without CD treatment (Fig. 2A). These results suggested that BK channel distribution on the cell membrane follows that of caveolin-1 and is regulated by the cellular cholesterol content.

BK Channels and Caveolin-1 Are Co-localized in the BAEC Membrane—To further determine whether BK channels are targeted to caveolae, BAECs were double-labeled with antibodies against the BK channel α-subunit and caveolin-1 followed by confocal immunofluorescence microscopic analysis (Fig. 3). The BAEC membrane was strongly labeled with both BK channels (Fig. 3A) and caveolin-1 (Fig. 3B). When these images were merged, there was clear colocalization of BK channel and caveolin-1.
caveolin-1 on the BAEC membrane but not in the cytoplasm or intracellular compartments (Fig. 3D).

**BK Channels Are Associated with Caveolin-1—**To determine whether BK channels in BAECs are physically associated with caveolin-1, we performed co-immunoprecipitation experiments. The caveolae-rich fraction (No. 2) was incubated with a polyclonal anti-caveolin-1 antibody to precipitate caveolin-1 and its associated proteins. The immune complexes were collected with protein G beads and analyzed by immunoblotting against anti-BK and monoclonal anti-caveolin-1 antibodies. These results indicated that the BK channels in the caveolae-rich fraction were co-precipitated with caveolin-1 (Fig. 4), suggesting that BK channels were targeted to the caveolae microdomains. In contrast, the negative control, which contained fraction 2 and protein G beads but not anti-caveolin-1 antibody, no BK channel or caveolin-1 was precipitated.

To further confirm the association between BK channels and caveolin-1, we performed in vitro GST pull-down assays using recombinant GST-caveolin-1 fusion protein incubated with the lysate of HEK293T cells expressing hSlo-V5. Fig. 5A (left panel) demonstrates the purity of the GST-caveolin-1 fusion protein, which is represented by a single dominant protein band with the corresponding molecular size in the Coomassie Blue-stained SDS-polyacrylamide gel. Efficient expression of hSlo-V5 in HEK293T cells, a highly transfectable derivative of the HEK293 cell line, which does not express detectable levels of endogenous BK channels and caveolin-1, was also confirmed by Western blot analysis (Fig. 5A, right panel). After incubation of the GST-caveolin-1 fusion protein with hSlo-V5-containing cell lysate or with control lysate, bound proteins were washed extensively, resolved, and analyzed by immunoblotting. Specific binding between recombinant hSlo-V5 protein and the GST-caveolin-1 fusion was detected. Conversely, no binding was detected between hSlo-V5 and GST alone, or between GST-caveolin-1 and the lysate of cells not transfected with hSlo-V5 (Fig. 5B). These results indicated that there is close interaction between the BK channel and caveolin-1.

**Interaction with Caveolin-1 Regulates BK Channel Function—**Cholesterol binds directly to caveolin-1 (38), and depletion of cholesterol prevents the formation of functional caveolae and inhibits functions of signaling molecules to this membrane microdomain (39). Hence, we tested the effect of cholesterol...
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FIG. 2. Western blot analysis of BAEC density gradient fractions. A, Western blot analysis of BAEC lysate density gradient fractions. An equal volume of each fraction was loaded onto each lane. Enrichment of caveolin-1 in fraction 2 indicated the successful separation of caveolae/lipid raft fraction (lower panel). The results showed that BK channels and caveolin-1 were co-fractionated to the low buoyant density fraction 2. B, clathrin and Golgi 58, the non-lipid raft plasma membrane and intracellular organelle marker proteins, respectively, are predominantly detected in the heavy fractions (fractions 6–8), but not in the caveolae-rich fraction (fraction 2). C, BAECs were treated with 10 mM CD for 1 h to deplete cellular cholesterol. The cell homogenates were then run on an OptiPrep density gradient as in control (no CD treatment). Fractions were assayed for BK channel and caveolin-1 by immunoblotting against anti-BK and anti-caveolin-1 antibodies. After treatment with CD, the low buoyant density fractions were devoid of BK channels and depleted in caveolin-1.

FIG. 3. Immunofluorescence imaging analysis of BK channel and caveolin-1 localization in BAECs. BAECs were fixed in methanol and double labeled with polyclonal anti-BK α-subunit antibody (1:100) and monoclonal anti-caveolin-1 antibody (1:200) for indirect immunofluorescence. Fluorescence images were acquired separately for Texas Red and fluorescein for caveolin-1 (B), and Hoechst 33258 for nucleus (C). BK channel and caveolin-1 were co-localized on the BAEC membrane (yellow) when the images were merged (D).

