Activity of BK$_{Ca}$ Channel Is Modulated by Membrane Cholesterol Content and Association with Na$^+$/K$^+$-ATPase in Human Melanoma IGR39 Cells

Nobuyoshi Tajima$^a$, Yutaka Itoika$^b$, Esa R. Korpi$^b$, Pentti Somerharju$^b$, and Reijo Kakela$^{a,1}$

From the Institute of Biomedicine, Departments of 4 Medical Biochemistry and Developmental Biology and 5 Pharmacology, University of Helsinki, Helsinki FI-00014, Finland

Interaction of large conductance Ca$^{2+}$- and voltage-activated K$^+$ (BK$_{Ca}$) channels with Na$^+$/K$^+$-ATPase, caveolin-1, and cholesterol was studied in human melanoma IGR39 cells. Functional BK$_{Ca}$ channels were enriched in caveolin-rich and detergent-resistant membranes, i.e. rafts, and blocking of the channels by a specific BK$_{Ca}$ blocker paxilline reduced proliferation of the cells. Disruption of rafts by selective depletion of cholesterol released BK$_{Ca}$ channels from these domains with a consequent increase in their activity. Consistently, cholesterol enrichment of the cells increased the proportion of BK$_{Ca}$ channels in rafts and decreased their activity. Immunocytochemical analysis showed that BK$_{Ca}$ channels co-localize with Na$^+$/K$^+$-ATPase in a cholesterol-dependent manner, thus suggesting their co-presence in rafts. Supporting this, ouabain, a specific blocker of Na$^+$/K$^+$-ATPase, inhibited BK$_{Ca}$ whole-cell current markedly in control cells but not in cholesterol-depleted ones. This inhibition required the presence of external Na$^+$. Collectively, these data indicate that the presence of Na$^+$/K$^+$-ATPase in rafts is essential for efficient functioning of BK$_{Ca}$ channels, presumably because the pump maintains a low intracellular Na$^+$ proximal to the BK$_{Ca}$ channel. In conclusion, cholesterol could play an important role in cellular ion homeostasis and thus modulate many cellular functions and cell proliferation.

Large conductance Ca$^{2+}$- and voltage-activated K$^+$ (BK$_{Ca}$) channels are widely expressed in excitable and nonexcitable cells of mammals. In neurons, BK$_{Ca}$ channels control firing frequency by contributing to repolarization of the action potential (1), and in smooth muscle cells, they participate in maintaining the balance between contraction and relaxation (2). Although the functions of BK$_{Ca}$ channels in excitable cells are relatively well known, those in nonexcitable cells are not.

In tumor cells, BK$_{Ca}$ channels regulate cell volume and shape and contribute to tumor progression and migration (3–5).

A number of ion channels have been shown to localize to specific plasma membrane microdomains enriched in cholesterol and sphingolipids, known as lipid or membrane rafts (6). Rafts are thought to operate as sorting platforms that bring together molecules for efficient cross-talk that controls cellular signaling cascades (7). In addition, biophysical properties of lipids in the immediate microenvironment of ion channels may also affect channel activity (8). Certain channels, such as the L-type Ca$^{2+}$ (9), K$_v$1.5 potassium (10), and TRPC1 channel (11), have been found in caveolae, flask-shaped membrane invaginations, considered as a subtype of rafts (12). A major structural component of caveolae is caveolin-1, which acts as scaffolding protein and plays multiple roles in angiogenesis, tumorigenesis, and cell migration, for example (13). BK$_{Ca}$ channels have two potential caveolin-binding motifs in the C terminus, and at least one of them can mediate association with caveolin-1 (14). In fact, BK$_{Ca}$ channels have been found to reside in caveolae of bovine vascular endothelial cells (15) and human myometrial smooth muscle cells (16). The $\alpha_1$ subunit of Na$^+$/K$^+$-ATPase has also been found in caveolae where it binds to caveolin-1 via specific motifs (17). Na$^+$/K$^+$-ATPase pumps K$^+$ into and Na$^+$ out of the cell thus creating concentration gradients of those ions over plasma membrane. Excitability of neurons and muscle cells is maintained by the resulting electrochemical gradient. In addition, Na$^+$/K$^+$-ATPase is also one of the main players in cellular ionic homeostasis essential for growth, differentiation, and survival of cells (18).

In this study, we addressed possible functional interaction of BK$_{Ca}$ channels with Na$^+$/K$^+$-ATPase and caveolin-1 in human melanoma IGR39 cells. Because all these proteins are assumed to reside in cholesterol-rich membrane domains, their responses to membrane cholesterol level were examined. Our results provide the first evidence for the proximity and functional interaction between the BK$_{Ca}$ channel and Na$^+$/K$^+$-ATPase. This interplay is suggested to depend on the proper microenvironment, i.e. caveolin-rich rafts. Perturbation of the rafts by cholesterol depletion interfered with the association of these proteins and led to altered activity of BK$_{Ca}$ channels.

**Experimental Procedures**

**Antibodies**—Rabbit antibodies used were as follows: anti-BK$\alpha$ (Alomone Laboratories, Jerusalem, Israel); anti-caveo-
lin-1 (N-20) and anti-Na+/K+-ATPase α1 (Cell Signaling Technology, Beverly, MA); and anti-Ki-67 (Novocastra, Newcastle, UK). The mouse antibodies were as follows: anti-BKα (L6/60) (University of California, Davis/NINDS/NIMH NeuroMab Facility, Davis, CA); anti-Na+/K+-ATPase α1 (sc-21712) and anti-β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-caveolin-1, anti-clathrin, and anti-calnexin (BD Transduction Laboratories). Alexa-conjugated secondary antibodies were purchased from Invitrogen.

Cell Culture—Human melanoma IGR1 and IGR39 cells, the former established from a metastatic tumor in groin lymph node and the latter from primary cutaneous tumor, were kindly provided by Dr. Stefan H. Heinemann (Friedrich Schiller University of Jena, Germany). Human glioma U251-MG cells were a gift from Dr. Keiko Funa (University of Gothenburg, Sweden). IGR1, IGR39, and HEK293 cells were grown in DMEM and U251-MG cells in Eagle’s minimum essential medium. The growth media were supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated in humidified atmosphere containing 5% CO2 at 37 °C and split when 80–90% confluent.

RNA isolation and RT-PCR—For reverse transcription-PCR (RT-PCR), 1 μg of total RNA was prepared using TRIzol (Invitrogen) and reverse-transcribed into cDNA with M-MuLV (Finnzymes, Espoo, Finland) and random primers (Invitrogen). PCR was performed by DNAzyme II DNA polymerase (Finnzymes) using a Takara PCR thermal cycler Dice (Takara, Ohtsu, Japan). The primer sequences for BKCa were 5′-CAG CAT TGG CCG TCA GTG TTC 5′ and 5′-CAT GCC TTT TGG TTA TTT TTC C-3′ (19) and for β-actin were 5′-CCA AGG CCA ACC GCC AGA AGA TGA C-3′ and 5′-AGG GTA CAT GGT GTG GCC GCC AGA C-3′ (20). After amplification, the RT-PCR product was analyzed by electrophoresis on 1% agarose gel.

Preparation of Cell Lysate and Total Membrane Fraction—Cells were lysed by a buffer containing 50 mM Tris-Cl, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 1% SDS, pH 7.5, supplemented with complete protease inhibitor mixture (Roche Applied Science), and centrifuged at 12,000 × g for 5 min. Supernatant was collected as cell lysate, and its protein concentration was measured using BCA protein assay kit (Bio-Rad). To prepare total membranes, cells were washed three times with ice-cold PBS and scraped into TNE buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.4) with the protease inhibitors. The cells were then homogenized by injecting 20 times through a 27-gauge needle and centrifuged at 1000 × g for 10 min. The supernatant was centrifuged at 200,000 × g for 1 h at 4 °C using SW41Ti rotor (Beckman Coulter, Fullerton, CA), and the final membrane pellet was resuspended in the lysis buffer.

