Published in final edited form as:
Glia. 2009 September ; 57(12): 1280–1295. doi:10.1002/glia.20847.

Ca^{2+}- and Thromboxane-Dependent Distribution of MaxiK Channels in Cultured Astrocytes: From Microtubules to the Plasma Membrane

J.W. OU[1], **Y. KUMAR**[2], **A. ALIOUA**[2], **C. SAILER**[5], **E. STEFANI**[2,3,4], and **L. TORO**[1,2,4,*]

[1]Department of Molecular and Medical Pharmacology, University of California at Los Angeles, Los Angeles, CA 90095

[2]Department of Anesthesiology, University of California at Los Angeles, Los Angeles, CA 90095

[3]Department of Physiology, University of California at Los Angeles, Los Angeles, CA 90095

[4]Brain Research Institute, University of California at Los Angeles, Los Angeles, CA 90095

[5]Division for Molecular and Cellular Pharmacology, Medical University, Innsbruck, Peter Mayr-Strasse 1, A-6020 Innsbruck, Austria

Abstract

Large-conductance, voltage- and Ca^{2+}-activated K^+ channels (MaxiK) are broadly expressed ion channels minimally assembled by four pore-forming α-subunits (MaxiKα) and typically observed as plasma membrane proteins in various cell types. In murine astrocyte primary cultures, we show that MaxiKα is predominantly confined to the microtubule network. Distinct microtubule distribution of MaxiKα was visualized by three independent labeling approaches: 1) MaxiKα-specific antibodies, 2) expressed EGFP-labeled MaxiKα, and 3) fluorophore-conjugated iberiotoxin, a specific MaxiK pore-blocker. This MaxiKα association with microtubules was further confirmed by *in-vitro* His-tag pulldown, co-immunoprecipitation from brain lysates, and microtubule depolymerization experiments. Changes in intracellular Ca^{2+} elicited by general pharmacological agents, caffeine or thapsigargin, resulted in increased MaxiKα labeling at the plasma membrane. More notably U46619, an analog of thromboxane A2 (TXA2) which triggers Ca^{2+}-release pathways and whose levels increase during cerebral hemorrhage/trauma, also elicits a similar increase in MaxiKα surface labeling. Whole-cell patch clamp recordings of U46619 stimulated cells develop a ~3-fold increase in current amplitude indicating that TXA2 stimulation results in the recruitment of additional, functional MaxiK channels to the surface membrane. While microtubules are largely absent in mature astrocytes, immunohistochemistry results in brain slices show that cortical astrocytes in the newborn mouse (P1) exhibit a robust expression of microtubules that significantly colocalize with MaxiKα. The results of this study provide the novel insight that suggests Ca^{2+} released from intracellular stores, may play a key role in regulating the traffic of intracellular, microtubule-associated MaxiKα stores to the plasma membrane of developing murine astrocytes.

Keywords

Traffic and targeting; microtubules; potassium channel; thromboxane A2; BK channel

Corresponding author: Ligia Toro, Dept. Anesthesiology, UCLA, BH-509A CHS, Box 957115, Los Angeles, CA 90095-7115, USA, Tel. 1-310 794-7809; Fax 1-310 825-5379, e-mail: E-mail: ltoro@ucla.edu.

Introduction

Astrocytes, the most prevalent glial cells in the central nervous system were originally thought to provide passive support functions such as maintaining brain architecture, scavenging excess neurotransmitters and debris, and constituting the blood brain barrier. However, recent studies have suggested that astrocytes serve a more active, functional role in the brain (Fields et al., 2002;He et al., 2007;Saloheimo et al., 2005). One of such roles is the astrocytic mediation of neuronal activity-dependent cerebrovascular tone, a function termed neurovascular coupling (Zonta et al., 2003a;Filosa et al., 2004;Straub et al., 2006). Perivascular astrocytes, which contain endfeet projections terminating on nearby cerebral blood vessels, are stimulated by neurotransmitter release from active adjacent neurons. This stimulation by neighboring neurons evokes glial Ca^{2+} increases which can subsequently affect vascular tone by the release of various vasoactive substances such as arachidonic acid metabolites (Zonta et al., 2003b;Mulligan et al., 2004;Li et al., 2003), prostacyclins, epoxyeicosatrienoic acids (Medhora et al., 2001), glutamate, adenosine, and ATP (Anderson et al., 2004). An additional manner of mediating vascular tone by astrocytes during neurovascular coupling is the release of K^+ from perivascular astrocytic endfeet resulting in vasodilation (Paulson et al., 1987;Hamel, 2006;Iadecola, 2004), a mechanism termed K^+ siphoning.

Astrocytes from either primary culture or brain slices express a wide variety of potassium channels (Oh Y, 1997;Barres et al., 1990;Hansson et al., 1994), much of which have yet to be well characterized. One class of K^+ channels, large-conductance, voltage- and Ca^{2+}-activated K^+ channels (MaxiK, BK), are ubiquitously expressed proteins found in a variety of tissues which are responsible for a broad spectrum of physiological roles such as blood flow, uresis, and neurotransmission (Lu et al., 2006). Recently, MaxiK channels have been identified as responsible for local K^+ efflux into the perivascular space coupling neuronal activity to arteriolar diameter. Astrocytic Ca^{2+} has a key role in this coupling as its increase promotes the Ca^{2+}-dependent activation of astrocytic MaxiK channels and K^+ efflux-mediated vasodilatation (Filosa et al., 2006). In line with the role of MaxiK channels in astrocytic function, MaxiK channels have been previously detected in cultured rat astrocytes by electrophysiological methods (Bychkov et al., 2001;Gebremedhin et al., 2003) and observed in the perivascular endfeet of rat astrocytes in brain slices (Price et al., 2002). However, there is virtually no data concerning the detailed subcellular localization of MaxiK channels and the factors regulating their expression in astrocytes. In our studies, we show that in cultured murine astrocytes, MaxiK channels are predominantly distributed intracellularly and associated with the microtubule network. In addition, a mechanism influencing MaxiK channel surface expression is an increase in intracellular Ca^{2+}. Furthermore, we show that thromboxane A2 mimics the Ca^{2+}-induced MaxiK surface expression, thereby providing a possible protective mechanism during pathological conditions of elevated thromboxane A2 levels such as cerebral hemorrhage.

Materials and Methods

Antibodies

Monoclonal α-tubulin antibody and anti-glial fibrillary acidic protein (GFAP) were from Sigma. Polyclonal MaxiK α-subunit antibody (MaxiKα) was from Alomone Labs [Lot # AN-05]. MaxiKα antibody was extensively characterized by immunoblotting using lysates from expression systems and smooth muscle where it labeled proteins of the appropriate molecular size (125 kDa monomer, 250 kDa dimer). Antibody specificity in immunoblots and immunocytochemistry was confirmed by antigen block. Secondary antibodies were from Molecular Probes (Invitrogen).

Astrocyte cultures

Primary astrocyte cultures were prepared from postnatal day 1 (P1) C57/BL6 mice (McCarthy et al., 1980). Briefly, cortices were dissociated (20 min, 37°C) with 0.25% Trypsin (Invitrogen) and centrifuged (208 × *g*, 5 min, 4°C). Cells were resuspended in DMEM containing 10% fetal bovine serum, 1.4 mM L-glutamine, 1% penicillin/streptomycin, plated into 25-cm^2 tissue culture flasks, and incubated at 37°C/5% CO_2. Upon confluency (7-10 days), glial cultures were shaken overnight (250 rpm, 37°C) to dislodge microglia and oligodendrocytes. Astrocytes were then detached with 0.05% Trypsin/EDTA and seeded onto poly-L-lysine (0.01%) coated coverslips for immunostaining or electrophysiology. Astrocyte identity was confirmed by positive GFAP immunolabeling (not shown).

Immunolabeling

Astrocytes or 10 μm cryopreserved coronal brain slices were fixed with pre-warmed 4% paraformaldehyde for 20 min, washed, and permeabilized in PBS + 0.2% Triton-X-100, pH 7.4 (3×10min). Non-specific labeling was blocked in PBS + 0.2% Triton-X-100 + 10% normal goat serum (1 hr, room temperature). Cells were double labeled (overnight, 4°C) with monoclonal α-tubulin (1 μg/ml) and polyclonal MaxiKα antibodies (1.6 μg/ml), or AlexaFluor-594 conjugated iberiotoxin (1 nM) diluted in PBS + 0.2% Triton-X-100 + 1% normal goat serum. Secondary antibodies AlexaFluor-488 and AlexaFluor-568 (2 μg/ml) were incubated for 1 hr at room temperature. Cells were subsequently washed 3 × 10 minutes in PBS and mounted using Prolong Gold anti-fade reagent (Invitrogen) for imaging.

Imaging and colocalization analysis

High-resolution laser confocal sections were acquired using an Olympus Fluoview FV5-PSU (60X objective lens; NA= 1.42). Image stacks were taken every 0.1 μm through the entire Z-plane of cells (dual fluorophore signals were sequentially acquired) and 3D blind deconvolved (Autodeblur & Autovisualize, Autoquant Imaging). Quantitative colocalization analysis was carried out by applying a grid delimiting 2×2 μm squares over each separate confocal plane. Squares with pixel intensity values larger than 0.25 of the maximum pixel intensity value of the whole image plane were selected and correlation coefficients (CC) of individual squares were calculated. The Protein Proximity Index (PPI) was calculated as ($N_{CC}>0$, $P<0.05/N_t$) where, $N_{CC}>0$, $P<0.05$ is the number of squares with $CC>0$ and $P_{sign\ test}$ value<0.05, and N_t is the total number of squares (Ropero et al., 2006).

Intensity analysis

Three cytosolic 10 μm diameter regions in each cell were randomly selected to measure average intensities using Metamorph software (Molecular Devices).

MaxiKα adenoviral vector

N-terminal c-Myc epitope tagged MaxiKα was fused at its C-terminus to enhanced green fluorescent protein (EGFP) (MaxiKα-EGFP) and expressed using AdEasy Adenoviral Vector System (Stratagene). Astrocytes were infected (multiplicity of infection=30) for 48 hrs prior to fixation and analysis.

