BK Channels Are Linked to Inositol 1,4,5-Triphosphate Receptors via Lipid Rafts

A NOVEL MECHANISM FOR COUPLING [Ca\textsuperscript{2+}] to ION CHANNEL ACTIVATION

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Glioma cells prominently express a unique splice variant of a large conductance, calcium-activated potassium channel (BK channel). These channels transduce changes in intracellular calcium to changes of K	extsuperscript{+} conductance in the cells and have been implicated in growth control of normal and malignant cells. The Ca\textsuperscript{2+} increase that facilitates channel activation is thought to occur via activation of intracellular calcium release pathways or influx of calcium through Ca\textsuperscript{2+}-permeable ion channels. We show here that BK channel activation involves the activation of inositol 1,4,5-triphosphate receptors (IP\textsubscript{3}R), which localize near BK channels in specialized membrane domains called lipid rafts. Disruption of lipid rafts with methyl-\beta-cyclodextrin disrupts the functional association of BK channel and calcium source resulting in a >50% reduction in K\textsuperscript{+} conductance mediated by BK channels. The reduction of BK current by lipid raft disruption was overcome by the global elevation of intracellular calcium to changes of K\textsuperscript{+} channel). These channels transduce changes in intracellular calcium to changes of K\textsuperscript{+} conductance in the cells and have been implicated in growth control of normal and malignant cells. The Ca\textsuperscript{2+} increase that facilitates channel activation is thought to occur via activation of intracellular calcium release pathways or influx of calcium through Ca\textsuperscript{2+}-permeable ion channels. We show here that BK channel activation involves the activation of inositol 1,4,5-triphosphate receptors (IP\textsubscript{3}R), which localize near BK channels in specialized membrane domains called lipid rafts. Disruption of lipid rafts with methyl-\beta-cyclodextrin disrupts the functional association of BK channel and calcium source resulting in a >50% reduction in K\textsuperscript{+} conductance mediated by BK channels. The reduction of BK current by lipid raft disruption was overcome by the global elevation of intracellular calcium through inclusion of 750 nM Ca\textsuperscript{2+} in the pipette solution, indicating that neither the calcium sensitivity of the channel nor their overall number was altered. Additionally, pretreatment of glioma cells with 2-aminoethoxydiphenyl borate to inhibit IP\textsubscript{3}R negated the effect of methyl-\beta-cyclodextrin, providing further support that IP\textsubscript{3}R are the calcium source for BK channels. Taken together, these data suggest a privileged association of BK channels in lipid raft domains and provide evidence for a novel coupling of these Ca\textsuperscript{2+}-sensitive channels to their second messenger source.

Gliomas are primary brain malignancies that derive from glial cells. These tumors are characterized by extensive cell migration and diffuse infiltration of normal brain making surgical removal almost impossible. Accumulating evidence indicates that glioma migration is supported by the activity of K\textsuperscript{+} and Cl\textsuperscript{-} channels (1–5). Specifically, ion flux through Cl\textsuperscript{-} and K\textsuperscript{+} channels along with obligated water is presumed to cause the profound changes in cell shape and cell volume as cells invade. In this vein, our laboratory, as well as others, has attempted to identify and characterize the underlying ion channels involved in this process. This led us to the molecular cloning of a novel large conductance calcium-activated K\textsuperscript{+} (BK) channel that is uniquely expressed in glioma cells and particularly up-regulated in highly invasive grade IV gliomas (6). A unique feature of BK channels is the ability to be activated by two independent stimuli, namely membrane voltage and the intracellular second messenger Ca\textsuperscript{2+}, whereas typical K\textsuperscript{+} channels are gated by voltage alone. Hence cell volume changes are likely to result from the activation of BK channels via changes in intracellular [Ca\textsuperscript{2+}]\textsubscript{i}. In the absence of large increases in [Ca\textsuperscript{2+}]\textsubscript{i}, BK channel activation requires very large depolarization of the membrane (approximately +80 mV), which is expected to be an uncommon occurrence in a nonexcitable cell such as a glioma cell. However, raising [Ca\textsuperscript{2+}]\textsubscript{i}, from a resting level of ~100 nM (7, 8) to the low micromolar range results in BK channel activation at the normal resting membrane potential for these cells (~40 mV (9)). Physiological conditions that raise Ca\textsuperscript{2+} throughout the cell sufficiently to activate glioma BK channels yet without compromising other Ca\textsuperscript{2+}-dependent processes are difficult to envision. One way to solve this problem is by localizing BK channels in close proximity to a Ca\textsuperscript{2+} source, i.e. a Ca\textsuperscript{2+} channel, Ca\textsuperscript{2+}-permeable receptor, or intracellular release pathway. Such a privileged association may be achieved through direct protein-protein interactions as has been demonstrated in rat brain where BK channels are linked to L-type voltage-gated calcium channels (10). Alternatively, privileged associations may occur through localization to membrane microdomains. Microdomains can be established through specialized membrane patches in the plasma membrane enriched in cholesterol and sphingolipids, and these are called lipid rafts (11). The existence of such raft domains within the plasma membrane and the idea that microdomains within the plasma membrane are suitable for the assembly of signaling complexes are now well accepted (for review see Ref. 12). Lipid rafts have been demonstrated to participate in protein trafficking and the assembly of signaling complexes, and indeed, a number of ion channels have been shown to localize to lipid raft domains. These include, for example, cardiac pacemaker channels, K\textsubscript{Ca} channels, L-type voltage-gated calcium channels, Kv, Kir, as well as Na\textsuperscript{+} channels, and in most instances, channel association with lipid rafts directly affects the biophysical properties of these channels (13–15). Similarly, K\textsubscript{Ca}, K\textsubscript{ATP}, P2X receptors, cyclic nucleotide-gated channels, and transient receptor poten-
tial channels (TRPC)² all localize to lipid rafts as part of signaling complexes (16–19).