Manipulation of Cellular Cholesterol Content—IGR39 cells were depleted or enriched with cholesterol (Sigma) by incubating them with methyl-β-cyclodextrin (MβCD, Sigma) or MβCD-cholesterol complex, respectively. To prepare MβCD-cholesterol complexes (molar ratio of 8.6:1), a cholesterol stock solution in chloroform/methanol (1:2 v/v) was dried under nitrogen and then in vacuum. Next, aqueous solution of MβCD was added, and the mixture was vortexed and sonicated for three times for 5 min. This stock solution was then diluted with serum-free DMEM to 50 μg/ml cholesterol. The cells were washed three times with the DMEM and incubated in humidified 5% CO2 at 37 °C in DMEM containing MβCD (5 mM) or MβCD-cholesterol complex (1.12, 0.13 mM).

Analysis of Protein Distribution between Detergent-soluble and -insoluble Membrane Fractions—IGR39 cells were washed twice in cold PBS, scraped into PBS containing protease inhibitors, and centrifuged at 2800 × g for 10 min. The cells were resuspended in 500 μl of TNE buffer and homogenized as described above. Next a detergent, Tween 20, was added to the suspension to obtain a 1% solution. An aliquot of this solution was adjusted to 40% sucrose in TNE and placed in an ultracentrifuge tube, and 4 ml of 35% sucrose and 4 ml of 5% sucrose were added on top, and the tube was centrifuged at 180,000 × g for 18 h in an SW41Ti rotor. Finally, 11 fractions were collected from the top and subjected to immunoblotting. All steps were performed at 4 °C. Soluble fractions 8–11 contained the non-raft marker clathrin and thus represent “non-rafts.” Fractions 1–6 contained the detergent-resistant membranes (DRMs) also known as “rafts.” Among these rafts, the fractions 4–5 were rich in caveolin-1, and thus are called “caveolin-rich rafts.”

Purification of Caveolin-rich Membrane Fraction—To obtain enough caveolin-rich membranes for co-immunoprecipitation (CoIP) assays, a high pH, detergent-free isolation method was used (17). Sodium carbonate was added to the cell homogenate to a concentration of 250 mM. A 2-ml aliquot was adjusted to 40% sucrose and placed in an ultracentrifuge tube. Four ml of 35% sucrose and 4 ml of 5% sucrose were added, and the tubes were centrifuged at 260,000 × g for 18 h in SW41Ti rotor. Caveolin-rich fractions 4 and 5 were combined, diluted with 4 ml of TNE, and centrifuged at 200,000 × g for 1 h in SW41Ti rotor. Pellets were used for CoIP.

Co-immunoprecipitation—CoIP was performed according to Alioua et al. (14). Caveolin-rich membrane pellets obtained as above were resuspended in a solution containing 150 mM NaCl, 50 mM Tris-HCl, 100 mM NaF, 5 mM EDTA, 1 mM Na3VO4, 10 mM HEPES, 0.1% Triton X-100, 0.25% sodium deoxycholate, and protease inhibitors (4 °C, pH 7.4). The lysate was centrifuged at 10,000 × g for 10 min at 4 °C, and an aliquot of the supernatant corresponding to 200 μg of protein was precleared by incubating with 40 μl of protein G-Sepharose (GE Healthcare) for 1 h. The cleared supernatant was incubated with 1 μg of an antibody for overnight and then with 20 μl of protein G-Sepharose for 3 h, washed, eluted with Laemmli buffer containing 1.4 mM mercaptoethanol, and finally used for Western blotting.

Cell Surface Biotinylation—Proportion of BKCa channels on the plasma membrane was studied by biotinylating the surface-exposed proteins. Cell surface protein isolation kit (Pierce) was used according to the manufacturer’s instructions.

Western Blotting—Proteins were separated by SDS-PAGE (6–12% gel) under reducing conditions and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were probed with primary antibodies that were de-
BK<sub>Ca</sub> Channel Interacts with Na<sup>+</sup>/K<sup>-</sup>-ATPase in Rafts

tected using secondary antibodies conjugated with horseradish peroxidase (Bio-Rad) and visualized with enhanced chemiluminescence reagent (GE Healthcare). The bands were quantified using NIH Image image processing program (rsb.info.nih.gov).

**Lipid Analysis**—Lipids in cells, plasma membrane preparations, and culture media were extracted according to Folch et al. (21). Total phospholipid and cholesterol were determined by standard methods (22, 23). To analyze the molecular species of lipids by mass spectrometry, aliquots were spiked with a mixture of 19 di-unsaturated phospholipid or saturated sphingolipid standards and infused in chloroform/methanol (1:2, v/v) containing 1% NH<sub>4</sub>OH (added just prior to the analysis) into a Quattro Micro triple quadrupole mass spectrometer (Micromass, Manchester, UK). The lipids were detected using class-specific detection modes (24, 25) and then identified and quantified as detailed previously (26, 27).

**Preparation of Plasma Membrane Blebs**—Plasma membrane blebs were obtained as described previously (28). Briefly, IGR39 cells were washed twice with PBS and incubated at 37°C in a buffer inducing membrane blebbing (10 mM HEPES, 150 mM NaCl, 2 mM CaCl<sub>2</sub>, 25 mM paraformaldehyde, 2 mM DTT, pH 7.4) for 2 h. Cell debris was removed by centrifuging at 2800 × g for 5 min. Supernatant was centrifuged further at 30,000 × g for 30 min, and the resulting bleb pellet was washed with PBS.

**Immunocytochemistry**—Cells were fixed in 4% paraformaldehyde for 10 min at room temperature, washed three times with PBS, blocked, and permeabilized for 30 min in PBS containing 0.2% Triton, 5% normal goat serum, and 3% BSA, and then incubated with antibodies overnight in a moist chamber at 4°C. Cells were then washed three times with PBS and labeled with Alexa 488 anti-rabbit or Alexa 543 anti-mouse antibodies for 2 h in the dark, washed, and incubated with 4,6-diamidino-2-phenylindole (DAPI, Invitrogen) for 5 min to stain nuclei. Confocal images of 6-μm nominal optical slice thickness were acquired with an LSM 510 Meta microscope (Carl Zeiss GmbH, Jena, Germany). Protein co-localization was determined by Pearson’s correlation analysis between the intensity values of green and red pixels in a dual-channel image (JACoP, rsb.info.nih.gov) (29).

**Cell Proliferation Assays**—Cells were plated on coverslips in 35-mm dishes at a density of 1 × 10<sup>4</sup> cells for 5 μm paraformaldehyde (Sigma) treatment and 5 × 10<sup>4</sup> cells for the high efficacy 10 μM ouabain (Sigma) treatments. After 24 h, the medium was replaced with one containing either paraformaldehyde or ouabain. After 1–3 days, the cells were fixed in 4% paraformaldehyde for 60 min at room temperature. After washing the Ki-67 antigen was exposed by microwave treatment in Tris/EDTA buffer, pH 8.0. Immunocytochemistry was performed as described above. The fraction of Ki-67-positive nuclei was determined by counting triplicate samples containing 200–500 cells.

**Electrophysiology**—Electrophysiological data were recorded at room temperature using an EPC 9 patch clamp amplifier (HEKA Electronik, Lambrecht, Germany), and the currents were filtered at 2.9 kHz and digitized at 10 kHz. Series resistance compensation was adjusted to 60%. Unless stated otherwise, holding potential was −60 mV, and currents were elicited to depolarize cells from −100 mV to +180 mV with an increment of 10 or 20 mV for 100 ms. Data acquisition and analysis were controlled with Pulse, PulseFit (HEKA), and IgorPro (WaveMetrics, Lake Oswego, OR) software. In whole-cell recordings, bath solution contained 135 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, and 10 mM HEPES, pH 7.4 (adjusted with NaOH). The nominally Ca<sup>2+</sup>-free internal solution contained 130 mM KCl, 2 mM MgCl<sub>2</sub>, 10 mM EGTA, and 10 mM HEPES, pH 7.4 (with KOH). To obtain ~600 nM free cytosolic [Ca<sup>2+</sup>], 8.8 mM CaCl<sub>2</sub> was added to the internal solution as calculated using MAXCHELATOR. To quantify the voltage dependence of BK<sub>Ca</sub> activation, the conductance was plotted against voltage normalized to that at 180 mV and then the following Boltzmann Equation 1 was fitted to the data,

\[
G = \frac{G_{180\, mV}}{1 + \exp(V_{1/2} - V)/k}
\]  
(Eq. 1)

where \(G_{180\, mV}\) is the conductance at +180 mV; \(V\) is the membrane potential; \(V_{1/2}\) is the half-activation voltage, and \(k\) is the slope factor. Single-channel recordings were performed in the excised inside-out or on-cell patch configuration. Current records were low pass filtered at 1 kHz and digitized at 10 kHz. For single-channel recordings, high symmetrical K<sup>+</sup> solutions were used. The bath solution contained 140 mM KCl and 10 mM HEPES for inside-out and 140 mM KCl, 1 mM MgCl<sub>2</sub>, and 10 mM HEPES for on-cell patch configuration, and the pipette solution contained 140 mM KCl, 2 mM MgCl<sub>2</sub>, and 10 mM HEPES (all adjusted to pH 7.4 with KOH). The probability of a single channel being open (\(P_o\)) was calculated using the expression shown in Equation 2,

\[
P_o = \sum N_o t_o / NT
\]  
(Eq. 2)

where \(T\) is the total recording time; \(N\) is the number of channels in the patch; \(N_o\) is the number of channels open, and \(t_o\) is the time during which \(N_o\) channels are open. To evaluate \(P_o\), continuous recordings of 1–3 min were performed, and recordings from patches containing 1–2 channels were analyzed. The maximum number of simultaneously open channels at high voltage (~250 mV) was defined as the number of channels per patch. Analysis of single-channel properties was performed using Clampfit 10.0 (Axon Instruments, Union City, CA).