FIG. 4. Co-immunoprecipitation of BK channels with caveolin-1. The low buoyant density caveolae-rich fraction (fraction 2) was incubated with (right lane) or without (left lane) polyclonal anti-caveolin-1 antibody (1.2 μg/ml) overnight at 4 °C. Thirty microliters of protein G-agarose was added to the samples, and the mixture was incubated for an additional h at 4 °C. The immunocomplexes were assessed by gel electrophoresis and Western blot analysis against polyclonal anti-BK antibody (upper panel) and monoclonal anti-caveolin-1 antibody (lower panel).

dearth of BK channel function in BAECs by exposing the cells to the cholesterol-binding antibiotic, filipin, which is a macrolide polyene antibiotic that binds cholesterol and can cause reversible disassembly of caveolae (36, 40). K+ currents in BAECs were recorded using whole cell patch clamp techniques. Exposure to 2.5 μg/ml filipin produced a slow rising but significant increase in K+ currents from 8.7 ± 1.4 pA/pF to 20.0 ± 2.7 pA/pF (n = 5, p = 0.002 versus baseline), and two-third of this filipin effect was sensitive to IBTX (filipin plus IBTX, 12.8 ± 1.8 pA/pF, p = 0.01 versus filipin alone) (Fig. 6). These results suggested that BK channel activity increased as membrane cholesterol was being removed from the cell. Exposure to filipin had no discernible effect for the first 8–10 min, suggesting there is a threshold of cellular cholesterol content below which alterations in BK channel function would become evident. These results, together with those presented in Fig. 2C, suggested that depletion of cholesterol in BAECs interrupted the formation of caveolae, resulting in the redistribution of BK channels to non-caveolae portions of the cell membrane, and this was accompanied by enhancement of the BK currents.

To further determine the functional role of the interaction between caveolin-1 and BK channels in BAECs, we examined the effects of cavtratin (28), a peptide that contains the caveolin-1
A structural scaffold for cell membrane microdomains

**A.** Representative electrophysiology study showing the effect of filipin (2.5 μg/ml) on whole cell K⁺ currents recorded with holding potential at −60 mV, testing potential at +80 mV, and 1 μM free Ca²⁺ in the pipette solution. Filipin produced an increase in K⁺ currents that was slow in onset. B, superimposed raw current tracings at baseline, with filipin (2.5 μg/ml) and filipin plus IBTX (100 nM). C, bar graphs showing group data on K⁺ current densities at baseline, with filipin, and with filipin plus IBTX (n = 5). *, p = 0.002 versus baseline; †, p = 0.01 versus filipin.

**FIG. 6. Effect of cholesterol depletion on BK channel activities.** A, representative electrophysiology study showing the effect of filipin (2.5 μg/ml) on whole cell K⁺ currents recorded with holding potential at −60 mV, testing potential at +80 mV, and 1 μM free Ca²⁺ in the pipette solution. Filipin produced an increase in K⁺ currents that was slow in onset. B, superimposed raw current tracings at baseline, with filipin (2.5 μg/ml) and filipin plus IBTX (100 nM). C, bar graphs showing group data on K⁺ current densities at baseline, with filipin, and with filipin plus IBTX (n = 5). *, p = 0.002 versus baseline; †, p = 0.01 versus filipin.

scaffolding domain and mimics caveolin-1 function, on BK channel activity. Under baseline conditions, BAECs exhibited very little IBTX-sensitive currents (Fig. 7A). BK currents could be activated by exposure to 1 μM ISO (Fig. 7B). With 10 μM of cavaratin in the pipette solution, a 1 μM ISO could no longer activate BK currents in BAECs (Fig. 7C). With a holding potential of −60 mV and a testing potential of +100 mV, the whole cell K⁺ current was 13.9 ± 4.0 pA/pF at baseline, 14.2 ± 4.4 pA/pF with ISO, and 12.2 ± 3.5 pA/pF with ISO plus IBTX (n = 5, p = not significant for all groups). In contrast, with 10 μM of the control scrambled peptide, AP-Cav-X (28), in the pipette solution, the effects of ISO remained intact: the whole cell K⁺ current was 7.8 ± 3.0 pA/pF at baseline, 37.1 ± 27.3 pA/pF with ISO (n = 4, p = 0.04 versus baseline), and 18.8 ± 8.0 pA/pF with ISO plus IBTX (n = 4, p = 0.02 versus ISO) (Fig. 7D). These results suggested that the caveolin-1 scaffolding domain exerted a negative regulatory effect on BK channel function.

**Caveolin-1 Knockdown Results in Activation of BK Channel in BAECs—**To further confirm the negative regulation of BK channels by caveolin-1, we knocked down caveolin-1 expression in BAECs using caveolin-1 siRNA. We found that the caveolin-1 siRNA efficiently suppressed caveolin-1 protein expression in a dose-dependent manner. Forty-eight hours after transfection with 20, 30, and 60 nM siRNA, caveolin-1 protein expression in BAECs was reduced by 85.0%, 90.0%, and 92.5%, respectively. In contrast, control siRNA had no effect (Fig. 8).