Protein Extraction

Freshly isolated brains from newborn mice were lysed by homogenization and sonication (3 × 10 seconds) in 1% NP-40 alternative, 1% Na-deoxycholate, 10% glycerol, 150 mM NaCl, 50 mM Tris, and protease inhibitors (lysis buffer) followed by incubation on ice (30 min). Cellular debris was removed by centrifugation (850 × g, 10 minutes). Protein concentration was determined by Lowry method.

His-tag pulldown assay

A MaxiKα carboxyl-terminus domain "tail" (Wei et al., 1994) (amino acids 679-1113, GenBank Accession #U11058, MaxiK$\alpha_{679\text{-}1113}$) was subcloned into the bacterial expression vector pET-28a (Novagen) to express six N-terminal histidine fusion protein (His-MaxiK$\alpha_{679\text{-}1113}$). His-MaxiK$\alpha_{679\text{-}1113}$ was expressed in E. coli BL21-Gold (DE3) cells (Stratagene), extracted under native conditions, and purified using Ni-NTA agarose beads (Qiagen). His-MaxiK$\alpha_{679\text{-}1113}$ bound to Ni-NTA agarose beads (bait) was incubated with whole brain lysates in binding buffer (20 mM Tris-HCl, pH 8.0,10 mM Imidazole, 300 mM NaCl, 10% glycerol). Non-specific binding was washed off with excess binding buffer containing 50 mM imidazole, and His- MaxiK$\alpha_{679\text{-}1113}$ bound proteins were eluted with 20 mM Tris-HCl, pH 8.0, 300 mM Imidazole, 300 mM NaCl, 10% glycerol. Elution fractions were subsequently analyzed by Western blot. Negative control consisted of incubating Ni-NTA agarose beads with bacterial lysates not expressing His-MaxiK$\alpha_{679\text{-}1113}$ prior to bead incubation with whole brain lysates.

Co-immunoprecipitation

Monoclonal anti-α-tubulin antibody (10μg) was bound to 40 μl protein A/G agarose beads (6 hrs, 4°C) followed by washing with lysis buffer (see above). The antibody-bead complex or beads alone (control) were incubated with newborn brain lysate (2 mg protein). After overnight incubation (4°C), samples were washed 3 times with lysis buffer and extracted in SDS sample buffer for Western blot analysis.

Western blot analysis

Samples were mixed with equal volumes of SDS loading buffer and dithiothreitol to a final concentration of 62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 0.01% bromophenol blue, and 42 mM dithiothreitol. Samples were separated on 10% SDS gels by polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred to nitrocellulose membranes together with prestained molecular weight standards (Li-Cor). Membranes were blocked (1 hr, room temperature) with 5% nonfat dried milk in Tris-buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH 7.4). Membranes were double labeled with antibodies against MaxiKα (0.8 μg/ml) and α-tubulin (1 μg/ml) in 1% nonfat milk/TBS containing 0.5% Triton X-100 and 0.1% Tween overnight at 4 °C, washed (TBS 3×10 min), and incubated with secondary antibodies Alexa Fluor 680 goat anti-rabbit IgG (0.133 μg/ml) or IRDyeTM 800 goat anti-mouse (0.066 μg/ml) (Rockland) for 1 h. Blots were washed (3 times, 10 min) and visualized by infrared fluorescence (Odyssey™, Li-Cor).

Electrophysiology

Whole-cell MaxiK currents were recorded with an Axopatch 5200A amplifier at room temperature using patch pipettes of ~2-3 MΩ. All currents were recorded from type I astrocytes as identified by morphological observation (flat, polygonal epithelial shape). Bath solution was (in mM): 135 Na-methanesulfonic acid (Mes), 5 K-Mes, 0.1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, and 5 Glucose (pH 7.4). Pipette solution was (in mM): 140 K-Mes, 0.1 $CaCl_2$, 2 $MgCl_2$, 0.1 Na-ATP, 0.1 Na-GTP, 0.145 EGTA, and 10 HEPES (pH 7.4) (pCa 6.35). To inactivate other voltage-activated currents, the holding potential (V_h) was set to 0 mV. Test pulses were from -70 to 160 mV in 10 mV increments. For time course recordings, V_h was −70 mV and 120 mV test pulses were given every 3 seconds. Whole-cell currents were normalized to cell capacitance (calculated from 10 mV pulses) to obtain current density. To subtract capacity transients from total current traces, recordings were scale subtracted off-line using currents elicited by pulses to -70 mV that showed no time-dependent ionic currents.

Statistics

Data is presented as mean ± SE. Statistical comparisons among groups were made using ANOVA followed by Student-Newman-Keuls test. A *P-value* < 0.05 was considered statistically significant and is marked with (*).

Results

MaxiK whole-cell currents are blocked by iberiotoxin and paxilline

Previous electrophysiological studies in rat astrocyte primary cultures have reported the conflicting information of macroscopic K^+ currents both sensitive to iberiotoxin (IbTx) block in striatal astrocytes (Bychkov et al., 2001) as well as IbTx insensitivity in cortical and hippocampal astrocytes (Gebremedhin et al., 2003). Additionally, paxilline (Pax), another well characterized MaxiK-specific inhibitor (Knaus et al., 1994), has been previously shown to block MaxiK currents in astrocyte derived glioma cells (Weaver et al., 2006) and in primary cultured rat hippocampal astrocytes (Yamaura et al., 2006). As no previous electrophysiological data exist detailing IbTx and Pax sensitivity in primary cultures isolated from murine cortical astrocytes, we investigated macroscopic K^+ current sensitivity to IbTx and Pax block. With IbTx added to the external bath solution and at a holding potential (V_h) of 0 mV to inactivate other voltage-sensitive channels, K^+ currents elicited by step depolarizations from -70 mV to +160 mV were not significantly blocked by 100 nM IbTx during the first 20 minutes of application (data not shown). A moderate but significant macroscopic K^+ sensitivity to IbTx was observed when a 5 μM concentration was applied after 5 minutes (Fig. 1A, upper right). In contrast, K^+ currents were instantaneously inhibited upon application of 2 μM Pax (Fig. 1A, lower right). The corresponding average current-voltage (IV) plot (Fig. 1B) and bar plot (Fig. 1C) of normalized current amplitude (to +120 mV) in response to pharmacological treatment indicate that 5 μM IbTx results in ~40% block of outward current (gray bar) while 2 μM Pax application results in a significantly greater ~80% outward current decrease.

Previous studies have found that dissociation rates of pore-blocking peptides with the MaxiK channel may vary dependent upon the channel's voltage state being slower at negative potentials (Anderson et al., 1988). In agreement with this concept, 5 μM IbTx results in a larger ~70% outward current block when currents to +120 mV are recorded from a V_h of -70 mV (Fig. 1C, D) than from a V_h of 0 mV (~40% block) (Fig. 1C) as the toxin would have more difficulty in dissociating from the channel at -70 mV, enhancing its blocking capacity. The resistance of MaxiK channels to IbTx block up to μM concentrations has been attributed to the expression of the MaxiK β4 auxiliary subunit which greatly slows down IbTx association with the channel (Meera et al., 2000). Thus, our findings of astrocyte macroscopic K^+ current apparent insensitivity up to μM IbTx concentrations argue in favor of the presence of the MaxiK α+β4 subunit complex in these cells. In further support of the presence of MaxiKβ4 in cultured murine astrocytes, the on-rate time constant for IbTx block in cultured murine astrocytes (Fig. 1D, $k_{on} = 90\ M^{-1}s^{-1}$) was ~4 orders of magnitude slower than for Maxi α subunit alone and ~1 order of magnitude slower than for MaxiK α+β4 subunits ectopically expressed in *Xenopus laevis* oocytes (Meera et al., 2000).

Immunolabeling of MaxiKα colocalizes to the microtubule network

MaxiK α-subunit specific antibodies were used to determine MaxiKα distribution in newborn murine cortical astrocytes in primary culture. Immunostaining of MaxiKα reveals the surprising finding that the typically surface membrane bound ion channel is predominantly distributed intracellularly in a punctated manner (Fig. 2A). Double labeling of MaxiKα with the microtubule marker, α-tubulin (Fig. 2B), revealed a high degree of overlap between channel labeling and the cytoskeletal architecture (Fig. 2C). To quantify the level of co-localization

between the two fluorophores, a 2×2 μm^2 grid of the images in Figs. 2A and 2B were analyzed as described in Methods. Figs. 2D and 2E show the correlation coefficient (CC) histogram of the total # of observations and the $P_{sign\ test}$ vs. CC relationship plot, respectively. Red bars in the histogram mark observations with positive correlation (CC>0), which are the large majority. The highlighted portion below the dotted line in the $P_{sign\ test}$ vs. CC plot depicts regions with a positive CC and with $P < 0.05$. Also, the vast majority of discrete regions with positive CC have $P<0.05$, which divided by the total # of observations result in a PPI of 0.87. To validate that the high degree of colocalization between MaxiK and α-tubulin was not due to overlay coincidence of a random expression pattern, PPI values were measured as a function of pixel shift in the x-axis. PPI was reduced from 0.87 to a baseline of 0.2 after 0.5 μm pixel shift of the x-axis (Fig. 2F), thus confirming that the high degree of colocalization between MaxiKα and the astrocytic microtubule network is not due to arbitrary fluorophore coincidence. The baseline PPI of 0.2 can be explained by a high signal density that precludes further PPI reduction by pixel shift.

Immunolocalization of IbTx-AlexaFluor594 colocalizes to astrocyte microtubules

Iberiotoxin, isolated from the venom of the scorpion *Buthus tamulus*, has been previously established as a MaxiK channel blocker that binds distinctly to the tetrameric pore (Galvez et al., 1990). To investigate whether the microtubule associated MaxiKα in cultured murine astrocytes is in its tetrameric conformation, we labeled primary astrocyte cultures with IbTx conjugated to AlexaFluor-594 (IbTx-A594). Analogous to MaxiKα and α-tubulin double labeling (Fig. 2A-C), IbTx-A594 revealed a similar punctated, intracellular expression pattern (Fig. 2G) that colocalizes to the microtubules (Fig. 2H) with a high degree of overlap (Fig. 2I). PPI analysis of IbTx-A594 colocalization with α-tubulin shows a PPI = 0.83 (Figs. 2J, K) similar to the PPI values between antibodies directed against MaxiKα and microtubules. Pixel shift analysis of images from Figs. 2G, H indicates a reduction of PPI value from 0.83 to 0.03 after 0.5 μm pixel shift in the x-axis (Fig. 2L). These results further confirm the association of astrocytic MaxiKα to the microtubule network. In addition, due to the proclivity of Ibtx binding to the fully formed pore of the MaxiK channel, these findings suggest that the intracellular MaxiKα subunit is arranged in a tetrameric conformation.