**Statistical Analysis**—Data are expressed as means ± S.E. Paired or unpaired two-tailed Student’s t tests were used, and \(p < 0.05\) was regarded as significant.

**RESULTS**

**Functional BK<sub>Ca</sub> Channels Are Present in Human Melanoma IGR39 Cells**—Two types of Ca<sup>2+</sup>-activated K<sup>+</sup> (K<sub>Ca</sub>) channels that do not depend on membrane depolarization are known to be expressed in human melanoma IGR1 and IGR39 cells, i.e. the intermediate conductance (hK<sub)i</sub> or hSK4) and small conductance (hSK1–3) K<sup>+</sup> channels (20, 30). We used RT-PCR to examine whether a BK<sub>Ca</sub> channel, which is activated by both membrane depolarization and cytosolic Ca<sup>2+</sup>, is expressed in these cells. The mRNA for the pore-forming α subunit of BK<sub>Ca</sub> was detected in IGR39 but not IGR1 cells.
revealed a bimodal distribution of BKCa, BKCa protein in total membranes and whole-cell lysates of IGR39 cells as a negative control, and β-actin as an internal control. Western blot analysis of the BKCa channel (−135 kDa) in cell lysate (20 μg) and total membranes (30 μg). C, representative immunofluorescence image of BKCa channels in IGR39 cells (scale bar, 20 μm). D, representative whole-cell currents (evoked by depolarization voltage steps from −100 to +180 mV for 100 ms in 10-mV increments) and current-voltage relationship (I/V) at 0 (○, n = 21) or 600 nM (●, n = 12) [Ca2+]i. E, inhibition of the whole-cell current by paxilline. Voltage steps are from −100 to +180 mV for 100 ms in 20-mV increments (n = 8).

(BKCa) Channels Contribute to Proliferation of IGR39 Cells—BKCa and some other K+ channels have been implicated in tumor proliferation, because they generate transient hypopolarization needed for the exit from the G1 phase of the cell cycle. This hypopolarization is accomplished by K+ efflux via the channels (31, 32). To find out whether BKCa channels of IGR39 cells are involved in proliferation, we monitored the proliferation rate by measuring the fraction of Ki-67-positive cells for 3 days in the presence of the BKCa channel blocker paxilline. Paxilline reduced cell proliferation rate by 52% during a 72-h incubation (Fig. 2, A and B). Thus, the BKCa channel activity contributes to cell proliferation.

Plasma Membrane BKCa Channels Reside in DRMs—Because BKCa channels have previously been found in caveolae of endothelial and smooth muscle cells (15, 16) and the channel contains two binding motifs for caveolin-1, we checked whether the caveolin-1 is expressed in IGR39 cells. Caveolin-1 was present in cell lysates and membranes of IGR39 cells but not detectable in those of IGR1 cells (Fig. 3A). To examine if BKCa channels are associated with caveolin-1, cell lysates were treated with a detergent, and DRMs were isolated using a sucrose density gradient. A strong immunoreactive band corresponding to BKCa was detected in DRM fractions 4–5, whereas only faint bands were visible in soluble fractions (Fig. 3B). Caveolin-1 was also enriched in the DRM fractions 4–5, although clathrin, a non-raft marker, was exclusively found in soluble fractions. Thus, we conclude that BKCa channels concentrate in the caveolin-rich DRMs of IGR39 cells.
BK<sub>Ca</sub> Channel Interacts with Na<sup>+</sup>/K<sup>+</sup>-ATPase in Rafts

**Cholesterol Depletion Dissociates BK<sub>Ca</sub> Channels from DRMs and Alters Their Intracellular Distribution**—Caveolin associates with cholesterol and also scaffolds other proteins (33). To study such interactions, plasma membrane cholesterol content can be manipulated with MβCD (34). To establish conditions that would allow selective depletion of cellular cholesterol content without affecting phospholipid composition of IGR39 cells, we carried out pilot experiments in which the concentration of MβCD or treatment time was varied. Treatment of cells with 5 mM MβCD for 30 min reduced cellular cholesterol content by 41% (Fig. 3, C and D), although no change in the composition of other lipids was observed (supplemental Fig. S2, A and B). To address cholesterol depletion of the plasma membrane, we determined the cholesterol content of the plasma membrane blebs isolated from cholesterol-depleted and control cells. Incubation with MβCD removed 68% of bleb cholesterol (Fig. 3D). Next, we studied whether cholesterol depletion influenced BK<sub>Ca</sub> distribution between the DRM and soluble fractions. The BK<sub>Ca</sub> channels as well as caveolin-1 did indeed shift toward soluble fractions (Fig. 3E). In DRMs, the amount of caveolin-1 was reduced from 50 to 31%, and a corresponding increase was found in combined soluble fractions (Fig. 3F and supplemental Fig. S3). Likewise, cholesterol depletion reduced the proportion of BK<sub>Ca</sub> in DRMs from 62 to 45% with a corresponding increase in soluble fractions (Fig. 3F and supplemental Fig. S3).

To test whether BK<sub>Ca</sub> channels and caveolin-1 associated with each other, we performed coIP experiments using the caveolin-rich membrane fractions of IGR39 cells (supplemental Fig. S4). Indeed, BK<sub>Ca</sub> channels were detected in the immunoprecipitate obtained using an antibody against caveolin-1 (Fig. 4A). Such co-precipitation was also detected when immunoprecipitation was carried out by using an antibody against BK<sub>Ca</sub> (Fig. 4A). Thus, a large fraction of BK<sub>Ca</sub> channels appears to reside in caveolin-rich rafts.

In control IGR39 cells, BK<sub>Ca</sub> and caveolin-1 had very similar bimodal intracellular distributions, i.e. they were located in the perinuclear regions and at the plasma membrane. Cholesterol depletion caused the channels to redistribute more evenly throughout the cell (Fig. 4B). Despite this major change in the subcellular distribution of caveolin-1 and BK<sub>Ca</sub>, correlation analysis of the immunocytochemistry images showed that the degree of co-localization between BK<sub>Ca</sub> α subunit and caveolin-1 did not decrease (Table 1 and Fig. 4B).

**Cholesterol Depletion Increases BK<sub>Ca</sub> Whole-cell Current by Altering Single-channel Activity**—Cholesterol depletion remarkably increased the whole-cell BK<sub>Ca</sub> current in IGR39 cells (Fig. 5A). Statistically significant difference in the current density between control and cholesterol-depleted cells emerged at +80 mV. At +140 mV, the current increased from 95 ± 3 pA/pF (n = 21) in control cells to 251 ± 10 pA/pF (n = 21) in cholesterol-depleted cells. In cells with 600 nM [Ca<sup>2+</sup>]<sub>in</sub>, cholesterol depletion also markedly increased current amplitude (supplemental Fig. S5A). To study whether the voltage dependence of BK<sub>Ca</sub> activation was affected by cholesterol depletion, the conductance was plotted against voltage, and the Boltzmann function was fitted to the data. Cholesterol depletion shifted the mean V<sub>1/2</sub> by −17.2 mV, although k remained at ~22 mV (Fig. 5B and supplemental Table S1). However, in the cells with 600 nM [Ca<sup>2+</sup>]<sub>in</sub>, cholesterol depletion caused only a minor shift of the mean V<sub>1/2</sub> by +1.5 mV, although k increased by 3.9 mV (Fig. 5B and supplemental Table S1).