Treatment of BAECs with 60 nM caveolin-1 siRNA resulted in activation of BK currents (Fig. 9, A and B). With a holding potential of −60 mV and a testing potential of +100 mV, the whole cell K⁺ currents were 30.2 ± 4.5 pA/pF at baseline (n = 6), and 19.4 ± 3.7 pA/pF with 100 nM IBTX (n = 6). In contrast, after treatment with 60 nM control siRNA, no IBTX-sensitive currents were observed in BAECs (Fig. 9, C and D). These results confirmed that caveolin-1 exerts a negative regulatory effect on BK channel function.

**DISCUSSION**

We reported several major findings in this study. First, we have provided compelling biochemical, immunohistochemical, and electrophysiological evidence that BK channels are targeted to the caveolae microdomains in BAECs. Second, there is physical interaction between the BK channel α-subunit and caveolin-1. Third, interaction with caveolin-1 exerts a negative regulatory effect on BK channel function. These findings indicate that caveolae targeting importantly regulates BK channel activities in vascular endothelial cells. These results also help to understand at least in part the observations that under baseline static culture conditions, BK channels in BAECs are inactive. Caveolin-1 may contribute to the suppression of BK channel activity, because both disruption of normal caveolae structure and knockdown of caveolin-1 protein expression activated the channel, whereas the presence of excessive caveolin-1 scaffolding domain peptide abrogated the increase of BK currents by β-adrenergic stimulation. These novel findings suggest that membrane microdomain targeting may represent a new mechanism of ion channel regulation and may help us further understand the fundamental mechanisms that regulate vascular endothelial function.

Ca²⁺-activated K⁺ channels in smooth muscle cells are important determinants in the regulation of endothelium-mediated vascular relaxation. However, how these channels are regulated in vascular endothelial cells remains unclear. Historically, BK channels have been identified in different cultured and freshly isolated vascular endothelial cells, but progress has been significantly impeded by the very low current densities (<4% in BAECs) (19). Under baseline culture conditions, we have found that BAECs exhibit practically no discernible BK currents. Yet, BK channel mRNA and protein are clearly expressed in these cells. These discrepancies suggest the presence of a negative regulatory mechanism of BK channel function in BAECs. A notable example of such negative regulatory mechanism is the targeting to caveolae microdomains, as in the case of endothelial nitric-oxide synthase. BK channels may be another example. Indeed, caveolin-1 acts as an inhibitor to many components of different signaling cascades, including Src, epidermal growth factor receptor, protein kinase C, G-protein α subunits (41), vascular endothelial growth factor receptor (42), Raf (43), MEK, ERK (43, 44), adenylyl cyclase (45), and protein kinase A (46).

Some ion channels are known to be targeted to lipid rafts and caveolae. These include the L-type Ca²⁺ channels (47, 48), the voltage-gated K⁺ channel Kv1.5 (31), and the voltage-gated Na⁺ channel (49). However, the functional significance of ion channel targeting to caveolae is unknown, and membrane microdomain targeting of BK channels has not been previously reported. In this study, BK channel targeting to caveolae in BAECs is supported by BK and caveolin-1 co-fractionation to low buoyant density cell fractions, by co-localization on BAECs membrane using immunofluorescence analysis, by co-immunoprecipitation using anti-caveolin-1 antibodies, and by GST-caveolin-1 fusion protein pull-down assays. Examination of the primary sequence of the BK channel showed that two consensus caveolin binding motifs (50) are present in the C terminus (1072YNMLCFGIY1080 and 602YTEYLSSAF610), indicating that these regions of the BK α-subunit might be sites for direct interaction with the caveolin-1 scaffolding domain. Thus, direct protein-protein interaction between caveolin and BK channels may provide a possible regulatory mechanism of BK channel function in vascular endothelial cells.
Three lines of evidence support the inhibitory nature of caveolin-1 on BK channel function. First, cholesterol-binding drugs, which deplete BAECs of cholesterol, prevent the formation of caveolae and the microdomain targeting of BK channels (Fig. 2C). Such treatment results in the activation of the BK currents in BAECs (n = 5), suggesting that the binding of BK channels to caveolin has negative regulatory effects on BK channel function. D, with 10 μM AP-Cuv-X, the scrambled control peptide, in the pipette solution, ISO was able to activate the BK current, similar to control (n = 4).