In light of these findings and a hypothesized requirement for glioma BK channels to localize closely to a source for Ca²⁺ entry or release, we set out to investigate whether BK channels in glioma cells may localize to microdomains and whether these may be established by lipid rafts. Using biophysical and biochemical methods, we indeed demonstrate that glioma BK channels localize preferentially to lipid raft microdomains where they assemble in proximity to the IP₃R and respond to IP₃-mediated intracellular calcium release. As a consequence, physiological activation of BK channels occurs via muscarinic acetylcholine receptors but only when lipid raft coupling of BK channels and IP₃Rs is intact.

EXPERIMENTAL PROCEDURES

Cell Culture and Drugs—The glioma cell lines D54-MG and U251-MG (World Health Organization, grade IV, glioblastoma multiforme) were a gift from Dr. D. Bigner (Duke University, Durham, NC). Cells were maintained in Dulbecco’s modified Eagle’s medium/F-12 (Media Tech, University of Alabama at Birmingham Media Preparation Facility) supplemented with 2 mM glutamine (Media Tech) and 7% fetal bovine serum (Birmingham Media Preparation Facility) supplemented with 2 mM glutamine (Media Tech) and 7% fetal bovine serum (Hyclone, Logan, UT), at 37 °C and 10% CO₂. Unless otherwise stated, all reagents were purchased from Sigma, and stock solutions were made according to manufacturer’s instructions. Methyl-β-cyclodextrin (MβCD) was made fresh daily by resuspending in either Normal Bath solution or media at a concentration of 5 mg/ml.

Drug Treatment—For all experiments using MβCD, treatment was carried out as follows. MβCD, at a concentration of 5 mg/ml, was resuspended in either Normal Bath solution or media lacking serum. In the case of electrophysiology experiments, MβCD was added to the bath and perfused onto cells for a total of 15 min. For all other experiments, cells were washed two times with serum-free media to ensure that any noncellular cholesterol was removed, and then MβCD-containing media were added to the cells for 15 min. Cells were then immediately used for experiments.

Electrophysiology—Recordings of whole cell currents were made using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) following standard recording techniques (20). For both perforated patch and whole cell patch recordings, patch pipettes were made with thin walled borosilicate glass (World Precision Instruments (TW150F-4), Sarasota, FL) using an upright puller (Narishige Instruments (PP-830), Tokyo, Japan), and had resistances of 3–5 meegohms. Current recordings were digitized on line at 10 kHz and low pass filtered at 2 kHz using a Digidata 1200 (Axon Instruments). pClamp 8.2 (Axon Instruments) was used to acquire and store data. Series resistance (Rₛ) was compensated to 80%, reducing voltage errors, and cells with a compensated Rₛ above 10 meegohms were omitted. U251 cells were plated on glass coverslips and allowed to adhere overnight. Cells were used between 1 and 4 days in culture.

Solutions—Our standard bath solution consisted of the following (in mM): 125 NaCl, 5 KCl, 1.2 MgSO₄, 1 CaCl₂, 1.6 Na₂HPO₄, 0.4 NaH₂PO₄, 10.5 glucose, and 32.5 Hepes acid; pH was adjusted to 7.4 using NaOH, and osmolarity was ~30 mosm. MβCD (5 mg/ml) was added directly to the bath solution. Pipette solutions contained the following (in mM): 145 KCl, 1 MgCl₂, 10 EGTA, 10 Hepes sodium salt, pH adjusted to 7.3 with Tris base. CaCl₂ was added directly to pipette solution. Cells were maintained in Dulbecco’s modified Eagle’s medium/F-12 (Media Tech, University of Alabama at Birmingham Media Preparation Facility) supplemented with 2 mM glutamine (Media Tech) and 7% fetal bovine serum (Birmingham Media Preparation Facility) supplemented with 2 mM glutamine (Media Tech) and 7% fetal bovine serum (Hyclone, Logan, UT), at 37 °C and 10% CO₂. Unless otherwise stated, all reagents were purchased from Sigma, and stock solutions were made according to manufacturer’s instructions. Methyl-β-cyclodextrin (MβCD) was made fresh daily by resuspending in either Normal Bath solution or media at a concentration of 5 mg/ml.