The increase in BK<sub>Ca</sub> whole-cell current by cholesterol depletion may be due to an increase in the number of functional BK<sub>Ca</sub> channels at the plasma membrane or altered single channel properties, i.e. increased conductance or activity. Cell-surface biotinylation showed that the fraction of BK<sub>Ca</sub> protein at cell surface was unaltered by cholesterol depletion (Fig. 5, C and D). Next, long term single-channel recordings were performed to check if alteration of channel properties increased whole-cell currents in cholesterol-depleted cells. Because excised membrane patches were found to be prone to mechanical damage, on-cell patch configuration was used. Cholesterol depletion only slightly increased the single-channel conductance from 174 ± 2 pS (n = 10) to 187 ± 3 pS at +100 mV (n = 12) but dramatically increased the open probability (P<sub>o</sub>) (Fig. 5, E and F), from 0.014 ± 0.003 (n = 10) in control cells to 0.094 ± 0.020 (n = 12) in cholesterol-depleted cells. We conclude that the increase of whole-cell current by cholesterol depletion was due to altered properties of individual channels in the plasma membrane, rather than altered distribution of channels between plasma membrane and intracellular compartments.

**Cholesterol Enrichment Increases BK<sub>Ca</sub> in DRMs and Decreases BK<sub>Ca</sub> Whole-cell and Single-channel Currents**—To complement the cholesterol depletion experiments, we examined how cholesterol enrichment of the cell membranes af-
fected $BK_{Ca}$ activity. When IGR39 cells were incubated with the MβCD-cholesterol complex for 120 min, cellular cholesterol content increased by 128% (Fig. 6, A and B) without detectable change in the contents of other lipids (data not shown). With 100 μg/ml cholesterol, no additional increase of cellular cholesterol was found (Fig. 6A). After treating the cells with MβCD-cholesterol complex, the cholesterol content of isolated membrane blebs, presumably reflecting that of the plasma membrane as a whole, increased by 120% as compared with the blebs from control cells (Fig. 6B). Next, we examined whether the distribution of $BK_{Ca}$ between sucrose density gradient fractions was affected by cholesterol loading.

As expected, cholesterol enrichment increased the proportion of $BK_{Ca}$ in DRMs from 46 to 67%, although that of caveolin-1 increased from 70 to 87% (Fig. 6, C and D, and supplemental Fig. S6).

Cholesterol loading of the cells resulted in a significant decrease in $BK_{Ca}$ current density (Fig. 7A). A statistically significant difference emerged at +80 mV, and at +140 mV the average current densities in control and cholesterol-enriched cells were 120 ± 6 pA/pF ($n = 19$) and 54 ± 3 pA/pF ($n = 19$), respectively. Cholesterol loading also decreased the average current density at 600 nM $[Ca^{2+}]_{\text{in}}$ (supplemental Fig. S5B). Next, the effect of cholesterol loading on the voltage-de-
BK<sub>Ca</sub> Channel Interacts with Na<sup>+</sup>/K<sup>+</sup>-ATPase in Rafts

FIGURE 4. BK<sub>Ca</sub> channel interacts with caveolin-1 in membrane rafts. A, Co-IP assay of caveolin-1 and BK<sub>Ca</sub> channels using caveolin-rich membranes isolated at high pH. BK<sub>Ca</sub> channels were detected in immunoprecipitates (IP) obtained with an antibody against caveolin-1 (upper blot), and caveolin-1 was detected in immunoprecipitates obtained with an antibody against BK<sub>Ca</sub> channel (lower blot). Note that BK<sub>Ca</sub> channels and caveolin-1 were not precipitated by IgG. B, immunocytostaining of caveolin-1 and BK<sub>Ca</sub> channels in the control and MβCD-treated cells (scale bar, 15 μm in the control, and 10 μm in the MβCD images).

TABLE 1
Correlation analysis of caveolin-1 (Cav1), BK<sub>Ca</sub> (BK), and Na<sup>+</sup>/K<sup>+</sup>-ATPase (NaK) co-localization in control and cholesterol-depleted (by MβCD) IGR39 cells based on Pearson’s coefficient

|          | Untreated | MβCD | n  | p      |
|----------|-----------|------|----|--------|
| BK-Cav1  | 0.69 ± 0.03 | 0.64 ± 0.02 | 5  | NS     |
| NaK-Cav1 | 0.71 ± 0.01 | 0.75 ± 0.02 | 4  | NS     |
| BK-NaK   | 0.71 ± 0.02 | 0.45 ± 0.04 | 5  | p < 0.05 |

*NS means not significant.

dependent activation of BK<sub>Ca</sub> channel was quantified using the Boltzmann equation. Cholesterol loading shifted the mean V<sub>1/2</sub> by +17.5 mV, and k increased by 3.8 mV (Fig. 7B and supplemental Table S1). In contrast, at 600 nM [Ca<sup>2+</sup>]<sub>i</sub>, no significant difference in these parameters between the cholesterol-loaded and control cells was found (Fig. 7B and supplemental Table S1).

The cholesterol-loaded cells displayed reduced BK<sub>Ca</sub> single-channel activities (Fig. 7C). The mean open probability was significantly decreased from 0.016 ± 0.002 (n = 10) in control cells to 0.008 ± 0.001 (n = 9) in cholesterol-loaded cells (Fig. 7D). The average single channel conductance at +100 mV was also slightly decreased from 175 ± 1 pS (n = 10) in the control to 161 ± 2 pS (n = 9) in cholesterol-loaded cells (Fig. 7D). Thus, the decrease in BK<sub>Ca</sub> whole-cell current upon cholesterol enrichment most probably results from altered single-channel properties rather than from altered surface concentration of the channels.

BK<sub>Ca</sub> Channels and Na<sup>+</sup>/K<sup>+</sup>-ATPase Interact in DRMs Depending on Membrane Cholesterol Level—Na<sup>+</sup>/K<sup>+</sup>-ATPase, which also contains caveolin-binding motifs in the α subunit, has been found in caveolae of epithelial cells and was suggested to be modulated by the level of cholesterol (17). Therefore, we examined whether Na<sup>+</sup>/K<sup>+</sup>-ATPase is present in the DRM fractions of IGR39 cells as well. In control cells, 70% of Na<sup>+</sup>/K<sup>+</sup>-ATPase was detected in caveolin-rich DRM fractions 4–5. In cholesterol-depleted cells, the amount of Na<sup>+</sup>/K<sup>+</sup>-ATPase in these fractions was reduced slightly (to 62%), but most Na<sup>+</sup>/K<sup>+</sup>-ATPase still remained in total DRMs (Fig. 8, A and B, and supplemental Fig. S7). Immunocytochemistry showed that Na<sup>+</sup>/K<sup>+</sup>-ATPase and caveolin-1 co-localized in the perinuclear regions (Fig. 8E), and correlation analysis of the images revealed that cholesterol depletion did not affect their co-localization (Table 1). Not unexpectedly, cholesterol loading of the cells increased the proportion of Na<sup>+</sup>/K<sup>+</sup>-ATPase in the DRMs from 75 to 89% (Fig. 8, C and D, and supplemental Fig. S7).

Presence of Na<sup>+</sup>/K<sup>+</sup>-ATPase in the caveolin-rich DRMs, where BK<sub>Ca</sub> was also found, led us to study interactions between Na<sup>+</sup>/K<sup>+</sup>-ATPase and BK<sub>Ca</sub> channel. Based on immunocytochemistry, both proteins appeared to be located around the nucleus and at the edge of lamellipodia of control cells (Fig. 8E). We performed CoIP assays using the caveolin-rich membranes from untreated cells. Na<sup>+</sup>/K<sup>+</sup>-ATPase co-purified with both BK<sub>Ca</sub> and caveolin-1 (Fig. 8, F and G). We confirmed that neither BK<sub>Ca</sub> nor Na<sup>+</sup>/K<sup>+</sup>-ATPase co-purified with the non-raft protein clathrin (supplemental Fig. S8). This result excludes the possibility that BK<sub>Ca</sub> and Na<sup>+</sup>/K<sup>+</sup>-ATPase co-purify merely because they are in the same membranes rather than associate in the rafts. Importantly, when cholesterol depletion redistributed BK<sub>Ca</sub> channels and Na<sup>+</sup>/K<sup>+</sup>-ATPase throughout the cells, the degree of co-localization of these proteins was reduced, as evidenced by significantly decreased Pearson’s coefficients of correlation in the immunostained images (Table 1). Thus, our results indicate that BK<sub>Ca</sub> channels reside in the same DRMs as Na<sup>+</sup>/K<sup>+</sup>-ATPase and caveolin-1 and that the proximity between BK<sub>Ca</sub> and Na<sup>+</sup>/K<sup>+</sup>-ATPase requires sufficient cholesterol content of the membrane.