Expression of MaxiK-EGFP in cultured murine astrocytes shows similar association with the microtubule network

To determine if exogenously expressed MaxiKα would follow a similar distribution pattern of predominant intracellular labeling along the microtubules as seen with native MaxiKα in cultured murine astrocytes, we infected primary astrocyte cultures with an adenoviral vector that would generate MaxiKα protein conjugated to EGFP for visualization purposes (Fig. 3A). To confirm the identity of the expressed EGFP labeled protein, transformed cultures were double labeled with MaxiKα specific antibodies (Fig. 3B). The nearly perfect colocalization value, PPI = 0.98 (Figs. 3D, E), of the overlay between MaxiKα-EGFP and MaxiKα antibody labeling observed in Fig. 3C indicates that the adenoviral expressed protein is indeed MaxiKα protein. Additionally, the extremely high PPI value of the double labeling also provides a reciprocal validation of the specificity of MaxiKα antibody used. Pixel shift analysis shows a PPI reduction of 0.98 to 0.2 after 0.5 μm pixel shift in the x-axis (Fig. 3F), thus confirming that the near-perfect colocalization between MaxiK antibody and MaxiK-EGFP is not arbitrary.

When MaxiK-EGFP expressing astrocytes (Fig. 3G) are double labeled against α-tubulin (Fig. 3H), the intracellularly punctated MaxiK-EGFP distribution shares a significant degree of colocalization to the microtubule architecture (Fig. 3I). The PPI value between MaxiK-EGFP and α-tubulin is 0.82 (Figs. 3J, K), with a pixel-shift PPI reduction from 0.82 to 0.2 after 0.5μm pixel shift in the x-axis (Fig. 3L). The results of MaxiK-EGFP expression colocalized

to the microtubule network mirror the findings found by MaxiK antibody and IbTx-A594 distribution. All three independent visualization strategies show MaxiK colocalization to α-tubulin have comparably high PPI values as seen in the bar plot comparison (see Fig. 4M, n = 10 for each group).

Microtubule depolymerization disrupts MaxiKα co-localization with the microtubule network

To investigate whether microtubule structural integrity has a bearing on the distribution of MaxiKα in cultured murine astrocytes, we examined MaxiKα → α-tubulin colocalization in response to microtubule depolymerization. Primary astrocyte cultures were treated with colchicine (100 μM), a well-characterized microtubule-depolymerizing agent, for 3 hours at 37°C. Colchicine treatment resulted in a diffuse labeling of α-tubulin, with filamentous polymerized microtubules no longer present (Fig. 4A). Double labeling of MaxiKα in colchicine treated astrocytes revealed a diffuse, punctated distribution of the channel (Fig. 4B) that no longer shares a high degree of colocalization with α-tubulin (Fig. 4C). PPI analysis of MaxiKα colocalization with α-tubulin in the colchicine treated cell in images Figs. 4A,B indicate a PPI = 0.13 (Figs. 4D,E). Pixel shift analysis (0-2 μm in the x-axis) of images from Figs. 4A, B show negligible change in the low PPI value (Fig. 4F) signifying arbitrary co-localization of MaxiKα with α-tubulin after colchicine treatment.

To further substantiate our findings, we treated primary astrocyte cultures with a different, well-characterized microtubule-depolymerizing agent, nocodazole. Nocodazole treatment (20 μM, 3 hours at 37°C) resulted in a similar diffuse labeling of α-tubulin with the notable absence of a filamentous microtubule network (Fig. 4G). In parallel with the colchicine treatment colocalization findings, double labeling with MaxiKα in nocodazole treated astrocytes (Fig. 4H) resulted in an insubstantial colocalization with α-tubulin (Fig. 4I). Quantification of co-localization between MaxiKα and α-tubulin in the nocodazole treated astrocyte from Fig. 4G-H exhibits a low PPI value (PPI = 0.2, Figs. 4J, K). Similar to the results found by pixel shift analysis of colchicine treated astrocytes, pixel shift of images from nocodazole treatment in Figs. 4G, H result in minimal PPI change (Fig. 4 L) indicating arbitrary co-localization of MaxiKα with α-tubulin after nocodazole induced microtubule depolymerization.

Bar plot summary of microtubule depolymerization experiments (Fig. 4N, n= 10 for each group) indicate that treatment with either colchicine or nocodazole causes a ~4-fold reduction in PPI values in comparison to untreated controls. PPI = ~0.2 for colchicine/nocodazole treatment groups (white and shaded bars) is comparable to 1 μm pixel-shifted images (black bar) indicating an arbitrary, baseline non-colocalization.

MaxiKα C-terminus “tail” interacts with microtubules *in vitro*

While the association between MaxiKα and α-tubulin observed in primary cultured astrocytes was demonstrated by high-resolution immunolocalization studies, we sought biochemical confirmation of this interaction in mouse whole brain lysates by means of His-tag pulldown assays. Histidine-tagged MaxiKα C-terminus “tail” protein ($MaxiK\alpha_{679\text{-}1113}$) was bound to Ni-NTA agarose beads and incubated with whole brain lysates extracted from newborn mice. As shown by immunoblots in Fig. 5A, elution fractions containing $His\text{-}MaxiK\alpha_{679\text{-}1113}$ “tail” bait protein (Fig. 5A, top row) were able to interact with endogenous α-tubulin (Fig. 5A, 2nd row). Ni-NTA agarose beads incubated with negative control bacterial lysates not containing the $His\text{-}MaxiK\alpha_{679\text{-}1113}$ “tail” fusion protein (to ensure the specificity of Ni-NTA agarose bead binding) prior to incubation with mouse whole brain lysates revealed, as expected, no $MaxiK\alpha_{679\text{-}1113}$ “tail” signals (Fig. 5A, 3rd row) and no α-tubulin pulldown (Fig. 5A, bottom row). His-pulldown experiments were repeated in triplicate yielding similar results indicating that MaxiKα interacts with α-tubulin *in vitro* and that the MaxiKα “tail” domain is sufficient for this interaction.

Native brain MaxiKα co-immunoprecipitates with α-tubulin

Having determined that the MaxiK$\alpha_{679\text{-}1113}$ "tail" interacts with α-tubulin *in vitro*, we next performed co-immunoprecipitation studies in newborn (P1) brain lysates (Fig. 5B) to investigate whether full-length native MaxiKα also interacts with α-tubulin in brain tissue. As evidenced by Western blot analysis in the input brain lysates (Fig. 5B, Lane 1), both native MaxiKα and α-tubulin are prominently expressed. Newborn brain lysates were subjected to immunoprecipitation with anti-α-tubulin monoclonal antibody (IP: α-tubulin) or without antibody as a negative control (IP: No Ab). As shown in Fig. 5B, α-tubulin was efficiently immunoprecipitated in lysates derived from newborn brain tissue (Lane 2 lower) along with MaxiKα (Lane 2 upper) indicating an interaction between the two proteins in native tissue. As expected for specific interactions, using beads alone resulted in no immunoprecipitated α-tubulin, nor MaxiKα signals (Fig. 5B, Lane 3). To ensure MaxiKα antibody specificity in our immunoblots, MaxiKα polyclonal antibody was preincubated with its antigenic peptide (5:1 ratio of antigenic peptide to antibody) prior to immunoblotting cell lysates. MaxiKα Ab antigen block resulted in the inability of the Ab to label MaxiKα in newborn brain cell lysates (Fig. 5B, Lane 4 upper) while this treatment did not affect binding specificity of anti-α-tubulin (Fig. 5B, Lane 4 lower).

Increased MaxiK surface expression in response to caffeine/thapsigargin application

Previous studies have shown that MaxiK channels are activated by the release of Ca^{2+} from sarcoplasmic reticulum (SR) stores (Nelson et al., 1995). In human myometrial smooth muscle cells, increases in intracellular Ca^{2+} released from SR stores elicited by caffeine application resulted in the translocation of the C-terminal end of MaxiKα splice variant, mK44, from intracellular stores to the cell membrane (Korovkina et al., 2006). To investigate whether microtubule-associated MaxiK channels would fare a similar response, we hypothesized that surface membrane expression of MaxiKα could be induced by release of Ca^{2+} from intracellular stores. To ensure SR was the source of Ca^{2+} release, all pharmacological treatments were conducted in Ca^{2+}-free buffers. After treatment of primary cultured astrocytes with 20 mM caffeine for 30 minutes at 37°C, double immunolabeling of MaxiKα (Fig. 6A) and α-tubulin (Fig. 6B) revealed an increased expression of MaxiKα at the plasma membrane. Intracellular colocalization between MaxiKα and α-tubulin remained consistent as observed in image overlay Fig. 6C, but MaxiKα surface expression at the cell membrane appeared distinct from the microtubule network as limited colocalization was seen at the periphery (white arrows, Fig. 6A,C). Caffeine application induced an approximate ~6-fold increase in the number of cultured astrocytes with MaxiKα labeling at the cell surface (Fig. 6G, # cells counted/condition = ~3,000, n=3).

To test an alternative mechanism of increasing intracellular Ca^{2+} concentrations thereby further bolstering the hypothesis that increasing cytosolic Ca^{2+} would induce increased expression of MaxiKα at the astrocyte plasma membrane, 1 μM thapsigargin (THAP) was added to the culture media of primary astrocyte cultures. THAP works to increase intracellular Ca^{2+} concentrations by effectively inhibiting the Ca^{2+} ion pump proteins of intracellular membranes located on the sarcoplasmic reticulum and endoplasmic reticulum (Lytton et al., 1991). Similar to the results of caffeine treatment, double immunolabeling of MaxiKα (Fig. 6D) and α-tubulin (Fig. 6E) showed that 1 μM THAP treatment for 30 minutes at 37° induced increased surface expression of MaxiKα (white arrows, Fig. 6D,F). Manual tally of number of cells with MaxiKα plasma membrane expression after THAP treatment indicated an approximate ~6-fold increase in the number of cultured astrocytes with MaxiKα labeling at the cell surface (Fig. 6G, # cells counted/condition = ~3,000, n=3).