² The abbreviations used are: TRPC, transient receptor potential channel; IP₃, inositol 1,4,5-triphosphate receptor; MβCD, methyl-β-cyclodextrin; 2-APB, 2-aminoethoxydiphenyl borate; LR, lipid rafts; PBS, phosphate-buffered saline; IP₃, inositol 1,4,5-triphosphate; VGCC, voltage-gated calcium channel; ACh, acetylcholine; mAChR, muscarinic acetylcholine receptor; TRITC, tetramethylrhodamine isothiocyanate.
BK Channel Coupling to IP$_3$R via Lipid Rafts

polyvinylidene difluoride paper (Millipore, Bedford, MA). Membranes were blocked in blocking buffer (5% nonfat dried milk, 2% bovine serum albumin, 2% normal goat serum in TBS plus 0.1% Tween 20 (TBST)). The primary antibodies anti-Cav-1 (sc-894, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-BK (Alomone, Jerusalem, Israel) were used at dilutions of 1:100 and 1:500, respectively. Blots were incubated in primary antibody for 1 h followed by a wash period (four times for 5 min). Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h, followed by another wash period (four times for 5 min) and developed using enhanced chemiluminescence (ECL; Amersham Biosciences) on Hyperfilm (Amersham Biosciences).

Biotinylation—The entire process was carried out at 4 °C. Cells were washed twice in standard bath solution containing 1 mM Ca$^{2+}$. After washing, sulfo-NHS-biotin (1.5 mg/ml; Pierce) was added and allowed to incubate for 30 min with occasional gentle rocking. Biotinylation was quenched by washing cells twice with standard bath solution containing 100 mM glycine plus an additional 15-min wash. Cells were then rinsed once in bath solution, collected, spun down, and resuspended in 0.5 ml of RIPA buffer supplemented with protease and phosphatase inhibitors (1:100; Sigma). Suspension was rotated for 30 min followed by centrifugation at 12,000 × g for 5 min. At this point protein concentration was measured, and 10 μg of protein was set aside as total lysate. The remainder of the sample was incubated with agarose-streptavidin beads (400-μl beads/ml supernatant; Pierce) for 2 h. Once again beads were centrifuged at 10,000 × g for 10 s, and the supernatant (representing the unbound fraction) was collected. The beads were gently washed four times with RIPA Buffer and resuspended in 100 mM glycine, pH 2.8, for 3 min with occasional vortexing to separate surface protein from the beads. Sample buffer was added immediately to neutralize pH. In the few cases where sample buffer was not enough to neutralize pH (as evidenced by a yellowish color), one drop of 1 N NaOH was added. Additionally, 6× sample buffer was added to the total lysate fraction, and all three samples were loaded onto a 4–20% gradient pre-cast SDS-polyacrylamide gel, separated, and transferred according to the Western protocol. All blots were probed with anti-actin (1:1000) as a negative control to ensure that the surface protein membrane fraction was not contaminated with intracellular protein, and (1:1000, Amersham Biosciences) on Hyperfilm (Amersham Biosciences).

Cells were washed two more times and mounted onto slides with Gel/Mount (Biomedia Corp., Forest City, CA). ×63 images were acquired with a Zeiss Axiovert 200 M (Göttingen, Germany).

Migration Assay—The day before the experiment was performed, an ~70% confluent dish of U251 cells were rinsed and supplied with serum-free media overnight. Cell culture inserts (BD Biosciences) with 8-μm pores were coated overnight with Vitronectin (BD Biosciences) at a concentration of 5 μg/ml in PBS. The following day, inserts were washed two times with PBS and blocked with 1% fatty acid–free bovine serum albumin for 1 h. Inserts were then washed two times in PBS, and 400 μl of Migration Assay Buffer (MAB, 0.1% fatty acid–free bovine serum albumin in serum-free media) was added to the bottom of each well. Cells were rinsed once in PBS and were lifted off the dish by the addition of 0.5 mM EGTA for ~20 min. U251 cells were rinsed twice by centrifugation and resuspended in MAB and counted. Forty thousand cells were plated on top of each filter and allowed to adhere for 30 min before drug was added. After addition of drug, cells were allowed to migrate for 5 h. Filters were then fixed and stained with Crystal Violet, and the tops were wiped clean of cells, and representative fields (five per filter) were counted with a Zeiss Axiovert 200 M microscope with a 10× objective.

Data Analysis—Results were analyzed using Origin (version 6.0, MicroCal Software, Northampton, MA) and Excel 2000 (Microsoft, Seattle). Significance was determined by one-way analysis of variance because all data showed normal distribution. All data reported are mean ± S.E., and * denotes p < 0.05 unless otherwise stated.

RESULTS

BK Channels in Glioma Cell Lines Localize to Lipid Raft Domains—The central hypothesis examined by this study is that glioma BK channels localize to privileged membrane sub-regions or microdomains where sufficient local changes in [Ca$^{2+}$], can be achieved to allow channel activation in response to physiological stimuli as cells invade. We further hypothesized that these microdomains may be established through privileged membrane regions termed lipid rafts (LRs). In a first series of experiments, we examined the possible association of BK channels with LRIs in glioma cells by labeling cells in culture with antibodies directed against the Maxi K$^-$ α-subunit and three common lipid raft markers, specifically caveolin-1, flotillin, and a fluorescently conjugated cholera toxin B subunit, which binds to GM1 ganglioside, a common component of lipid rafts (21). Representative examples from two different cell lines, one (GBM-62) derived directly from a patient biopsy, are shown in Fig. 1. In both cell lines, BK channel immunoreactivity is found diffusely throughout the cell. In addition, however, both BK channels and each of the LR proteins caveolin-1 (Fig. 1A and supplemental Fig. 1A) and flotillin (Fig. 1B and supplemental Fig. 1B) as well as the cholera toxin B subunit (Fig. 1C and supplemental Fig. 1C) appeared to be found in hot spots at the protruding edges of the cell. The cells in Fig. 1B appeared to have been migrating away from one another probably having just recently divided. Interestingly, the BK and LR hotspots in both of these cells seem to localize to the leading edge, or lamel-
lipodia, of each cell, indicating that these channels may associate with LRs in distinct regions in migratory cells. These immunocytochemical stainings suggest that BK channels may indeed be found in lipid raft microdomains in glioma cells.