Inhibition of Na<sup>+</sup>/K<sup>+</sup>-ATPase Decreases BK<sub>Ca</sub> Current in Control but Not in Cholesterol-depleted Cells—The indirect association of BK<sub>Ca</sub> channels and Na<sup>+</sup>/K<sup>+</sup>-ATPase in caveolin-rich DRMs could have functional consequences (see under “Discussion”). Accordingly, we performed electrophysiological experiments to see if the BK<sub>Ca</sub> channel current is affected when Na<sup>+</sup>/K<sup>+</sup>-ATPase is inhibited by ouabain. To obtain a more reproducible signal, Rb<sup>+</sup> instead of K<sup>+</sup> was used as the current carrier. Aside from this replacement, very similar data were obtained with these two cations (data not shown). Paxiline (1 μM) inhibited the Rb<sup>+</sup> current almost completely, thus
confirming that this current was due to the BK<sub>Ca</sub> channels (data not shown). We recorded whole-cell Rb<sup>+</sup>/H<sub>11001</sub> current using a single step from a holding potential of −60 mV to +140 mV every 20 s, and then we applied 100 μM ouabain. In control cells, BK<sub>Ca</sub> current was reduced within <1 min after addition of ouabain and decreased gradually reaching a minimum (35 ± 1% of the control current) in ~5 min (Fig. 9, A and B). As expected, this effect of ouabain was irreversible. Strikingly, in cholesterol-depleted cells, ouabain had no significant effect (6 ± 3% inhibition) on BK<sub>Ca</sub> current (Fig. 9, A and B). We concluded that most of functional BK<sub>Ca</sub> channels in untreated cells seem to reside in the cholesterol- and caveolin-rich DRMs where they are strongly inhibited by oua-
bain, Na<sup>+</sup>/K<sup>+</sup>-ATPase inhibitor. Cholesterol depletion segregated BK<sub>Ca</sub> channels from Na<sup>+</sup>/K<sup>+</sup>-ATPase, and consequently the indirect channel inhibition by ouabain was lost. It is noteworthy that ouabain did not have any direct effect on BK<sub>Ca</sub> current, because no inhibition was observed in the cholesterol-depleted cells.

Increase of Intracellular [Na<sup>+</sup>] Due to Na<sup>+</sup>/K<sup>+</sup>-ATPase Inhibition Is Responsible for the Reduction of BK<sub>Ca</sub> Current of Untreated Cells—To examine whether the reduction of BK<sub>Ca</sub> current by ouabain is due to diminished outward pumping of Na<sup>+</sup> by the inhibited Na<sup>+</sup>/K<sup>+</sup>-ATPase, we replaced Na<sup>+</sup> with D-mannitol in the bath solution and recorded whole-cell Rb<sup>+</sup>/H<sub>11001</sub> current as explained above. Importantly, ouabain had no sig-

FIGURE 5. Cholesterol depletion increases BK<sub>Ca</sub> channel activity. A, representative whole-cell currents in control (○) and cholesterol-depleted (●) cells. Voltage steps from −100 to +180 mV for 100 ms in 20-mV increments are shown. I/V curves represent mean whole-cell currents recorded for control (○, n = 21) and MβCD-treated (●, n = 21) cells. B, voltage dependence of the normalized conductance of control (○, zero), 600 nM [Ca<sup>2+</sup>]<sub>i</sub> and MβCD-treated (●, zero, ■, 600 nM [Ca<sup>2+</sup>]<sub>i</sub>) cells based on the data in A and supplemental Fig. S5A, respectively. A Boltzmann function was fitted to the data. Solid and dashed lines represent the fits for control and MβCD-treated cells, respectively. C, biotinylation assay for BK<sub>Ca</sub> channels exposed at the cell surface. The cells were incubated in FBS-free medium with or without 5 mM MβCD for 30 min and then labeled with membrane-impermeant biotinylating reagent sulfo-NHS-SS-biotin for 30 min at 4 °C followed by Western blot. Endoplasmic reticulum marker, calnexin, was used as a negative control. D, bar graph shows the fraction of BK<sub>Ca</sub> channels at cell surface in control (n = 3) and MβCD-treated (n = 3) cells. E, representative recordings of single-channel current at +100 mV in control and MβCD-treated cells. Lower traces show a part of the upper trace magnified. Horizontal bars on the left indicate closed state of the channel. F, comparison of unitary channel conductance in control (Cont) (E, n = 10) and MβCD-treated (●, n = 12) cells (left) and mean open probability (P<sub>o</sub>) in control (○, n = 10) and MβCD-treated (●, n = 12) cells (right) at +100 mV. **, p < 0.01 compared with control cells.
significant effect (2 ± 1% inhibition) on the BK<sub>Ca</sub> current under these conditions (Fig. 10, A and B) thus indicating that extracellular Na<sup>+</sup> is needed for the ouabain-induced inhibition of BK<sub>Ca</sub> current in untreated cells. To confirm that a rise in intracellular Na<sup>+</sup> concentration (dependent on external Na<sup>+</sup> source when the pump is not active) inhibits BK<sub>Ca</sub> current, we measured the Rb<sup>+</sup> current by the BK<sub>Ca</sub> channel in cells being in D-mannitol solution at varying [Na<sup>+</sup>]<sub>i</sub>. Rise in [Na<sup>+</sup>]<sub>i</sub> caused strong reduction of the BK<sub>Ca</sub> current (Fig. 11, A and B). The average current densities at +140 mV at 0, 1, 10, and 20 mM [Na<sup>+</sup>]<sub>i</sub> were 251 ± 8 (n = 11), 153 ± 7 (n = 11), 97 ± 5 (n = 11), and 69 ± 2 pA/pF (n = 10), respectively (Fig. 11, B and C). Thus, the local [Na<sup>+</sup>]<sub>i</sub> needed to induce the observed 65% reduction of the BK<sub>Ca</sub> current at +140 mV would be ~10 mM. Because extracellular Na<sup>+</sup> was essential for ouabain to inhibit the BK<sub>Ca</sub> current, a rise in [Na<sup>+</sup>]<sub>i</sub> due to the blocking of Na<sup>+</sup>/K<sup>+</sup>-ATPase by ouabain, is the most likely explanation for the decreased BK<sub>Ca</sub> current.

DISCUSSION

This study provides the first evidence that a human melanoma cell harbors functional BK<sub>Ca</sub> channels in caveolin-rich membrane rafts. In IGR39 cells, approximately one-half of the functional BK<sub>Ca</sub> channels were found in rafts. Manipulation of cellular cholesterol content altered the distribution of BK<sub>Ca</sub> channels between raft and non-raft domains, which significantly affected their function. Cholesterol depletion released the channels from rafts and increased their activity, whereas cholesterol enrichment had an opposite effect. Importantly, both effects were reversible. For example, the elevated BK<sub>Ca</sub> whole-cell current of cholesterol-depleted cells decreased again when the medium was supplemented with cholesterol (supplemental Fig. S9). These data suggest that during the manipulations of membrane cholesterol content the BK<sub>Ca</sub> channels maintained their integrity.

The greatly increased BK<sub>Ca</sub> activity in cholesterol-depleted cells suggests that in nonmanipulated IGR39 cells, the BK<sub>Ca</sub> channels residing in caveolin-rich rafts are partially inhibited. Rafts may appese BK<sub>Ca</sub> channels by accommodating them in domains with strictly controlled ion milieu, specific protein-protein interactions, or specific lipid environment. Dissociation of rafts may then release BK<sub>Ca</sub> channels from such inhibitory molecular interactions. In contrast to our study, in human glioma cells BK<sub>Ca</sub> current was reported to decrease...
when the cells were depleted of cholesterol by MβCD (35). However, in colon epithelial cells, the BKCa current was found to increase in response to MβCD, as happened in our study with melanoma cells (36). Cholesterol level may modulate interactions of BKCa channels with nearby proteins, which may differ depending on the cell type, resulting in different indirect responses.