Thromboxane A2 receptor agonist, U46619, increases MaxiKα expression at the plasma membrane

As glial cells possess a broad spectrum of neuroligand receptors that are linked to intracellular Ca^{2+} mobilization (Finkbeiner, 1993), we sought to identify a potential physiological signaling candidate that would elicit increased MaxiKα surface expression in astrocytes via cytosolic Ca^{2+} modulation. Thromboxane A2 (TXA2), a metabolite of arachidonic acid produced by cyclooxygenase and TXA2 synthase, is responsible for a variety of cellular responses including platelet aggregation and contraction in various types of smooth muscle (Huang et al., 2004). Previous studies have identified the presence of TXA2's cognate prostanoid receptor, thromboxane A2 receptor (TPR) in astrocytes (Nakahata et al., 1992;Kitanaka et al., 1995). Although Ca^{2+} mobilization in response to TPR activation has been demonstrated in cultured astrocytes (Kitanaka et al., 1996), the physiological significance and function of TPR signaling in glia remains undetermined. To explore the hypothesis that TPR activation induces increased MaxiKα surface expression, we investigated the effect of TPR agonist U46619 in primary astrocyte cultures. Similar to the results of caffeine/thapsigargin treatment, 100 nM U46619 application for 6 hours at 37° resulted in increased plasma membrane expression of MaxiKα at the cell surface (Fig. 7C). Double immunolabeling of MaxiKα (Fig. 7A) and α-tubulin (Fig. 7B) revealed an approximate ~6-fold increase in the number of cells with prominent MaxiKα surface expression in comparison to vehicle treated controls (Fig. 7G shaded vs. open bars).

In the cultured astrocytes exhibiting increased MaxiKα plasma membrane expression, analysis of intracellular MaxiKα labeling intensity reveals a slight yet statistically significant reduction of immunolabeling intensity in cell cultures stimulated with U46619, caffeine, or thapsigargin (Fig. 7H; 10 cells/condition, n=3). These data support the hypothesis that the MaxiKα comprising the increased plasma membrane distribution in response to stimulation may be recruited and translocated from the intracellular pools of MaxiKα associated with the microtubule network. Consistent with the view that microtubules serve as a reservoir and conduit for MaxiKα trafficking to the surface, U46619, caffeine, or thapsigargin treatment of astrocytes under depolymerized microtubule conditions (colchicine treatment) failed to exhibit an increase in the number of cells expressing MaxiKα at the surface membrane (Fig. 7G, blue bars).

Preincubation of astrocytes with BAPTA-AM prevents increases in MaxiKα surface expression

We further investigated the role of Ca^{2+} in caffeine/thapsigargin/U46619 induced MaxiKα cell surface expression. To provide additional support for the hypothesis that Ca^{2+} is a mediating factor in astrocyte activity dependent MaxiKα cell membrane expression, primary cultured astrocytes were preincubated with BAPTA-AM, a cell permeable Ca^{2+} chelator. Astrocytes preincubated with 50 μM BAPTA-AM for 30 minutes prior to U46619 application exhibited significantly less MaxiKα surface expression in comparison to vehicle preincubation. Representative images of BAPTA-AM + U46619 treated cultured astrocytes are shown in Figs. 7D-F. Similarly, 50 μM BAPTA-AM preincubation was also able to effectively prevent MaxiKα cell membrane expression induced by caffeine and thapsigargin application. The bar plot summary comparing the average number of cells expressing cell-surface MaxiKα (Fig. 7G) reveal that BAPTA-AM preincubation (red bars) causes a ~6-fold reduction in the number of astrocytes expressing surface MaxiKα after treatment with U46619/caffeine/thapsigargin (compare with shaded bar in Fig. 7G, and with black and gray bars of Fig. 6G). BAPTA-AM preincubation prior to cell stimulation reduces the number of cells exhibiting MaxiKα surface expression to levels comparable to that of untreated controls (Fig. 7G, red vs. open bars) and BAPTA-AM alone (Fig. 7G, red vs. black bars) treated cultured astrocytes (~5%). These results suggest that the changes in intracellular Ca^{2+}, as a result of pharmacological/physiological

stimulation, may be necessary for increased surface expression of MaxiK channels in cultured murine astrocytes.

U46619 treatment increases outward macroscopic K^+ currents

To determine whether the increase of astrocytic MaxiKα labeling at the plasma membrane in response to U46619 exposure resulted in an increase in functional MaxiK channels, we recorded macroscopic K^+ currents in 1 μM U46619 stimulated primary astrocytes using the same protocol as in Fig. 1A. K^+ currents elicited by step depolarizations from -70 mV to +160 mV (V_h = 0 mV) were recorded in vehicle treated cells (Fig. 8A, upper trace) or cultured astrocytes treated with 1 μM U46619 for 6 hours prior to current recording (Fig. 8A, lower trace). The average current density-voltage (I-V) plot (Fig. 8B, n = 6 cells per condition) shows a ~3-fold increase in K^+ current amplitude in U46619 treated astrocytes suggesting that the increased MaxiKα labeling observed by immunocytochemistry form functional channels resulting in increased outward K^+ current.

MaxiKα co-localizes with α-tubulin in the cerebral cortex of newborn mice

Previous studies have well-established that microtubule expression is low in mature astrocytes of adult brains (Peters et al., 1991). Our own confocal images of adult mouse cortex double labeled with GFAP (Fig. 9A, red) and α-tubulin (Fig. 9B, green) reiterated this long-held finding. In the overlay image of GFAP and α-tubulin in the cerebral cortex region in the adult mouse brain (Fig. 9C), GFAP labeling is distinct from α-tubulin with minimal fluorophore coincidence indicating that GFAP expressing mature astrocytes exhibit a minimal expression of microtubules. In contrast, newborn mice brain sections (postnatal day 1) simultaneously labeled with both GFAP (Fig. 9D, red) and α-tubulin (Fig. 9E, green) showed robust immunoreactivity of α-tubulin in GFAP expressing astrocytes. In confocal images taken with identical acquisition parameters as in the images of adult brains (Figs. 9A-C), high prevalence of yellow colocalization between the two fluorophores in Fig. 9F indicate that *in-vivo* GFAP-positive astrocytes in newborn mice exhibit a strong expression of microtubules.

To determine if the GFAP-positive *in-vivo* astrocytes in the newborn mouse brain were immunoreactive for MaxiKα, brain slices were simultaneously labeled with both GFAP monoclonal antibodies and MaxiKα polyclonal antibodies. Unlike adult MaxiKα brain distribution exhibiting minimal colocalization between GFAP and MaxiKα save for perivascular astrocytes (Price et al., 2002), cortical regions of newborn mice brain labeled with MaxiKα (Fig. 9G, red) and GFAP (Fig. 9H, green) display a high degree of co-localization in the large majority of GFAP-positive astrocytes (Fig. 9I).

While confocal images in brain tissue may lack the resolution capacity to differentiate individual microtubules as observed in images of primary cultured cells, our confocal data still reveals a substantial co-localization of MaxiKα with the microtubule network in newborn mouse brain slices (Figs. 9J-O). Consistent with our colocalization findings between MaxiKα and α-tubulin in primary cultured cortical astrocytes, cortical tissue sections from P1 newborn mice brains simultaneously immunolabeled for MaxiKα (Figs. 9J, M 6x zoom) and α-tubulin (Figs. 9K, N 6x zoom) exhibited a considerable co-localization between the two fluorophores in the image overlays (Figs. 9L, O 6x zoom).

Discussion

MaxiK channels are involved in a wide assortment of physiological and pathophysiological processes which may in part be regulated by their cellular and subcellular distribution (Gribkoff et al., 2001). Previous studies on MaxiK distribution in the brain have largely spotlighted neuronal form and function (Sailer et al., 2006;Gu N et al., 2007;Misonou et al., 2006) with

sparing emphasis on MaxiK in glia. As there has been a recent emerging research focus on understanding the complexities of integrated brain function and dysfunction via interactions of the neurovascular unit, the multi-cellular interplay between neurons, astrocytes, oligodendrocytes, microglia, and cerebral vasculature (Lok J et al., 2007;Abbott et al., 2006;Park et al., 2003), the knowledge of glial MaxiK subcellular distribution and regulatory traffic mechanisms are important elements in understanding cellular activity in the brain.

The findings presented in this study provide the first direct demonstration that the α-subunit of MaxiK channels in cultured murine cortical astrocytes are distributed intracellularly, associated with the microtubule network. Furthermore, we put forward the novel finding that surface expression of astrocytic MaxiK is regulated by changes in intracellular Ca^{2+} concentration. To reveal MaxiKα channel subcellular distribution in cultured astrocytes, we implemented three independent visualization methodologies: 1) Specific antibodies directed against the α-subunit of MaxiK, 2) AlexaFluor 594 conjugated iberiotoxin, and 3) Expression of EGFP conjugated MaxiKα delivered by adenoviral infection. Quantitative colocalization analysis of all three visualization strategies confirmed the major finding of MaxiKα association to the astrocytic microtubule network. Iberiotoxin binding of intracellular, microtubule associated MaxiK suggest that the channel is in its tetrameric conformation. Additionally, previously published findings of MaxiKα immunolabeling in other brain cells such as neurons in culture and in brain slices indicate a predominant plasma membrane distribution with no reports of cytosolic, microtubule associated MaxiK channels (Sailer et al., 2006;Misonou et al., 2006). Thus, our finding of the intracellular, robust microtubule distribution of MaxiKα in cultured astrocytes may be a unique attribute to this particular cell type.