To further verify this association, we examined this question biochemically. To do this we adapted a well described lipid raft isolation protocol (22) that separates whole cell lysates into water, detergent-soluble, and detergent-insoluble fractions at 4 °C based on their relative solubilities in aqueous solutions with or without detergent. Based on this isolation technique, as described in greater detail under “Experimental Procedures,” cytosolic constituents were isolated in the water-soluble fraction (Fig. 2A, W) because they exist in an aqueous environment in the cell. Following removal of the cytosolic fraction, we were left primarily with membrane and cytoskeletal elements that we further dissociated into a detergent-soluble fraction (Fig. 2A, D) containing membrane-associated proteins capable of being solubilized in Triton X-100 and detergent-insoluble proteins. The detergent-insoluble fraction consists of cytoskeletal proteins as well as proteins associated with regions of the membrane exhibiting a large enough concentration of lipid to render them insoluble in detergent at 4 °C, i.e., lipid rafts. The remaining proteins were further separated into 9 fractions by discontinuous gradient centrifugation. All 11 isolated fractions were run on an SDS-polyacrylamide gel and then Western-blotted to probe for both lipid raft markers and BK channels. As depicted in Fig. 2A, Caveolin-1 (Cav-1), a common lipid raft-associated protein used to biochemically define the lipid raft containing gradient fractions, was found in the second density gradient fraction. This lane was herein referred to as the lipid raft fraction because lipid rafts are buoyant and thus their constituents are typically found in the less dense fractions of the gradient. In addition to precipitating in the 2nd detergent-insoluble fraction, a portion of Cav-1 was also found in the detergent-soluble fraction. This lane was herein referred to as the lipid raft fraction. The merged image of each immunocytochemistry is shown in the right-hand panel and illustrates overlap of both the BK channel and each of the three lipid raft markers suggesting that these channels localize to the lipid rafts in these cells. Scale bar represents 20 μm.

![Image 1](https://example.com/image1)

**FIGURE 1. BK channels and lipid raft markers co-localize.** Glioblastoma cells derived from a patient biopsy were cultured for 2 days and then fixed to probe for subcellular localization of BK channels and lipid raft markers. All cells were probed with monoclonal antibodies to the MaxiK + (BK) α-subunit as indicated in the left-hand panels of A–C. BK channel immunostaining was compared with the localization of Cav-1 (A), flotillin (B), and the B subunit of cholera toxin (C). ×63 images were obtained via wide field microscopy. Western blots were then probed for BK channels and lipid raft markers suggesting that these channels localize to the lipid rafts in these cells.

![Image 2](https://example.com/image2)

**FIGURE 2. BK channels associate with lipid rafts.** A biochemical isolation of lipid rafts was performed on U251MG cells by isolating water soluble (W) and detergent-soluble (D) proteins from whole cell lysates. The remaining cellular constituents were subjected to ultracentrifugation, and nine fractions (1–9) were collected and run along side the water- and detergent-soluble proteins on an SDS-polyacrylamide gel. Western blots were then probed for BK channels and Cav-1. As illustrated in the Control blot, Cav-1 is found in the 2nd fraction which is typically considered the lipid raft fraction. BK channel protein is also found in this fraction indicating the BK channels localize to lipid rafts in glioma cells. The same isolation protocol was performed after 15 min of treatment with MβCD at 5 mg/ml, and subsequent Western blotting of one such experiment shows that disruption of lipid rafts removes BK channel protein from the lipid raft fraction of the isolation. B, immunocytochemistry before and after the same treatment as in A demonstrates further that MβCD is capable of removing BK channels from lipid rafts. Control immunostaining in the top row shows the normal distribution of BK channels in U251 cells, similar to that seen in the GBM-62 cells. Disruption of lipid rafts with MβCD results in a redistribution of BK channel immunoreactivity diffusely throughout the cell. C, glioma cells were immunostained with TRITC-conjugated phalloidin (red) to visualize actin filaments and 4′,6-diamidino-2-phenylindole (DAPI) (blue) to label nuclei. Immunolabeling was performed on cells treated with or without MβCD and demonstrates no change in actin labeling following disruption of lipid rafts.
solutions has been well documented (23). Several studies have shown that MβCD, in addition to being able to solubilize cholesterol, can also actively remove and sequester cholesterol from membrane (24–26). To demonstrate its usefulness as an LR-disrupting agent, U251 glioma cells were treated with MβCD at a concentration of 5 mg/ml for 15 min at 37 °C prior to performing the same biochemical and immunocytochemical assays described above. As shown in Fig. 2A, treatment with MβCD resulted in a complete shift of BK channel protein from the lipid raft fraction to the detergent-soluble fraction of the membrane. Likewise, immunostaining of Cav-1 and BK channels (Fig. 2B) illustrates that BK channel and Cav-1 protein coexist in an ordered arrangement with both proteins being found in hot spots on the lamellipodia and in perinuclear regions indicative of endoplasmic reticulum localization. However, immunostaining of cells treated with MβCD demonstrated a redistribution of channels in the membrane, disrupting the tight organization found under normal conditions indicating that MβCD does indeed interrupt an association between BK channels and lipid rafts. Furthermore, immunolabeling of U251 glioma cells with phalloidin illustrates no difference in the distribution of actin filaments following MβCD treatment. This indicates that MβCD does not interfere with the intracellular actin architecture.