Caveolin is known to bind cholesterol and act as a scaffolding protein assembling functional protein complexes in rafts. In IGR39 cells, manipulation of cellular cholesterol content caused caveolin-1 to redistribute between DRMs and soluble fractions as expected (Fig. 3, A and B, and Fig. 6, C and D). CoIP assays demonstrated that BKCa and caveolin-1 are present in the same domains (Fig. 4A). BKCa channels have been suggested to bind to caveolin-1 directly via their C-terminal caveolin-binding motifs (14). In that report, when expressed in HEK cells, caveolin-1 reduced the number of BKCa channels at the cell surface, resulting in decreased BKCa current. However, this was not the case in IGR39 cells, in which the disruption of caveolin-rich rafts and partial dissociation of the putative functional units of proteins did not change the fraction of BKCa at the cell surface (Fig. 5, C and D). Although we cannot fully exclude the possibility that caveolin-1 may down-regulate surface expression of BKCa in IGR39 cells, disruption of membrane rafts by MβCD treatment (30 min) did not affect the number of BKCa channels at the plasma membrane. Thus, in our study, increased open probability and conductance are the most likely reasons for the stimulation of BKCa activity in cholesterol-depleted cells, although decreased open probability and conductance underlie the inhibition of BKCa channels in cholesterol-loaded cells (Fig. 5, E and F, and Fig. 7, C and D).

Although the molecular details underlying the changes in single-channel activity in response to cellular cholesterol level are not clear, manipulation of cellular cholesterol content may have affected the physicochemical properties of the plasma membrane surrounding the BKCa channel, such as lateral stress and curvature, which could affect channel activity as has been reported for gramicidin and sodium channels (37). In fact, it has been reported that cholesterol content of plasma membrane correlates inversely with the BKCa activity because of altered membrane fluidity that affects kinetic properties of the channels (38). The MβCD treatment (5 mM, 30 min) used to lower the cholesterol content in our study did not result in detectable changes in cellular contents of other membrane lipids (supplemental Fig. S2). Another possibility is that cholesterol depletion makes the membrane thinner, thus reducing the free energy difference between the closed and open state of the channel. Indeed, bilayer thickness has been found to modulate BKCa activity in vitro (39). Yet another possibility is that cholesterol binds to BKCa channels. Sensitivity of Kir 2.1 channel to cholesterol was recently shown to depend on a specific C-terminal cytosolic domain of the channel (40). Because cholesterol and caveolin-1 form a scaffold for other proteins, interaction with caveolin-1 is also a possi-

![FIGURE 7. Cholesterol enrichment decreases BKCa channel activity. A, representative whole-cell currents in control (○) and cholesterol-enriched (●) cells (voltage steps from −100 to +180 mV for 100 ms with 20-mV increments). I/V curves represent average whole-cell currents recorded from control (○, n = 19) and cholesterol-enriched (●, n = 19) cells. B, voltage dependence of the normalized conductance of control (C; zero, ○) and cholesterol-enriched (●; 600 nM [Ca\(^{2+}\)]\(_{1/2}\)) cells based on the data in A and supplemental Fig. S8, respectively. A Boltzmann function was fitted to the data. Solid and dashed lines represent the fits for control and cholesterol-enriched cells, respectively. C, representative recordings of single-channel currents in control (○, n = 10) and cholesterol-enriched (●, n = 9) cells (**, p < 0.05; **, p < 0.01).

![FIGURE 7. Cholesterol enrichment decreases BKCa channel activity. A, representative whole-cell currents in control (○) and cholesterol-enriched (●) cells (voltage steps from −100 to +180 mV for 100 ms with 20-mV increments). I/V curves represent average whole-cell currents recorded from control (○, n = 19) and cholesterol-enriched (●, n = 19) cells. B, voltage dependence of the normalized conductance of control (C; zero, ○) and cholesterol-enriched (●; 600 nM [Ca\(^{2+}\)]\(_{1/2}\)) cells based on the data in A and supplemental Fig. S8, respectively. A Boltzmann function was fitted to the data. Solid and dashed lines represent the fits for control and cholesterol-enriched cells, respectively. C, representative recordings of single-channel currents in control (○, n = 10) and cholesterol-enriched (●, n = 9) cells (**, p < 0.05; **, p < 0.01).
factor modulating BKCa activity, as reported previously for N-type calcium channels (41).

Since its discovery in 1957 (42), numerous studies have been conducted to elucidate the role of Na+/H+ -ATPase in normal physiological as well as pathophysiological processes. By using energy derived from ATP hydrolysis, Na+/H+ -ATPase pumps Na+ out of and K+ into the cytoplasm (43). Besides establishing gradients of Na+ and K+ across the plasma membrane to maintain membrane potential, Na+/K+ -ATPase also plays an important role in regulating intracellular homeostasis of various other ions, osmolarity, and pH. Recently a novel function was suggested for the Na/K-ATPase in the control of plasma membrane cholesterol distribution (44). In IGR39 cells, most (70%, Fig. 8) of the Na+/H+ -ATPase was found in rafts, and manipulation of the cellular cholesterol content caused only a minor shift between raft and non-raft fractions, suggesting that strong protein-protein interactions keep them in rafts (Fig. 8, A–D, and supplemental Fig. S7). In line with this, Na+/K+ -ATPase and caveolin-1 co-immunoprecipitated (Fig. 8F). Consistently, an association of Na+/K+ -ATPase 1 and caveolin-1 via specific binding motifs has been reported previously for various epithelial cell lines (17, 45). Immunocytochemistry experiments showed that in untreated cells Na+/K+ -ATPase and caveolin-1 co-localized, and following cholesterol depletion, they were redistributed throughout the cell (Fig. 8E). However, according to correla-
BKCa Channel Interacts with Na\(^{+}/K^{+}\)-ATPase in Rafts

**FIGURE 9.** Functional association of BKCa channels and Na\(^{+}/K^{+}\)-ATPase. 
A, effect of ouabain on whole-cell BKCa currents in control or M\(\beta\)CD-treated cells. BKCa currents were elicited by pulses from −60 to +140 mV every 20 s. Representative currents were recorded before addition of ouabain (a) and after addition of ouabain for control (○, upper left) or M\(\beta\)CD-treated (●, upper right) cells (b and c, respectively). Bath solution was perfused with dissolved ouabain at 0.65 ml/min by gravity. Lower plot of A shows the time course of BKCa currents for ouabain addition in control or M\(\beta\)CD-treated cells. Scaled peak current amplitude is plotted as a function of time. Rb\(^{+}\) was used as a current carrier for BKCa current measurements as in Fig. 9. NaCl (130 mM) in the bath solution was replaced by D-mannitol (240 mM), and the pH in the bath solution was adjusted to 7.4 with Tris base. 

B, relative inhibition (%) of BKCa currents by ouabain in control (○, n = 15) and M\(\beta\)CD-treated (●, n = 11) cells (***, p < 0.001).

**FIGURE 10.** Involvement of extracellular [Na\(^{+}\)] in BKCa current inhibition by ouabain. A, effect of ouabain on whole-cell BKCa currents in untreated cells in the absence of extracellular Na\(^{+}\). BKCa currents were elicited by pulses from −60 to +140 mV every 20 s. Representative currents recorded before (a) and after (b) addition of ouabain are shown. Lower plot of A displays the time course of BKCa current upon ouabain addition. Scaled peak current amplitude is plotted as a function of time. Rb\(^{+}\) was used as a current carrier for BKCa current measurements as in Fig. 9. NaCl (130 mM) in the bath solution was replaced by D-mannitol (240 mM), and the pH in the bath solution was adjusted to 7.4 with Tris base. B, inhibition of BKCa currents by ouabain (○, n = 11).