Further evidence of this association was attained by the significant decrease of colocalization between MaxiKα and astrocytic microtubules upon depolymerization of microtubules with either colchicine or nocodazole. These findings suggest that MaxiKα interaction with microtubules is dependent on the structural integrity of filamentous microtubules rather than disseminated tubulin monomers. Additional biochemical confirmation of MaxiKα interaction with α-tubulin was demonstrated with the C-terminus “tail” of MaxiKα successfully pulling down α-tubulin from brain lysates. This finding suggests that the microtubule association domain of MaxiKα is located within the intracellular C-terminus “tail” domain.

In addition to the *in-vitro* interaction between MaxiKα and α-tubulin, native brain tissue MaxiKα was co-immunoprecipitated with microtubules providing further biochemical confirmation of MaxiKα→α-tubulin association *in-situ*. Whether this interaction is direct or indirect as mediated by a third party protein remains to be seen. Previous studies have reported MaxiK channel interaction with the light chain of microtubule associated protein 1A (MAP1A) in rat brain lysates and in Purkinje cells from adult mouse cerebellum (Park et al., 2004) but our colocalization and biochemical experiments yielded negative results with MAP1A (data not shown) suggesting that a different third party protein may be mediating the binding of MaxiKα to microtubules in cultured murine astrocytes.

Previous studies have demonstrated that MaxiK channel activity is regulated by pools of deep cytosolic Ca^{2+} (Young et al., 2004). In human uterine myocytes, application of caffeine to release the stores of deep cytosolic Ca^{2+} evokes the translocation of a proteolytic fragment of a MaxiKα splice variant likely from the endoplasmic reticulum to the surface membrane (Korovkina et al., 2006). In parallel to the findings in human uterine myocytes, we have found that application of caffeine to cultured murine astrocytes also causes an increase in surface expression of MaxiKα but in this case, from the microtubule network. As application of thapsigargin also evokes a similar ~6-fold increase in number of cells with plasma membrane expression, this would suggest that MaxiKα translocation and surface expression is regulated by increases in cytosolic Ca^{2+} rather than a caffeine-specific activity, i.e. ryanodine receptors.

Interestingly, MaxiKα activity in glioma cells is linked to inositol 1,4,5-triphosphate receptors (Weaver et al., 2007); whether MaxiKα targeting in glioma cells is affected by Ca^{2+} from this or other source remains to be established. Further positive identification of intracellular Ca^{2+} as a mediating factor for MaxiKα surface expression in normal cultured astrocytes was demonstrated by the inhibition of plasma membrane distribution by preincubation with membrane permeable BAPTA-AM to chelate cytosolic Ca^{2+}.

The decrease in intracellular MaxiKα by increased cytosolic Ca^{2+} in the presence of caffeine and thapsigargin (and U46619) coupled with the lack of post-stimulation increase in surface expression under depolymerized microtubule conditions support the idea that MaxiKα associated with the microtubule network serve as a reservoir awaiting an increase in cytosolic Ca^{2+} for initiation of transport to the plasma membrane. Additionally, because MaxiKα associated to the microtubule network is in its tetrameric conformation, a potential functional MaxiKα role in the cytoplasm cannot be discarded at this time.

As efflux of K^+ through MaxiK channels distributed in the endfeet of perivascular astrocytes has been proposed as a potential regulating factor in neurovascular coupling (Filosa et al., 2006), our finding that increases in cytosolic Ca^{2+} increases MaxiKα surface expression may provide clues into recruitment mechanisms which would have an impact on K^+ siphoning especially in the newborn brain. Recent studies (Metea et al., 2007) have demonstrated that K^+ efflux from depolarized retinal glial endfeet have no effect on the diameter of retinal arterioles, thereby suggesting that K^+ siphoning may not contribute to neurovascular coupling in this particular cell system. While untested as of yet in cortical glia, our finding that elevations in deep pools of cytosolic Ca^{2+} regulates surface expression of MaxiKα in cortical astrocytes may provide an additional potential element that can affect glial K^+ influence on vascular tone. One could predict that depending on the numbers of MaxiKα at the plasma membrane that would in turn define the amount of K^+ being released to the perivascular space, the vascular response could be vasodilation (K^+ at levels to open smooth muscle inward rectifier K^+ channels causing hyperpolarization) or vasoconstriction (if K^+ is high enough to cause depolarization) (Straub et al., 2007).

Comparable to the effects of caffeine/thapsigargin stimulation, our studies have shown that application of thromboxane A2 receptor agonist, U46619, causes a similar increase in MaxiKα plasma membrane distribution in cultured murine astrocytes. This finding provides a prospective physiological candidate which may impact neurovascular coupling by recruiting additional MaxiK channels to the surface membrane thereby increasing glial K^+ efflux to affect the adjacent cerebral vasculature. Reciprocally, as the physiological consequences of thromboxane A2 signaling in glial cells currently remains unclear, linking TXA2 to neurovascular coupling may help to shed light on the matter.

This model of Ca^{2+}-regulated, astrocytic MaxiKα surface expression via microtubule transport observed in the primary culture test system may not be a major operative mechanism in normal, mature astrocytes *in-vivo* as mature astrocytes are not known to contain a substantial amount of microtubules under normal physiological conditions (Hajos et al., 1984;Peters et al., 1991) and reiterated in our immunolabeling experiments in adult brain tissue sections. Rather, as astrocytic microtubules are prominently expressed during neonatal brain development (Vaughn et al., 1967) as well as during incidences of brain injury leading to astrocytosis (Vaughn et al., 1970), our proposed model of intracellular Ca^{2+} evoked changes in astrocytic MaxiKα distribution may serve a more appreciable role during the cellular organization of early brain development or during brain injury and disease. As evidenced by our immunohistochemistry results in cortical sections from the newborn mice brain, the substantial co-localization of MaxiKα with the microtubule network would support the position that Ca^{2+}-dependent MaxiK trafficking and surface expression during neonatal/fetal brain

architecture formation may play a part in the developmental role of mature astrocytes in neurovascular coupling.

Although the proliferative nature and general characteristics of the primary cultured immature astrocytes may not be entirely reflective of mature astrocytes *in-vivo*, the cellular behavior of this model system may provide invaluable data and open avenues of study apropos to characterizing physiological and pathophysiological reactive astrocytes. As a variety of pathophysiological cerebral conditions which arouse reactive astrocytes (i.e. intracerebral hemorrhage, stroke and hypertension) also evoke changes in thromboxane activity (van Kooten et al., 1999;Mayhan, 1992;Saloheimo et al., 2005) which can affect interactions in the neurovascular unit, our findings suggesting the relationship between TXA2 and astrocytic MaxiKα may be helpful in future research directed at understanding brain dysfunction and trauma. In an additional, congruent avenue of pathophysiological astrocyte-relevant research, gliomas and astrocytomas, brain tumors derived from unchecked astrocyte proliferation, have been previously shown to express MaxiK channels (Ransom et al., 2001;Weaver et al., 2006). As this category of brain tumors has been previously reported to not only express thromboxane receptors (Honma et al., 2006) but release thromboxane as well (Murphy et al., 1990), our findings of MaxiKα response to U46619 stimulation warrants further study into the relationship between thromboxane and MaxiK channels in astrocyte-derived brain tumors.

Acknowledgments

This work was supported by NIH grants HL54970 (LT), HL088640 (ES), and AHA National Center grant 0435084N (AA).

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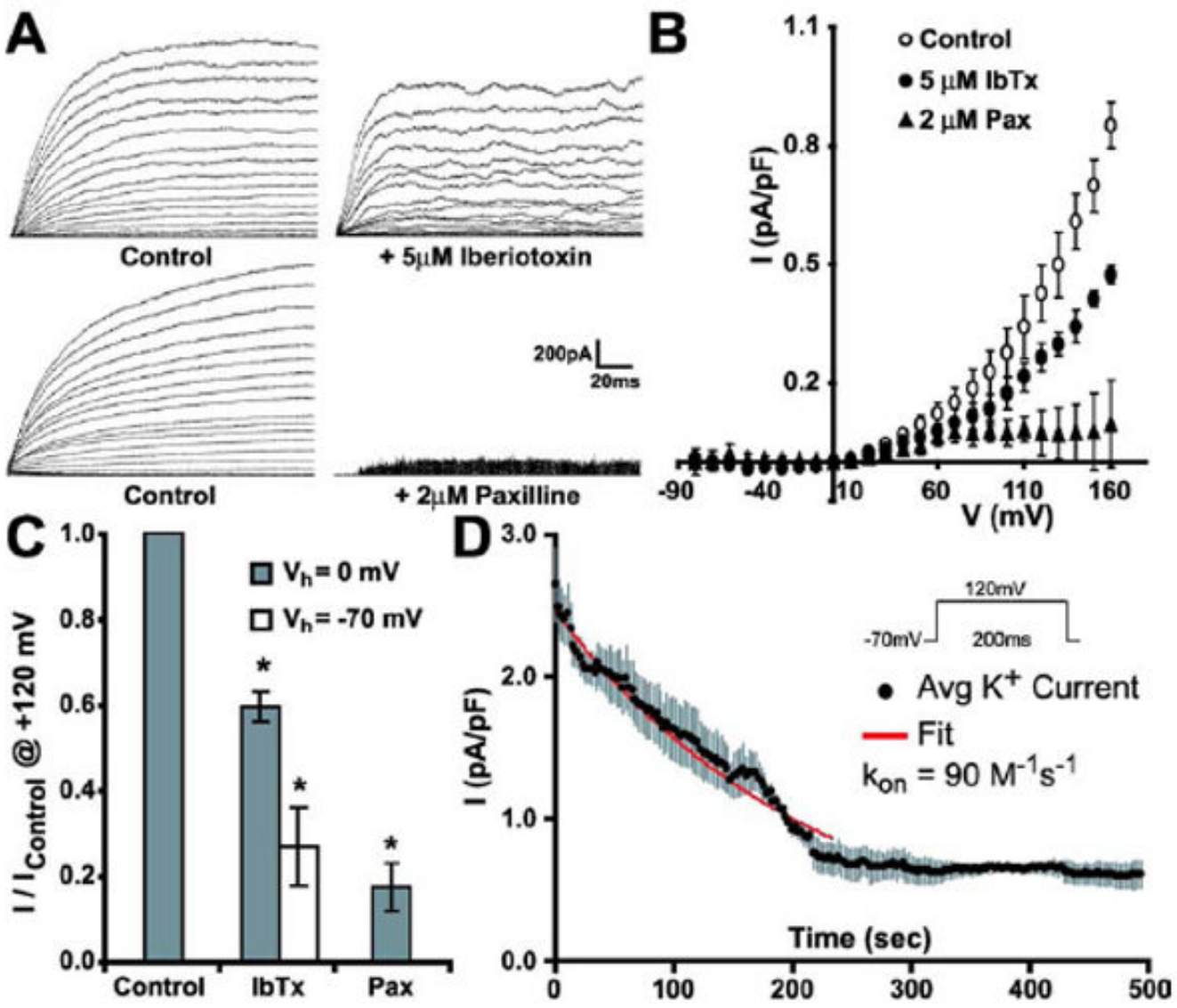

Figure 1.