Three lines of evidence support the inhibitory nature of caveolin-1 on BK channel function. First, cholesterol-binding drugs, which deplete BAECs of cholesterol, prevent the formation of caveolae and the microdomain targeting of BK channels (Fig. 2C). Such treatment results in the activation of the BK currents, suggesting that the redistribution of BK channels from caveolae to a non-raft membrane would relieve the channel of inhibition by caveolin-1. Some caveolae-targeted proteins such as the β2-adrenergic and adenosine A1 receptors exit the caveolae upon agonist stimulation (51, 52), whereas others, such as the muscarinic M2 receptors, translocate into caveolae upon activation (53). To determine whether BK channels egress from caveolae upon agonist stimulation, we compared ISO (1 μM, 10 min)-treated and non-treated BAECs by gradient
centrifugation as described under “Experimental Procedures.” Immunoblot analysis showed no significant redistribution of BK channels and caveolin-1 compared with control cells (data not shown). Hence, activated BK channels remain in caveolae, similar to endothelial nitric-oxide synthase (54). Second, exposure of BAECs to cavtatin results in suppression of BK channel activation by ISO. Because cavtatin contains the caveolin scaffolding domain and mimics caveolin-1 function, these results suggest that the presence of excessive scaffolding domain peptide would keep the BK channel bound and prevent it to be activated by β-adrenergic stimulation. Third, transiently and specifically knockdown of caveolin-1 protein expression results in BK channel activation. These results suggest that caveolae targeting inhibits BK channel function under static culture conditions. Because the endothelium is not an excitable tissue, endothelial ion channels do not need to be in a constant active state. Caveolae targeting may provide reservoir function whereby dormant BK channels are positioned in the vicinity of important signaling molecules, allowing tight and efficient channel activation upon stimulation by agonists or other signals. This concept is supported by the recent finding that BK channels bind directly with the β2-adrenergic receptor, forming a macromolecular complex that includes AKA79/150 and protein kinase A (55).

The exact mechanism underlying the β-adrenergic stimulation of BK channel in BAECs is not clear. BK channels are known to be activated by both cAMP-dependent protein kinase and direct G protein mechanisms (56–58). Cavtatin and binding with the caveolin scaffolding domain could interfere with protein kinase A phosphorylation of the BK channel or with the direct interaction between the BK channel and activated Gα. Recently, the voltage-gated Na+ channel was found to be targeted to caveolae in cardiac myocytes (49), and this might be important for the direct G protein activation of the Na+ channel, which might involve the presentation of intracellular Na+ channels from sub-membrane caveolae to the membrane surface. Whether BK channel activation by β-adrenergic stimulation involves a similar mechanism is unclear. There are, however, important differences between the BK channel regulation in BAECs and Na+ channel regulation in cardiac myocytes. Caveolae targeting was not inhibitory to Na+ channel function, and binding of anti-caveolin-3 antibodies would completely block the G-protein activation of the channel, suggesting that

**FIG. 8.** Specific knockdown of caveolin-1 expression in BAECs by siRNA. BAECs at 70% confluence were transfected with 20, 30, and 60 nM caveolin-1 siRNA, or control siRNA. 48 h after transfection, the cells were lysed and analyzed by Western blotting against caveolin-1 and actin antibodies.

**FIG. 9.** Effect of caveolin-1 knockdown on BK channel activities. Whole cell K+ currents in cultured BAECs were elicited with a holding potential of −60 mV and testing potentials of −60 to +150 in 10-mV steps, and with 1 mM free Ca2+ in the pipette solution. A, K+ currents in BAECs under baseline conditions 48 h after transfection with 60 nM caveolin-1 siRNA (left panel, n = 6). The currents were partially inhibited by 100 nM IBTX (right panel, n = 6). B, group data of current-voltage relationships in BAECs after caveolin-1 knockdown by siRNA. BAECs exhibited IBTX-sensitive currents. C, K+ currents in BAECs under baseline conditions 48 h after transfection with 60 nM control siRNA (left panel, n = 6), and the currents were not inhibited by 100 nM IBTX (right panel, n = 6). D, group data of current-voltage relationships in BAECs transfected with control siRNA. BAECs exhibited no IBTX-sensitive currents.
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Na\(^+\) channel targeting to caveolae might facilitate its activation by the G-proteins. Whether \(\beta\)-adrenergic activation of BAEC BK channels involves protein kinase A-dependent or G-protein direct effects and how \(\beta\)-adrenergic stimulation affects BK-channel-1 interaction remain to be determined.

In conclusion, this is the first report that vascular endothelial BK channels are targeted to caveolae microdomains. In addition, BK channels closely interact with caveolin-1, which exerts profound effects on BK channel function. Targeting of the BK channel to caveolae represents a new mechanism of ion channel regulation and would provide the spatial organization to facilitate signal modulation of BK channels. Our results indicate that interaction with caveolin-1 inhibits BK channel function, and BK channels are activated by disruption of caveolae structure or knockdown of caveolin-1 protein expression. These results may help to provide new insights, not only on the regulation of BK channels in endothelial cells, but also on the fundamental mechanisms that regulate endothelial and vascular function.

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