In untreated IGR39 cells, BKCa channels were found in the same DRM fractions as Na\(^{+}/K^{+}\)-ATPase (Fig. 8G). As evidenced by immunocytochemistry, upon cholesterol depletion, these proteins were not only redistributed but also segregated away from each other (Fig. 8E and Table 1), which entails that their association requires a normal cholesterol level. We speculate that although the association between caveolin-1 and the BKCa channel/ATPase is relatively strong, the interaction between BKCa channel and Na\(^{+}/K^{+}\)-ATPase when associated directly or indirectly in rafts is weaker and requires cholesterol-rich raft environment and possibly also caveolin-1 as the scaffolding protein. Supporting this view, whole-cell record-ings showed that the response of BKCa current to ouabain was very different in control and cholesterol-depleted cells (Fig. 9, A and B). Since the observed ~65% inhibition of BKCa current amplitude by ouabain corresponds to the fraction of BKCa in rafts isolated in the sucrose gradient assay (47–62%, Fig. 3, C and D), it entails that most BKCa channels in rafts were affected by ouabain. These data thus strongly support the view that BKCa channels are functionally associated with Na\(^{+}/K^{+}\)-ATPase in the caveolin-rich rafts of IGR39 cells. Supporting such interactions, the β1 subunit of Na\(^{+}/K^{+}\)-ATPase was recently shown to associate with the BKCa α subunit in chick ciliary ganglion neurons, as well as to affect the current of heterologous BKCa channels (46).

Why does ouabain, an inhibitor of Na\(^{+}/K^{+}\)-ATPase, affect BKCa current? We propose that local ionic homeostasis close to the caveolin-rich rafts harboring the BKCa channels is controlled by Na\(^{+}/K^{+}\)-ATPase. The human Na\(^{+}/K^{+}\)-ATPase is highly sensitive to and typically irreversibly inhibited by ouabain (47, 48). Addition of 100 μM ouabain has been reported to completely block endogenous Na\(^{+}/K^{+}\)-ATPase current of HEK and HeLa cells within 20–50 s (48, 49). Thus, 100 μM ouabain apparently halts ion pumping by Na\(^{+}/K^{+}\)-ATPase...
**BKCa Channel Interacts with Na⁺/K⁺-ATPase in Rafts**

![Diagram](image)

**FIGURE 11.** Rise in intracellular [Na⁺] decreases the BKCa whole-cell current in concentration-dependent manner. A, representative whole-cell Rb⁺ current by BKCa channels at different [Na⁺] in. The Rb⁺-base pipette solution (cf. in Fig. 8) was supplemented with 1, 10, and 20 mM NaCl, and a bath solution containing 0.5-mannitol (240 mM) instead of NaCl was used (cf. Fig. 9). Voltage steps were from −100 to +180 mV in 20-mV increments, each for 100 ms. B, /V curves that represent mean whole-cell currents recorded in cells with 0 (●, n = 11), 1 (▲, n = 11), 10 (●, n = 11), and 20 mM (○, n = 10) [Na⁺] in. C, comparison of mean current amplitude at +140 mV using the data in A. The curve represents single-exponential fit to the data.

rapidly, which results in gradual intracellular depletion of K⁺ and accumulation of Na⁺. The BKCa channels in rafts may then be affected by the low K⁺ and high Na⁺ concentrations close to the inner leaflet of the membrane. Since this ouabain-induced inhibition of the BKCa current is an indirect response, the ~1 min delay in this response found in this study was to be expected. Previous studies with human umbilical vein endothelial cells also suggest that inhibition of Na⁺/K⁺-ATPase and subsequent accumulation of intracellular Na⁺ decreases BKCa current (50). We found that in untreated cells in the complete absence of extracellular [Na⁺], ouabain failed to inhibit BKCa channel current (Fig. 10), and when [Na⁺] was raised intracellularly in the pipette, the BKCa current was suppressed in a concentration-dependent manner (Fig. 11). The results infer that inhibition of Na⁺/K⁺-ATPase by ouabain leads to an accumulation of Na⁺ close to the microdomains harboring BKCa channels, which inhibits the channel function. Segregation of BKCa channels and Na⁺/K⁺-ATPase upon disruption of rafts allows the BKCa channels to escape from this inhibitory milieu (supplemental Fig. S10).

There is evidence that Na⁺/K⁺-ATPase can mediate extracellular signals elicited by ouabain and other cardiac glycosides by recruiting signaling proteins such as Src family kinases in rafts (51, 52). Binding of cardiac glycosides to Na⁺/K⁺-ATPase may activate signaling cascades employing inositol 1,4,5-trisphosphate receptors and phospholipase C, thus releasing Ca²⁺ from endoplasmic reticulum (52, 53). However, it is unlikely that intracellular Ca²⁺ release would be responsible for the modulation of BKCa channel activity by ouabain in IGR39 cells, because in that case the current amplitude of BKCa should have been increased, but it did not. Thus, in IGR39 cells, Na⁺/K⁺-ATPase seems to primarily control Na⁺ homeostasis close to BKCa channels rather than act via signaling pathways affecting Ca²⁺. Interestingly, cardiac glycosides have also been found to prevent proliferation of cancer cells (54). In accordance, ouabain treatment for 72 h markedly reduced the number of proliferating IGR39 cells, i.e. from 80 to 13% (data not shown). This could be due to the inhibition of BKCa (indirectly via Na⁺/K⁺-ATPase) by ouabain. However, involvement of other mechanisms/pathways cannot be excluded.

The results of this study have some intriguing physiological implications. Most importantly, the stability of rafts and thus the activity of BKCa channels could be modulated by cholesterol status *in vivo*. For instance, the activity of BKCa in the vascular endothelium and colon epithelium has been shown to be influenced by their cholesterol content (15, 36, 55). The functional interaction of BKCa channel with Na⁺/K⁺-ATPase, a novel finding of this study, suggests that BKCa can also be modulated by the activity of Na⁺/K⁺-ATPase. Like BKCa, the activity of Na⁺/K⁺-ATPase is sensitive to membrane cholesterol content (56). Remarkably, even a minor (1–2 mol%) change in cholesterol content alters the activity of Na⁺/K⁺-ATPase in model membranes as much as 30% (57). The activity of BKCa channels *in vivo* could thus be modulated by the cellular cholesterol status either directly or indirectly via the activity of Na⁺/K⁺-ATPase.

The activity of reconstituted Na⁺/K⁺-ATPase also depends on the degree of phospholipid acyl chain unsaturation (58). It was proposed that phospholipid unsaturation affects membrane thickness and/or lateral organization thereby modulating the activity of Na⁺/K⁺-ATPase. Such a modulation could operate *in vivo* as well because a diet rich in n-3 polyunsaturated fatty acids has been shown to reduce the cholesterol content of colon epithelial membranes by 46% in mice (59). Possible alterations in BKCa channel activity in these conditions remain to be studied.

BKCa channels are also strongly implicated in the control of cell proliferation and apoptosis as well as diverse cancers, such as gliomas and breast, gastric, pancreatic, ovarian, and prostate cancers (4, 60–64). For example, glioma cells express specific BK channels at a high level, and these channels seem to play a crucial role in glioma migration and metastasis (3, 5). Activation of the gene encoding for the BK channel α subunit (KCNA1) occurs in prostate cancer cells, and the proliferation of these cells is inhibited by iberiotoxin, a specific inhibitor of BKCa channels (4). In this study, we found that paxiline, another specific inhibitor of the BKCa channel, markedly decreased the proliferation of IGR39 melanoma cells (Fig. 2, A and B). Consistently, ouabain, which inhibited the BKCa current indirectly via the Na⁺/K⁺-ATPase, did the same.

The effects of BK channel activity on cell proliferation may be mediated either by K⁺ currents *per se* or by a transmembrane Ca²⁺ influx to which the BKCa channel could contribute by hyperpolarizing the membrane thus increasing the driving force for Ca²⁺ entry (31, 32, 65–67). The downstream pathways are not well known, but in the latter case a rise in [Ca²⁺], activates transcription factors regulating the cell cycle (64, 68) and may also serve as a positive feedback loop further activating the BKCa channels.
Finally, BK$_{Ca}$ channels have also been implicated in many other (path)physiological processes, including endothelial dysfunction, neurological disorders, and alcohol sensitivity of the brain (5, 69, 70). Provided that the functional association between the BK$_{Ca}$ channel and Na$^+$/K$^+$-ATPase observed in this study also exists in endothelial cells and neurons, our findings might constitute a basic mechanism for physiological regulation by altered cholesterol levels.