**Lipid Raft Disruption Reduces Whole Cell BK Currents**—To examine whether the BK channel association with LR has any functional significance, we next performed electrophysiological experiments before and after acute disruption of lipid rafts. Specifically we recorded whole cell BK currents in glioma cells while continuously bath-applying MβCD (5 mg/ml) for a total of 15 min. A representative example of one such experiment before and after 15 min of MβCD is illustrated in Fig. 3A. Cells were voltage-clamped at −40 mV, and BK currents were activated by stepping the membrane in 20-mV increments to +180 mV before and after application of MβCD. To verify that the MβCD effect was unique to BK currents, we recorded inward currents mediated by Kir channels in U251 cells, which were activated by stepping the membrane to negative potentials between −160 and +20 mV following a 200-ms prepulse at 0 mV. As shown in the representative traces in Fig. 3A, following 15 min of MβCD treatment, BK currents were reduced by −50%, whereas Kir currents were unaffected. Cumulative data from 13 cells for BK currents and 10 cells for Kir currents are shown in Fig. 3, B and C, respectively. As is evident in the raw traces, treatment with MβCD selectively reduced whole cell BK currents without affecting Kir currents in the same cell, ruling out nonspecific effects of the drug and, importantly, suggesting that intact LR may be required for maximal BK channel function.

**Lipid Raft Disruption Does Not Cause Channel Internalization or Alter Ca2+ Sensitivity of the Channel**—The above illustrated whole cell recordings were all conducted 15 min following MβCD treatment. To determine the time course of the MβCD-induced reduction in whole cell BK currents in more detail, we performed whole cell voltage clamp recordings as described above using only a single step to +140 mV from a holding potential of −40 mV every 30 s for a 15-min period. Fig. 4A shows a representative example in which the peak BK current recorded in response to a voltage step to 140 mV was normalized at every given time point to the starting value, preceding the application of MβCD. As is clearly evident from this plot, MβCD caused a rapid decrease in BK currents that reached a maximum in approximately 5 min after which current amplitudes remained relatively constant for the duration of the experiment. This rapid time course of MβCD-induced BK current reduction may indicate that either BK channels are being rapidly internalized, as lipid rafts are known to be involved in protein trafficking, or that lipid rafts in glioma cells localize BK channels near a Ca2+ source, and this association is being disrupted. To examine the first possibility, an internalization of BK channels, we performed biochemical studies in which cell surface protein can be selectively tracked through cell surface biotinylation as described previously (7, 8). Results from one such experiment, illustrated in Fig. 4B, demonstrate that no change in either plasma membrane BK channel protein (membrane fractions, control, and MβCD) or whole cell BK channel protein (total fractions, control, and MβCD) occurred following treatment with MβCD.

If the alternative explanation were true, i.e. that lipid raft disruption dissociates BK channels from their Ca2+ source, we should be able to overcome the effect of MβCD simply by raising [Ca2+], to a level above that required to physiologically activate BK channels. To address this question we first needed to determine the [Ca2+]i required to activate BK channels at the resting membrane potential. We did this by performing perforated patch recordings with amphotericin B that allows us to monitor membrane potential without manipulating [Ca2+]i. After achieving whole cell access, current clamp recordings...
FIGURE 4. MjBCD does not cause internalization or a change in calcium sensitivity of BK channels. A, whole cell patch clamp recordings were performed by clamping the membrane potential at −40 mV and stepping to ±140 mV every 30 s, while bath-applying MjBCD. The resultant current was normalized to the average current obtained before drug application and plotted as a function of time. The data indicate that MjBCD induces a rapid decrease in BK current that levels off after 5 min and remains constant for the duration of the experiment. B, confluent cultures of U251 cells were treated either in control or MjBCD-containing media and incubated in the presence of biotin at 4 °C to prevent endocytosis (n = 3). Whole cell lysates were collected and incubated with streptavidin beads to immunoprecipitate biotinylated membrane protein. Western blotting was performed on total and membrane fractions, and blots were probed for BK channel protein and Na+/K+/ATPase, which was used as a loading control. As illustrated, there is no change in the amount of protein on the membrane after treatment with MjBCD. C, perforated patch clamp recordings using amphoterin in B to leave [Ca^{2+}]_i, undisturbed. Ionomycin was bath-applied to selectively permeabilize the membrane to calcium, and baths with increasing calcium concentrations were then applied while monitoring the resting membrane of the potential cells. A representative trace demonstrates that 500 nM calcium is required to activate BK channels as evidenced by the hyperpolarization of the membrane potential that can be inhibited by 2 μM paxilline. D, cumulative data from the experiments in B (n = 6) and C (n = 10). Whole cell voltage clamp recordings were performed before and after bath application of MjBCD with either 750 nM Ca^{2+} (E) or 150 nM Ca^{2+} in the pipette solution to mimic high and basal calcium levels, respectively. Data indicate that in the presence of 750 nM [Ca^{2+}]_i, MjBCD is incapable of reducing BK currents, whereas 150 nM [Ca^{2+}]_i is incapable of overcoming this effect. **, p ≤ 0.01.