Pharmacological identification of MaxiK currents in cultured cortical astrocytes. (A, upper and lower left) Control outward K^+ currents elicited by -70 to +160 mV pulses; V_h = 0 mV. K^+ currents after application of MaxiK inhibitors (A, upper right) iberiotoxin (IbTx, 5 μM) or (A, lower right) paxilline (Pax, 2 μM). (B) Average current density-voltage curves before (control) and after application of 5 μM IbTx or 2 μM Pax; V_h = 0 mV (n = 3 for each group). (C) Mean current amplitude reduction in response to IbTx or Pax application. IbTx blockade is shown at V_h of -70 mV and 0 mV. (D) Average time course of 5 μM IbTx-induced MaxiK current blockade (n=3) from V_h = -70 mV. Red line is the fit to a first order bimolecular blocking reaction scheme (Meera et al., 2000).

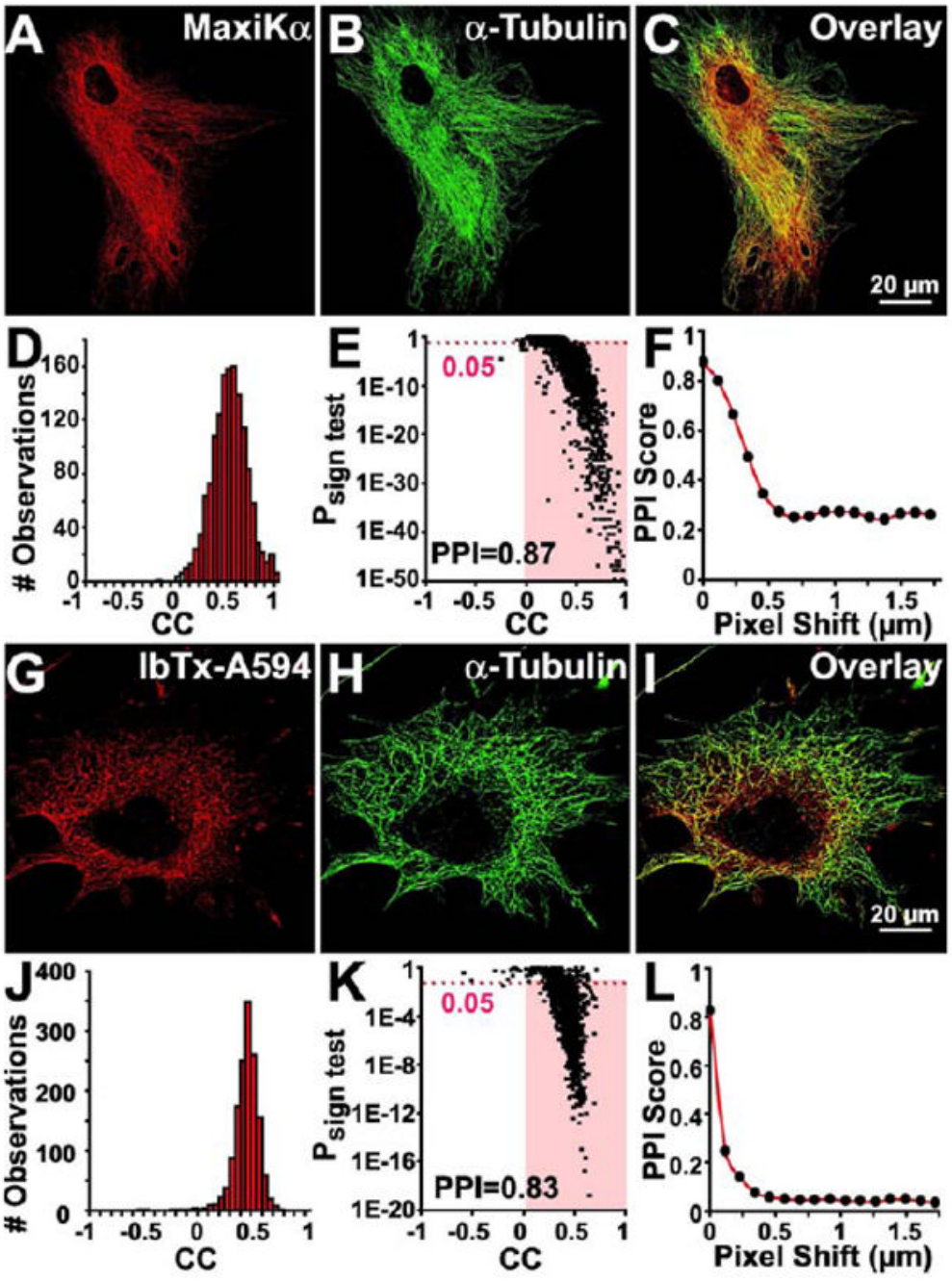

Figure 2.

Quantification of the subcellular localization of MaxiKα to the microtubule network of cultured astrocytes. Double labeled astrocytes with anti-MaxiKα (A, red) and anti-α-tubulin (B, green) or with IbTx-A594 (G, red) and anti-α-tubulin (H, green). Concurrent dual fluorophore labeling (yellow) in overlays (C, I) indicates co-localization of MaxiKα and IbTx-A594 with α-tubulin. Colocalization analysis of MaxiKα with α-tubulin (D-F) and IbTx-A594 with α-tubulin (J-L). Correlation coefficient (CC) frequency distribution histograms for MaxiKα→α-tubulin (D) and IbTx-A594→α-tubulin (J). Columns in red have CC>0. Corresponding $P_{sign\ test}$–CC plots (E, K); highlighted area are squares with CC>0 and a P<0.05. PPI as a function of pixel shift

in the x-axis of one plane for MaxiKα→α-tubulin (F) and Ibtx-A594→α-tubulin (L). In this and following figures, images are single confocal planes.

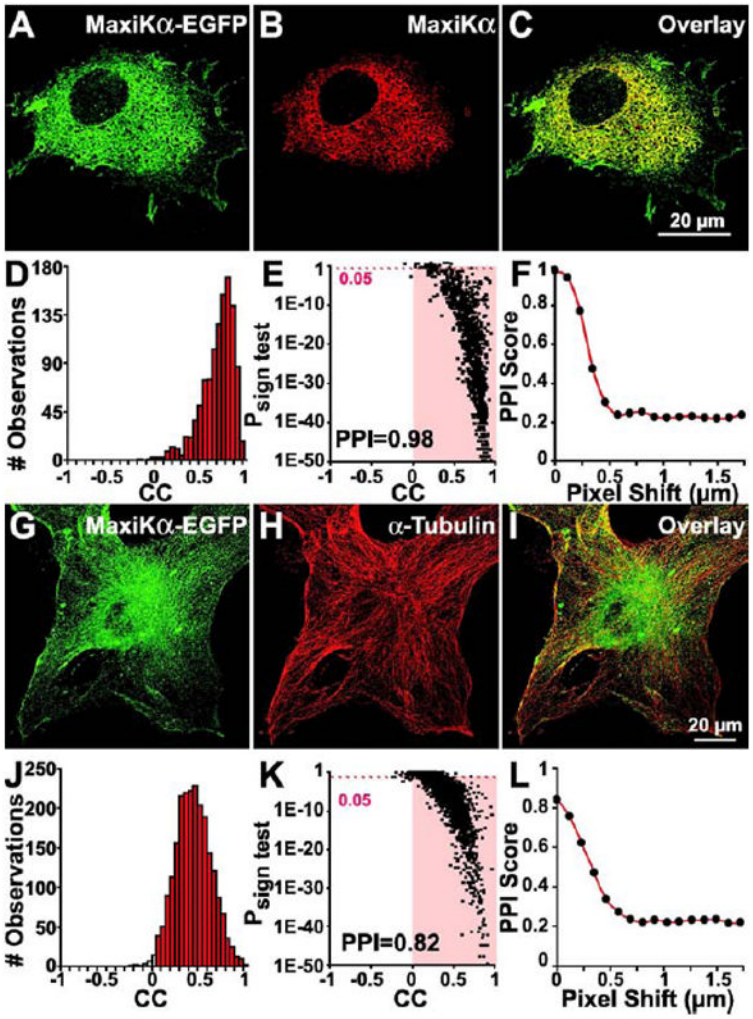

Figure 3.

Expressed MaxiKα-EGFP localizes to the microtubule network in cultured astrocytes. Astrocytes were infected with MaxiKα-EGFP recombinant adenovirus (A, G in green) and co-labeled with anti-MaxiKα (B, red) or anti-α-tubulin (H, red) antibodies. Concurrent dual fluorophore labeling (yellow) in overlays (C, I) indicates colocalization. Colocalization analysis of anti-MaxiKα antibody with MaxiKα-EGFP signals (D-F) and anti-α-tubulin antibody with MaxiKα-EGFP signals (J-L) show a high protein proximity index (PPI). Correlation coefficient (CC) frequency distribution histograms are for anti-MaxiKα→MaxiKα-EGFP (D) and for anti-α-tubulin→MaxiKα-EGFP (J) signals. Columns in red have CC>0. Corresponding $P_{sign\ test}$–CC plots (E, K); highlighted area are squares with CC>0 and a P<0.05. PPI as a function of pixel shift in the x-axis of one plane for anti-MaxiKα→MaxiKα-EGFP (F) and anti-α-tubulin→MaxiKα-EGFP (L).