Acknowledgments—We thank Lea Armassalo and Tarja Grundström for excellent technical assistance, the Molecular Imaging Unit of Biomedicum Helsinki for assistance, and Dr. Elina Ikonen and Martin Hermansson for helpful comments on this manuscript.

REFERENCES
1. Salkoff, L., Butler, A., Ferreira, G., Santi, C., and Wei, A. (2006) Nat. Rev. Neurosci. 7, 921–931
2. Ghatta, S., Nimmagadda, D., Xu, X., and O’Rourke, S. T. (2006) Pharmacol. Ther. 110, 103–116
3. Kraft, R., Krause, P., Jung, S., Basrai, D., Liebmann, L., Bolz, J., and Patt, S. (2003) Pflügers Arch. 446, 248–255
4. Bloch, M., Ousingsawat, I., Simon, R., Schraml, P., Gasser, T. C., Michatsch, M. J., Kunzelmans, K., and Bubendorf, L. (2007) Oncogene 26, 2525–2534
5. Sontheimer, H. (2008) Exp. Biol. Med. 233, 779–791
6. Simons, K., and Ikonen, E. (1997) Nature 387, 569–572
7. Simons, K., and Toomre, D. (2000) Nat. Rev. Mol. Cell Biol. 1, 31–39
8. Schmidt, D., Jiang, Q. X., and MacKinnon, R. (2006) J. Biol. Chem. 281, 715–735
9. Balijepalli, R. C., Foell, J. D., Hall, D. D., Hell, J. W., and Kamp, T. J. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 7935–7940
10. Lam, R. S., Shaw, A. R., and Duszyk, M. (2004) Biochim. Biophys. Acta 1667, 241–248
11. Lundbaek, J. A., Birn, P., Hansen, A. J., Søgaard, R., Nielsen, C., Girshman, J., Bruno, M. J., Tape, S. E., Egebjerg, J., Greathouse, D. V., Mattice, G. L., Koppel, R. E., and Andersen, O. S. (2004) J. Gen. Physiol. 123, 599–621
12. Bolotina, V., Omelyanenko, V., Heyes, B., Ryan, U., and Bregestovski, P. (1989) Pflügers Arch. 415, 262–266
13. Yuan, C., O’Connell, R. J., Jacob, R. F., Mason, R. P., and Treistman, S. N. (2007) J. Biol. Chem. 282, 7276–7286
14. Cai, T., Wang, H., Chen, Y., Liu, L., Gunning, W. T., Quintas, L. E., and Xie, Z. (2009) J. Biol. Chem. 284, 14881–14890
15. Skou, J. C. (1957) Biochim. Biophys. Acta 23, 394–401
16. Kaplan, J. H. (2002) Annu. Rev. Biochem. 71, 511–535
17. Chen, Y., Cai, T., Wang, H., Li, L., Loreaux, E., Lingrel, J. B., and Xie, Z. (2009) J. Biol. Chem. 284, 14881–14890
18. Cai, T., Wang, H., Chen, Y., Liu, L., Gunning, W. T., Quintas, L. E., and Xie, Z. J. (2008) J. Cell Biol. 182, 1153–1169
19. Yuan, C., Cai, T., Tian, J., Ivanov, A. V., Giovannucci, D. R., and Xie, Z. (2008) Exp. Biol. Med. 233, 1667, 241–248
20. Lam, R. S., Shaw, A. R., and Duszyk, M. (2004) Biochim. Biophys. Acta 1667, 241–248
21. Mundel, P., Biena, G., Taglietti, V., Cazzaniga, E., and Parenti, M. (2005) Biophys. J. 89, 2443–2457
22. Skou, J. C. (1957) Biochim. Biophys. Acta 23, 394–401
23. Kaplan, J. H. (2002) Annu. Rev. Biochem. 71, 511–535
24. Chen, Y., Cai, T., Wang, H., Li, L., Loreaux, E., Lingrel, J. B., and Xie, Z. (2009) J. Biol. Chem. 284, 14881–14890
25. Cai, T., Wang, H., Chen, Y., Liu, L., Gunning, W. T., Quintas, L. E., and Xie, Z. J. (2008) J. Cell Biol. 182, 1153–1169
26. Ila, S., and Dryer, S. E. (2009) FEBS Lett. 583, 3109–3114
27. Mijatovic, T., Van Quaquebeke, E., Delest, B., Debeir, O., Darro, F., and Kiss, R. (2007) Biochim. Biophys. Acta 1776, 32–57
28. Kocksckämper, J., Gisselmann, G., and Gilsch, H. G. (1997) Biochim. Biophys. Acta 1335, 197–208
29. Yamamoto, S., Askev, G. R., Heiny, J., Masaki, H., and Yatani, A. (1996) J. Physiol. 270, C457–C464
30. Liang, G. H., Kim, M. Y., Park, S., Kim, J. A., Choi, S., and Suh, S. H. (2008) Pflügers Arch. 457, 67–75
31. Tian, J., Cai, T., Yuan, Z., Wang, H., Liu, L., Haas, M., Maksimova, E., Huang, X. Y., and Xie, Z. J. (2006) Mol. Biol. Cell 17, 317–326
32. Li, Z., and Xie, Z. (2009) Pflügers Arch. 457, 635–644
33. Yuan, Z., Cai, T., Tian, J., Ivanov, A. V., Giovannucci, D. R., and Xie, Z. (2005) Mol. Biol. Cell 16, 4034–4045
34. Newman, R. A., Yang, P., Pavlus, A. D., and Block, K. I. (2008) Mol. Interv. 8, 36–49
35. Kuhlmann, C. R., Gast, C., Li, F., Schäfer, M., Tillmanns, H., Waldecker, B., and Wiecha, J. (2004) J. Am. Soc. Nephrol. 15, 868–875
36. Cornelius, F. (2001) Biochemistry 40, 8842–8851
37. Cuebas, F. J., Jameson, D. M., and Sotomayor, C. P. (2006) Biochemistry 45, 13855–13868
38. Cornelius, F. (2008) Biochemistry 47, 1652–1658
39. Ma, D. W., Seo, J., Davidson, L. A., Callaway, E. S., Fan, Y. Y., Lupton, J. R., and Chapkin, R. S. (2004) FASEB J. 18, 1040–1042
40. Ouadid-Ahoud, H., Roudbaraki, M., Abidouche, A., Delcourt, P., and Prevatt, E. (2004) Biochim. Biophys. Res. Commun. 316, 244–251
41. Liu, S. I., Chi, C. W., Lui, W. Y., Mok, K. T., Wu, C. W., and Wu, S. N. (1998) Biochim. Biophys. Acta 1368, 256–266
42. Weaver, A. K., Liu, X., and Sontheimer, H. (2004) J. Neurosci. Res. 78, 224–234
43. Jäger, H., Dreker, T., Buck, A., Giehl, K., Gress, T., and Grissmer, S. (2004) Mol. Pharmacol. 65, 630–638
44. Han, X., Wang, F., Yao, W., Xing, H., Weng, D., Song, X., Chen, G., Xi, L., Zhu, T., Zhou, J., Xu, G., Wang, S., Meng, L., Iadecola, C., Wang, G., and Ma, D. (2007) Apoptosis 12, 1837–1846

BK$_{Ca}$ Channel Interacts with Na$^+$/K$^+$-ATPase in Rafts
BKCa Channel Interacts with Na+/K+-ATPase in Rafts

65. Wang, Z. (2004) Pflugers Arch. 448, 274–286
66. Kuhlmann, C. R., Schaefer, C. A., Kosok, C., Abdallah, Y., Walther, S., Lüdders, D. W., Neumann, T., Tillmanns, H., Schäfer, C., Piper, H. M., and Erdogan, A. (2005) Planta Med. 71, 520–524
67. Luedders, D. W., Muenz, B. M., Li, F., Rueckleben, S., Tillmanns, H., Waldecker, B., Wiecha, J., Erdogan, A., Schaefer, C. A., and Kuhlmann, C. R. (2006) J. Cardiovasc. Pharmacol. 47, 365–370
68. Berridge, M. J., Lipp, P., and Bootman, M. D. (2000) Nat. Rev. Mol. Cell Biol. 1, 11–21
69. Crowley, J. J., Treistman, S. N., and Dopico, A. M. (2003) Mol. Pharmacol. 64, 365–372
70. Lee, U. S., and Cui, J. (2010) Trends Neurosci. 33, 415–423