were used to monitor the “resting” membrane voltage. Cells were then perfused in Ca^{2+}-free bath containing 5 μM iodonium that selectively permeabilizes the membrane to calcium allowing us to equilibrate [Ca^{2+}]_i with [Ca^{2+}]_bath. The Ca^{2+} channel and lipid rafts colocalize the two, then inhibition of the Ca^{2+} channel should reduce BK currents, and this reduction should be insensitive to MjBCD. If release occurred from intracellular stores, depletion of these stores should disrupt BK
whether calcium release from intracellular stores fulfilled this role. To assess this possibility directly, we depleted intracellular Ca\(^{2+}\) stores with 100 nM thapsigargin, an irreversible inhibitor of the sarcoplasmic reticulum Ca\(^{2+}\) -ATPase pump, for 30 min at 37 °C. We then performed whole cell patch recordings of BK currents, again doing so before and after disruption of lipid rafts with MβCD treatment. Importantly, recordings were carried out 5 min after gaining whole cell access to allow for any transient increase in [Ca\(^{2+}\)\(_i\)], caused by thapsigargin treatment to be dialyzed by pipette solution. As shown in Fig. 6A (n = 15), thapsigargin pretreatment completely inhibited the effect of MβCD on BK currents. Raw traces from one experiment are shown in Fig. 6B and demonstrate that after 15 min of MβCD treatment, there is no change in whole cell BK currents. Calcium released from intracellular stores can occur through one of two independent pathways. The first of these is through activation of ryanodine receptors by ryanodine, caffeine, or heparin and has been well characterized as the mechanism for calcium release responsible for contraction in skeletal and smooth muscle (29). The second mechanism for release of intracellular calcium is through activation of IP\(_{3}\)R by IP\(_{3}\) production following activation of the phospholipase C-\(\gamma\) signaling pathway and has been directly linked to [Ca\(^{2+}\)\(_i\)] elevations that occur in response to various growth factors and neurotransmitters (30). We used selective pharmacological blockers to determine whether either of these pathways might serve as the Ca\(^{2+}\) source for BK channels. As depicted in Fig. 5C, MβCD was still capable of reducing whole cell BK currents in glioma cells pre-treated with 50 \(\mu\)M ryanodine, a compound that at low concentrations (<10
μM) can activate ryanodine receptors, but at high concentrations (>20 μM) will inhibit the receptor. Conversely, pretreatment with 50 μM 2-APB, a nonspecific inhibitor of the IP₃R, prevented the MβCD-induced decrease in whole cell BK currents completely, indicating that IP₃Rs are the probable calcium source for BK channels in lipid rafts in glioma cells.

**Physiological Stimulation of IP₃Rs Activates BK Channels in Lipid Rafts**—Pharmacology indicates that BK channels may be coupled to IP₃Rs via lipid rafts in glioma cells. We next sought to activate IP₃Rs using a physiological stimulus. Previous evidence from our own laboratory has indicated that stimulation of muscarinic acetylcholine receptors (mAChRs) with muscarine or acetylcholine (ACh) elicits an increase [Ca²⁺], that subsequently activates BK channels in U373 glioma cells (2). Additionally others have demonstrated the presence of the M₃ mAChR in glioma cells (32) that has been shown to induce inositol phosphate hydrolysis and intracellular calcium release (33, 34). Accordingly, we were interested in whether muscarinic receptor signaling in U251 glioma cells might also activate BK channels through production of IP₃ and whether this signaling was dependent on lipid raft integrity. We loaded cells with fura-2AM, a calcium-sensitive ratiometric dye, and we simultaneously monitored [Ca²⁺], while performing perforated patch recording with amphotericin B to monitor resting membrane potential without altering intracellular calcium dynamics. Bath application of 50 μM ACh induced an increase in intracellular calcium. Moreover, all cells that responded to ACh with an increase in [Ca²⁺] (six of nine cells examined) also exhibited a concurrent hyperpolarization of the resting membrane potential that was completely abolished in the presence of 2 μM paxilline, a specific inhibitor of BK channels (Fig. 7, A and E). We also bath-applied 50 μM 2-APB for 5 min prior to application of ACh to induce a calcium response. In four of five cells examined, 2-APB treatment did not change [Ca²⁺], or the membrane potential, as illustrated in the representative trace (Fig. 7, B and E). This indicates that the calcium response elicited by ACh was because of IP₃ hydrolysis and activation of IP₃Rs. Furthermore, these data support our finding that IP₃-mediated Ca²⁺ release is likely the calcium source for BK channels. Finally, we sought to address the dependence on lipid raft integrity on this signaling cascade. As a first step we biochemically isolated LR membrane fractions as before and examined the localization of the M₃ mAChR. As shown in Fig. 7C (n = 3), antibodies directed against the receptor recognized bands in the lipid raft fraction as determined by reprobing the same blot for Cav-1. Furthermore, as demonstrated by the representative trace in Fig. 7D and cumulative data in Fig. 7E, disruption of lipid rafts prevented both the ACh-induced rise in [Ca²⁺], and the BK channel-induced hyperpolarization of the membrane. Taken together the data presented here support the hypothesis that BK channels are coupled to IP₃-mediated calcium release and can be physiologically activated by ACh via the Gq-coupled receptor, M₃, and that this signaling cascade requires intact lipid rafts.