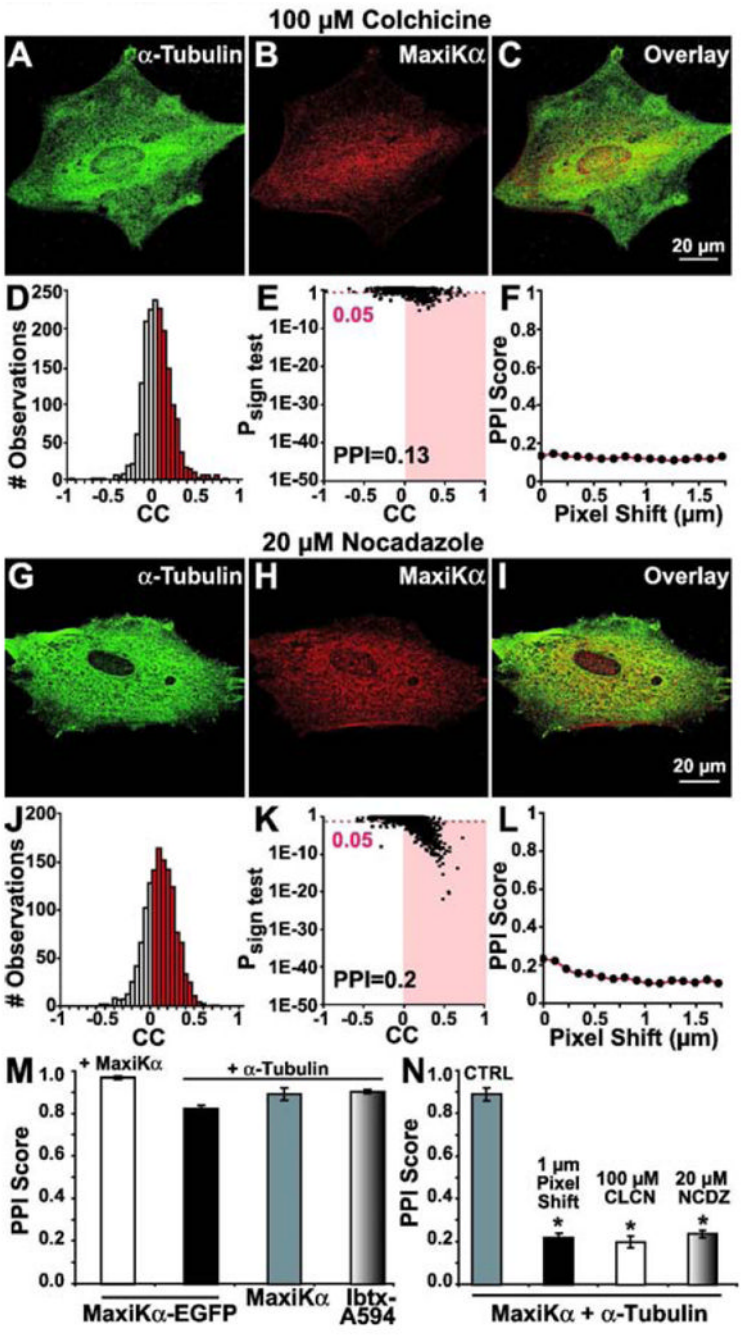

Figure 4.

Microtubule depolymerization disrupts MaxiKα colocalization with α-tubulin. Cultured astrocytes were incubated (3 hours @ 37°C) with 100 µM colchicine (A-C) or 20 µM nocodazole (G-I) and double labeled with anti-α-tubulin (A, G in green) and anti-MaxiKα (B, H in red) antibodies. Reduction of yellow labeling in overlays (C, I) (versus Figs. 2-3) indicates disruption of colocalization. Analysis shows a low PPI of MaxiKα and α-tubulin after colchicine (D-F) and nocodazole (J-L) treatments. (M) Mean PPI scores of independent MaxiKα labeling methods (black bar = MaxiKα-EGFP; gray bar = MaxiKα antibody; shaded bar = IbTx-A594) with α-tubulin and between MaxiKα antibody signals with those of MaxiKα-EGFP (open bar). (N) Mean PPI scores after microtubule depolymerization by

colchicine (CLCN, open bar) and nocodazole (NCDZ, shaded bar) versus untreated astrocytes (CTRL, gray bar) show a significant reduction in PPI similar to that of 1 μm x-axis pixel shift (black bar). n=10 cells per condition.

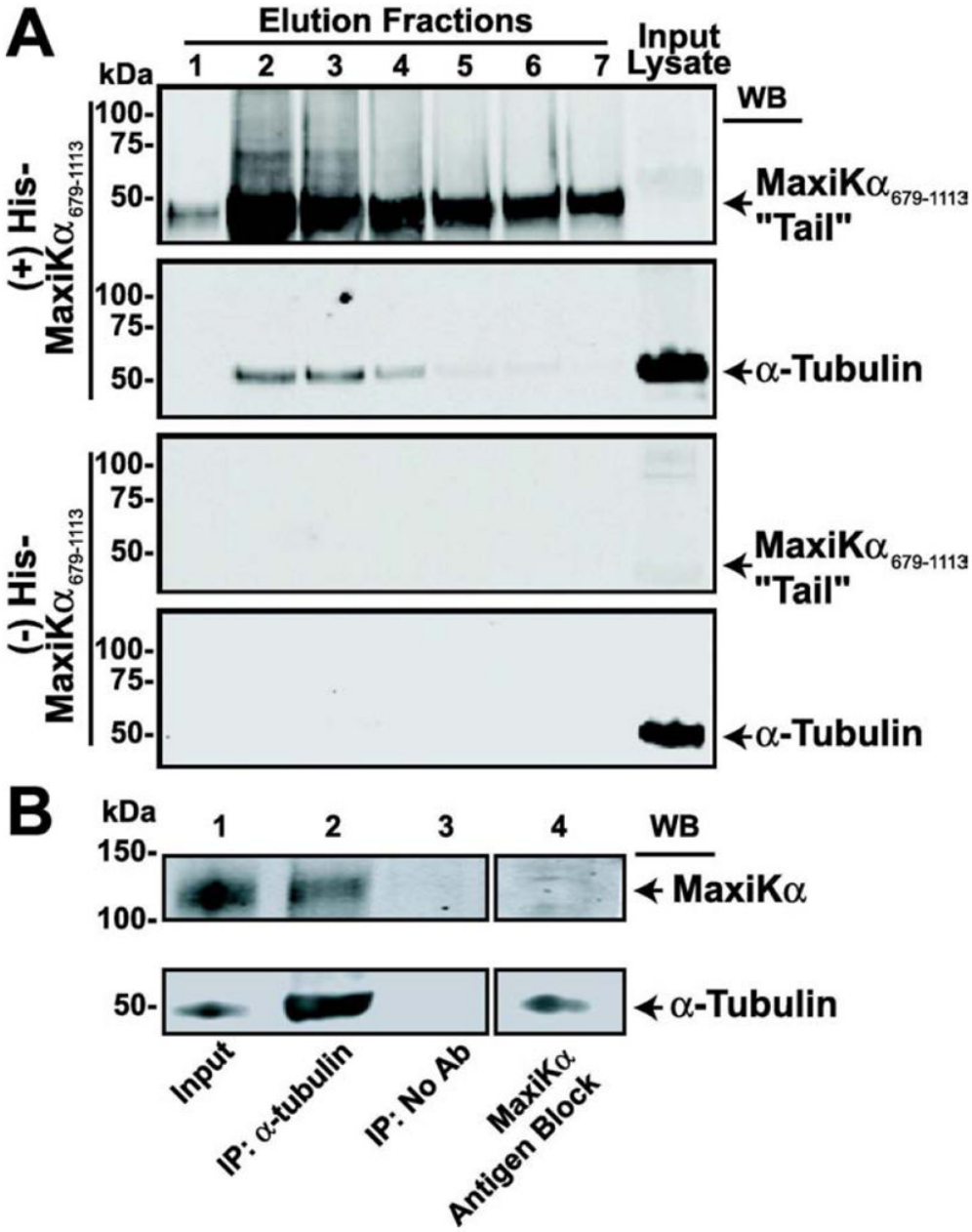

Figure 5.

MaxiKα interacts with α-tubulin. (A) Western blot analysis of pull-down assay. His-MaxiKα$_{679\text{-}1113}$ "tail" in elution fractions 1-7 (A, top row) pulls down α-tubulin (A, 2nd row). Without His-MaxiKα$_{679\text{-}1113}$ "tail" bait protein (A, 3rd row), α-tubulin is not pulled down (A, last row). Proteins in input lysates are also shown. (B) MaxiKα co-IP by α-tubulin in newborn brain lysates. Immunoprecipitated proteins were immunoblotted with polyclonal MaxiKα (B, upper row) and monoclonal α-tubulin antibodies (B, lower row). Lane 1, immunoblots of cell extracts (input) used for IP. Lane 2, immunoprecipitated proteins by α-tubulin antibody probed with anti-MaxiKα and anti-α-tubulin antibodies. Lane 3, control reactions in the absence of anti-α-tubulin antibodies (No Ab). Lane 4, lysate immunoblotted with anti-MaxiKα antibodies preincubated with antigenic peptide (antigen block, control). Experiments were repeated in triplicate yielding similar results.

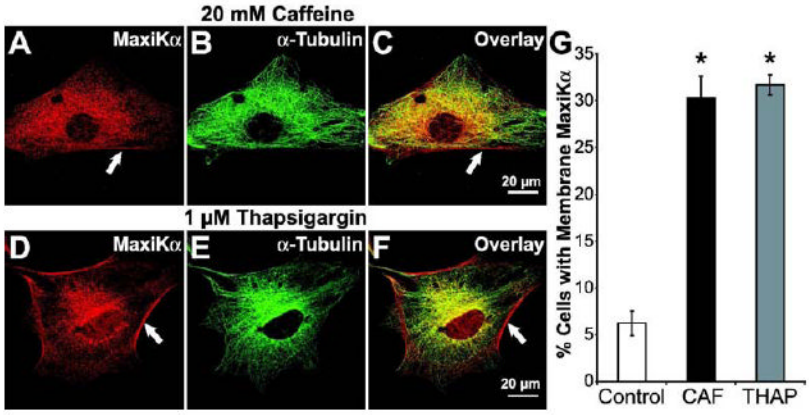

Figure 6.