**IP₃Rs Localize Near BK Channels**—Pharmacology indicated that IP₃-mediated Ca²⁺ release was the calcium source for BK channels and that this coupling is dependent on lipid rafts. If IP₃Rs are indeed the source of calcium required for activation of BK channels, then one would expect that they should localize to the same regions of glioma cells as both BK channels and lipid raft markers. To analyze the subcellular distribution of IP₃Rs and to compare this to the location of BK channels we co-labeled cells with antibodies directed against both proteins. We then examined the subcellular distribution of each protein using a spinning disk confocal microscope. As shown in the image in Fig. 8A and earlier in Fig. 1, BK channels localize diffusely throughout the cell as well as in hot spots at what appears to be the lamellipodia or leading edge of the cell. IP₃Rs, as would be expected, seemingly to primarily be concentrated in an intracellular compartment, most likely the ER. However, in addition to being found in this intracellular compartment, immunostaining also revealed that IP₃Rs localize to the exact same hotspots as BK channels, as evidenced. Additionally, Western blotting of a lipid raft isolation as performed earlier demonstrates that IP₃Rs can be found in the LR fraction as shown in Fig. 8B.

This led us to investigate the mechanism by which BK channels in lipid rafts are coupled to IP₃Rs in glioma cells. One possible option for this LR-dependent coupling is a direct protein-protein interaction between the IP₃R and BK channels that would indicate that the BK channel association with lipid rafts acts to bring the channel into close proximity with the IP₃R. To assess this possibility, we immunoprecipitated BK channel protein from whole cell lysates of U251 glioma cells with a monoclonal antibody directed against the C-terminal region of the peptide. We then collected and ran total lysate, unbound and bound samples on an SDS-polyacrylamide gel and probed for IP₃Rs. Blots were stripped and reprobed with polyclonal BK channel antibodies to verify that we had indeed pulled down the ion channel and actin antibodies to illustrate that our bound sample was not contaminated with nonbound proteins. Fig. 8C shows a representative blot from one such experiment illustrating a lack of IP₃R protein in the bound lane indicating that they do not co-immunoprecipitate and therefore do not directly bind to BK channels. Instead, they appear to localize in the vicinity of the channel in microdomains established by lipid rafts.

**DISCUSSION**

The data shown here demonstrate that BK channels in glioma cells localize to lipid raft microdomains where they are assembled into a signaling complex with a specific Ca²⁺ source that allows for channel activation. Specifically, we show that BK channels in glioma cells localize to the same fraction of an Opti-prep density gradient as the well characterized lipid raft-associated protein Cav-1. This result was further supported by immunocytochemistry demonstrating that BK channels and Cav-1 seem to localize to the same regions of the cell. Moreover, disruption of lipid rafts with MβCD caused an ~50% reduction in whole cell BK currents that was not because of channel internalization and could be completely prevented by raising [Ca²⁺], implying that lipid raft disruption also interrupts an association between BK channels and their Ca²⁺ source. Through the use of pharmacology we eliminated [Ca²⁺] influx as the source for BK channels, and we demonstrated that release of intracellular calcium via the IP₃R appears to be the
FIGURE 7. Physiological activation of mAChRs stimulates IP$_3$-mediated calcium release and activation of BK channels dependent on lipid rafts. Perforated patch clamp recordings were performed using amphotericin B to leave [Ca$^{2+}$], undisturbed in cells loaded with Fura2-AM. A, 50 μM ACh was bath-applied while simultaneously monitoring both membrane potential and [Ca$^{2+}$]. The top half of the graph shows the $F_{340}/F_{380}$ ratio generated during the recording and the concurrent response of the resting membrane potential. ACh induced a rise in calcium as evidenced by an increase in the ratio and concomitant hyperpolarization of the membrane potential to $-75$ mV that was completely inhibited by the subsequent application of 2 μM paxilline, a specific inhibitor of BK channels. B, similar experiment as in A except 50 μM 2-APB, an inhibitor of the IP$_3$R, was applied prior to ACh. In the presence of 2-APB, ACh was unable to elicit a calcium response or a simultaneous hyperpolarization of the membrane indicating that the calcium release because of activation of mACHRs is via IP$_3$. Furthermore, this calcium release pathway is what is responsible for the activation of BK channels. C, again simultaneous calcium imaging and perforated patch recording experiments were performed. Cells were bathed continuously in MjCD prior to application of ACh. Disruption of lipid rafts completely inhibited the calcium response to ACh and the concomitant hyperpolarization. D, cumulative data from the experiments carried out in A–D. Experiments were performed on at least five cells in each condition. Resting membrane potentials in response to ACh in the presence or absence of paxilline, 2-APB, and MjCD were determined 1 s following perfusion of ACh and were averaged. Data represent mean ± S.E.

**A**

**B**

**C**

**D**

likely source of calcium for BK channels in lipid rafts. Finally, we show that ACh, a ligand of muscarinic ACh receptors, stimulates [Ca$^{2+}$], release via IP$_3$ production that activates BK channels and that this is absolutely dependent on lipid raft integrity. These findings are significant in that they provide a novel mechanism for BK channel activation that is independent of cell depolarization and instead places BK channels near a Ca$^{2+}$ source that is sufficient for channel activation.

BK channels are unique ion channels capable of responding to either changes in membrane potential, changes in [Ca$^{2+}$]$_i$, or a combination of both. Unlike neurons or muscle cells, glioma cells do not experience the large membrane depolarizations associated with action potential propagation or the large Ca$^{2+}$ sparks following muscle stimulation. Even in excitable cells evidence exists that BK channels form signaling complexes with specific calcium sources. Indeed, Doheny et al. (35) demonstrated that BK channels in uterine myocytes are functionally coupled to β$_2$-adrenoceptors, and BK channel activation is responsible for the β$_2$-adrenoceptor-induced uterine relaxation because inhibition of BK channels prior to activation of the receptor completely inhibits relaxation. Additionally, β$_2$-adrenoceptors have been shown to form signaling complexes with BK channels and L-type VGCCs, functionally coupling the influx of calcium through the L-type channel to activation of BK channels (36). Interestingly, a very recent study has suggested that Ca$^{2+}$ wave propagation in astrocytes is absolutely dependent on IP$_3$ signaling in lipid raft domains (37). Additionally, BK channels have been found to localize to astrocytic end feet (38), where they aid in blood-brain barrier signaling in response to these same Ca$^{2+}$ waves (39). Given the findings in this study, it seems likely that a similar mechanism is in place in astrocytes and that BK channels likely localize to lipid rafts to ensure their activation in response to IP$_3$-mediated calcium waves.

Unlike other mechanisms for assembly of signaling complexes, lipid rafts provide a scaffold where proteins can move in and out freely, making it possible for BK channels to have multiple signaling partners depending on the cellular context. Glioma cells are characterized by rapid proliferation and migration/ invasion. These cellular processes can be initiated by very different signals. Evidence exists that multiple growth factor receptors implicated in both proliferation and migration diffuse into or out of lipid raft microdomains upon ligand binding (for review see 40). Additionally, inositol 1,4,5-triphosphate production has been shown to occur exclusively in lipid rafts (41). Together, these phenomena may imply that any receptor that binds ligand while either localized to lipid rafts or is subsequently translocated into lipid rafts following binding may activate BK channels in that same raft, initiating K$^+$ fluxes required for migration. This idea is supported by our immunocytochemistry data demonstrating that BK channels, IP$_3$Rs, and LR markers all localize to what appear to be lamellipodia or the leading edge of glioma cells. Furthermore, independent studies from our own laboratory suggest that chloride channels associate with lipid rafts (42) and that these channels are also involved in glioma cell migration (1–5). Interestingly, immunolabeling from this latter study indicates that chloride channels also localize to the leading edge of migratory glioma
cells, and thus it is tempting to hypothesize that BK channels, chloride channels, IP$_3$Rs, and growth factor receptors may localize to lamellipodia where together they form a lipid raft-dependent signaling complex specifically involved in facilitating the coordinated K$_{\text{Cl}}$ and Cl$_{\text{Cl}}$ effluxes thought to be required for migratory volume changes.

Two previous studies conducted in lipid bilayers have demonstrated that the lipid content of membrane has drastic effects on the biophysical properties of BK channels (43, 44). Specifically, increasing the cholesterol content of the bilayer increases the mean closed time of the channel thereby attenuating channel activity. Interestingly, these same observations of channels inserted into artificial membrane appear to hold true in cellular systems. Several recent studies have demonstrated that BK channels in colonic epithelia, vascular endothelia, and human myometrium all localize to lipid rafts. Moreover, in each of these studies BK currents were not present until after disruption of these membrane microdomains with M$_{\text{g}}$CD (45–47). In these same cell systems, as with several other smooth muscle cell types, however, lipid rafts also appear to serve a similar function as in glioma cells, i.e. they localize BK channels to the same regions of the cell as ryanodine receptors and L-type voltage-gated calcium channels responsible for generating calcium sparks in response to electrical stimulation (31, 48, 49).
BK Channel Coupling to IP₃R via Lipid Rafts

In this study, however, we demonstrate for the first time that BK channels in glioma cells are functionally coupled to [Ca²⁺], through IP₃Rs via lipid rafts. This finding is important because different signaling pathways can activate different mechanisms of [Ca²⁺] mobilization, and it implies that in glioma cells BK channels rely heavily on phospholipase C-γ-dependent signaling for their activation. Therefore, examining G-protein-coupled receptors, receptor tyrosine kinases, and other receptors that directly stimulate phospholipase C-γ, inducing IP₃R-dependent calcium release, may provide additional insight into the function of BK channels in the context of glioma cell invasion. These studies may therefore provide novel ways to interfere with the diffuse spread of gliomas in the surrounding healthy brain.

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