Increases in intracellular Ca^{2+} increases MaxiKα expression at the plasma membrane of cultured astrocytes. Astrocytes double labeled with anti-MaxiKα (A, D red) and anti-α-tubulin (B, E green); concurrent dual fluorophore labeling (yellow) in overlays (C, F). After 20 mM caffeine (A-C) or 1 μM thapsigargin (D-F) MaxiKα was distinctly observed at the plasma membrane (white arrows). Note in the overlays that MaxiKα signal in the periphery (plasma membrane) did not coincide with the microtubule signals. (G) Mean percentage of cells (~3000 cells/coverslip, manual count, n=3 independent cultures) with MaxiKα expression at the plasma membrane after caffeine (CAF) or thapsigargin (THAP) treatment. n=10 cells/condition.

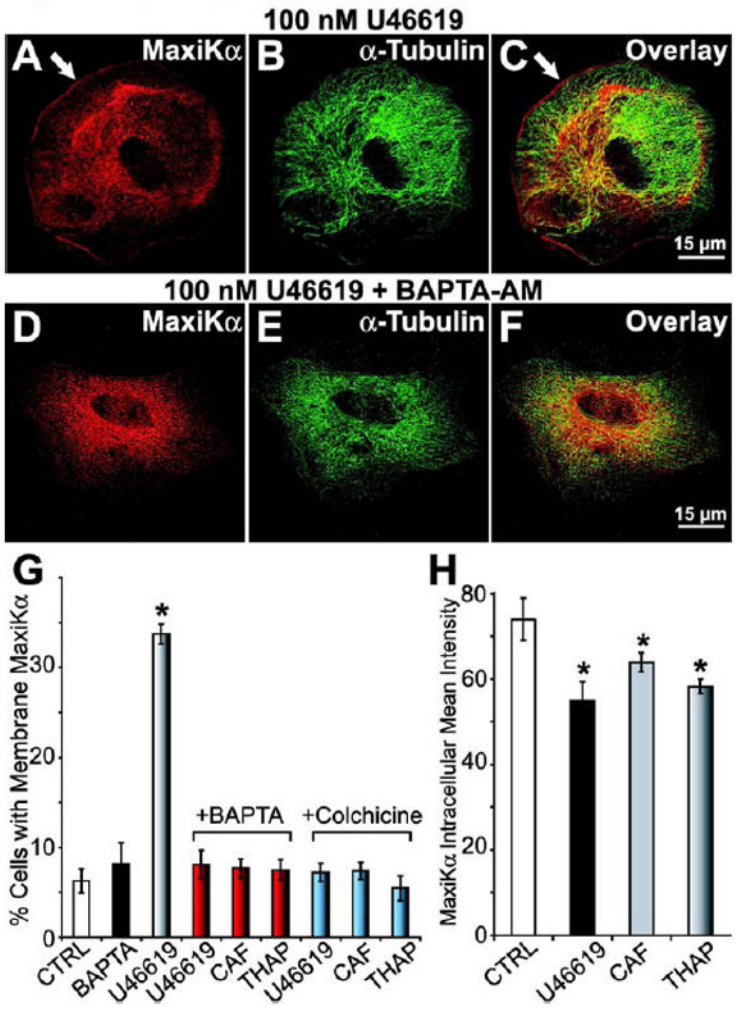

Figure 7.

Increases in intracellular Ca^{2+} caused by Thromboxane A2 agonist U46619 favors MaxiKα distribution at the plasma membrane. Astrocytes double labeled with anti-MaxiKα (A, D red), anti-α-tubulin (B, E green) antibodies, and overlays (C, F). U46619 (100 nM) (A-C) induces MaxiKα expression at the plasma membrane (white arrow). Preincubation with 50 μM BAPTA-AM (D-F) inhibits U46619-induced MaxiKα plasmalemmal targeting. (G) Mean percentage of cultured astrocytes (~3000 cells/coverslip, manual count, n=3 independent cultures) with plasma membrane associated MaxiKα after treatment with vehicle (CTRL, open bar), U46619 (shaded bar) or BAPTA (black bar) alone. Preincubation of BAPTA (red bars) prior to stimulation with U46619, caffeine, or thapsigargin significantly reduced the percentage of membrane distributed MaxiKα to control levels. Preincubation of 100 μM colchicine prior to cell stimulation also prevented the increase in percentage of cells with membrane distributed MaxiKα (blue bars). (H) Mean intensities of intracellular MaxiKα expression after vehicle (CTRL), 100 nM U46619 (black bar), 20 mM caffeine (CAF, gray bar), or 1 μM thapsigargin (THAP, shaded bar) (n = 10 cells/group).

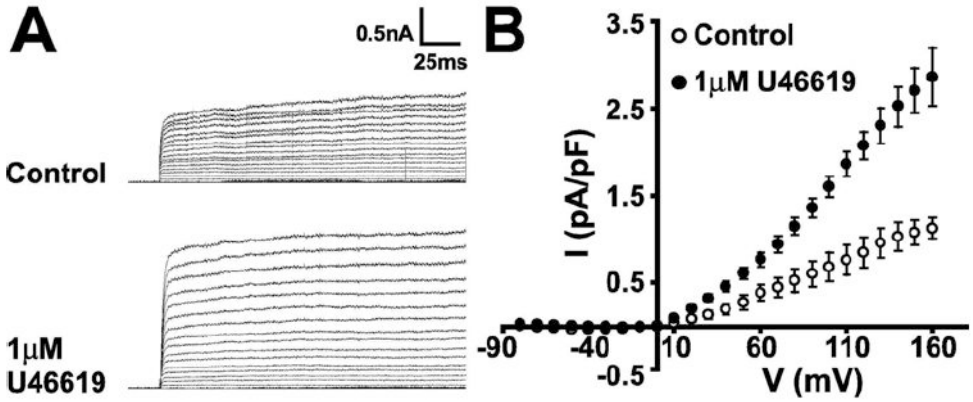

Figure 8.

U46619 stimulation of cultured murine astrocytes results in increased K^+ currents. (A, upper) Control whole-cell K^+ currents recorded during step depolarization from -70 to +160 mV every 10 mV. V_h = 0 mV. (A, lower) K^+ currents were significantly greater in ~ 50% of cultured astrocytes stimulated with 1 μM U46619. (B) Average current density-voltage curves for untreated cultured astrocytes (control) and for astrocytes responding to stimulation with U46619 (n = 6 cells/group).

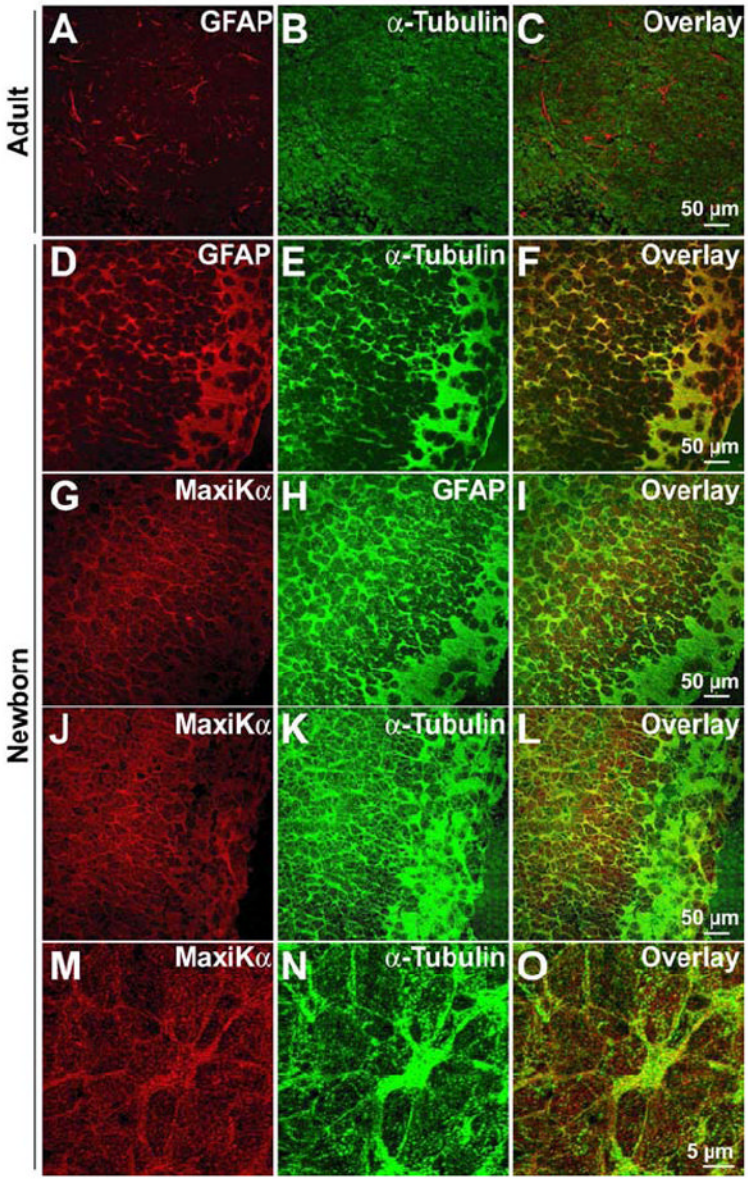

Figure 9.

MaxiKα co-localizes with α-tubulin in the newborn cerebral cortex. Cryosections from adult mouse brains (A-C) and newborn (P1) mouse brains (D-O). Co-immunolabeling of GFAP (A) and α-tubulin (B) in adult cortical sections reveal minimal colocalization (C) indicating the lack of α-tubulin expression in mature astrocytes. In contrast, double immunolabeling of GFAP (D) and α-tubulin (E) in P1 mouse cortices show significant colocalization in the image overlay (F). Newborn brain slices labeled with MaxiKα (G) and GFAP (H) exhibit a high degree of co-localization (I) indicating a substantial expression of astrocytic MaxiKα *in situ*. Comparable to the data found in cultured murine cortical astrocytes, newborn brain cortex sections labeled with MaxiKα (J, 6x zoom M) and α-tubulin (K, 6x zoom N) exhibit significant co-localization (L, 6x zoom O).