Direct interaction between BKCa potassium channel and microtubule-associated protein 1A

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Abstract The BKCa channel, a potassium channel that is allosterically activated by voltage and calcium, is expressed in both excitable and non-excitable cells. The channel plays an important role in regulating membrane excitability. The channel activity can be modulated by post-translational modifications such as phosphorylation. Recently, hippocampal BKCa channels were shown to be directly modulated by assembly/disassembly of the submembranous actin cytoskeleton. Here, we report that the BKCa channel physically interacts with the light chain of microtubule associated protein 1A (MAP1A). The light chain was isolated in a yeast two-hybrid screen of a human brain cDNA library. The specificity of the interaction was demonstrated in biochemical experiments utilizing GST fusion protein pulldown assays and reciprocal co-immunoprecipitations from rat brain. Furthermore, utilizing immunofluorescence, the BKCa channel and MAP1A co-localize in the Purkinje cell layer of the cerebellum. These studies identify a novel interaction between the C-terminal tail of the BKCa channel and the light chain of MAP1A, which enables channel association with and modulation by the cytoskeleton.

Keywords: BKCa; Potassium channel; Cytoskeleton; Microtubule associated protein; Macromolecular complex

1. Introduction

Calcium (Ca^{2+}) activated potassium (K^+) channels are a family of K^+-selective ion channels that respond in an allosteric fashion to membrane voltage and intracellular Ca^{2+} [1–5]. The large conductance voltage gated K^+ channels (BKCa; Maxi K) channels are found in a large variety of excitable and non-excitable cells [3]. In brain, BKCa channels are localized to the cell bodies and nerve terminals [6]. In neurons, the channels are partly responsible for the resetting of the membrane potential after an action potential. In smooth muscle, BKCa channels are responsible for hyperpolarizing the membrane, thereby indirectly reducing contractility [7,8].

BKCa channels encoded by the gene Slo1 [9] are regulated extensively by alternative splicing, phosphorylation and associated proteins, such as β subunits. The α subunit is composed of 7 transmembrane segments, including a S4 voltage sensor and P region to form the selectivity filter along with a long C-terminus, which encompasses nearly 2/3 of the protein [9–12]. The C-terminus contains domains (Ca^{2+} bowl, regulator of conductance of K^+ domain; RCK) [11,13,14] that are responsible for regulation by Ca^{2+} and Mg^{2+}, although a recent report suggested that Ca^{2+} sensitivity can be maintained even after C-terminus truncation [15]. However, C-terminal truncation led to a markedly reduced surface expression of the BKCa channel on the oocyte/HEK cell membrane, suggesting that it might be involved in trafficking to the cell membrane.

Emerging evidence suggests that ion channels can recruit regulatory proteins, which can modulate their function [16,17]. BKCa channels are known to associate with β subunit, potentially through the N-terminus, leading to specific regulation of channel function [18–21]. In Drosophila, utilizing yeast two hybrid screening, the C-terminus of dSlo was shown to be associated with Slo binding protein (Slob) [22], which then recruits 14–3–3 [23,24]. BKCa channels have also been shown to associate with β 2 adrenergic receptor (β 2AR) in brain and smooth muscle [25]. Thus, it is apparent that the BKCa channel can form a macromolecular complex that can regulate its activity. To identify novel BKCa binding partners, we performed a yeast two hybrid screen of a rat brain cDNA library with a bait corresponding to the distal C-terminus of the BKCa channel and isolated the light chain of the microtubule associated protein 1A (MAP1A).

The function, localization and trafficking of many ion channels can be regulated by their interaction with the cytoskeleton [26,27]. BKCa channels can be modulated by dynamic assembly and disassembly of the submembranous actin cytoskeleton [28]. Disruption of actin filaments after brief treatment with cytochalasin D markedly decreases the activity of BKCa channels in brain [28], but increased channel activity in rabbit coronary artery smooth muscle [29]. Microtubules and their associated proteins are important determinants of neuronal and non-neuronal cellular morphology and function [30]. High molecular weight microtubule associated proteins (MAP), MAP1A and MAP2, form thin projections from

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microtubule surfaces and have been implicated in crosslinking microtubules and cytoskeletal components [31]. MAP1B is important for neurite formation in the developing brain [32], whereas MAP1A may be required for neuronal maturation and stabilization. The MAP1A mRNA encodes a heavy chain (HC; 276 kDa) in frame with light chain 2 (LC2; 24 kDa). LC2 is cleaved from the MAP1A polyprotein and assembles non-covalently with the N-terminal regions of the HC. The components of a mature MAP1A consist of MAP1A HC, LC2, LC3 (produced from a distinct single copy gene) and LC1 (27 kDa; cleaved from MAP1B).

We found that LC2 binds to the C-terminal region of BKCa channels in vitro and in vivo, enabling the association of the BKCa channels with MAP1A. Furthermore, we demonstrated the co-localization of the BKCa channel in cerebellar Purkinje cells. Our findings suggest that the BKCa channel–MAP1A association may play an important role in BKCa localization in brain and potentially other cellular systems.

2. Materials and methods

2.1. Y2H assay

Yeast two hybrid cDNA library screening/analysis was performed using the Matchmaker system as per the manufacturer's instructions. Rat BKCa C-terminal segment comprising of amino acid residues 746–1144 (S9–S10 region) was subcloned in frame into DNA-BD/bait vector (pGBKT7; Clontech) and transformed into AH109 strain. Lack of auto-activation was confirmed. The transformed AH109 were mated with the Y187 strain, pre-transformed with AD vectors containing human brain cDNA library inserts (Clontech). The mated strains were screened, using SD/-Ade/-His/-Leu/-Trp in the presence of 3 μM 3-amino-1,2,4-triazole, and positive clones were subsequently plated onto X-α-Gal to detect α-galactosidase. Isolated clones were purified, transformed into DH5α and sequenced.

2.2. GST fusion protein construction

BKCa C-terminus and MAP1A-LC2 cDNA digested from the Clontech MATCHMAKER library AD vector were subcloned into the pGEX-4T1 (Amersham) expression vector. Competent BL21 cells were transformed with the above vectors, and GST-fusion protein expression was induced using IPTG. BL21 were lysed with sonication and fusion proteins were purified with glutathione sepharose beads (Pharmacia Biotech). The isolated bead pellets were washed four times with 1 ml phosphate buffer (PBS).

2.3. GST pulldowns

Rat brain lysates were homogenized in modified RIPA buffer containing 1% Triton X-100 (v/v), 20 mM EDTA, 10 mM EGTA, 10 mM Tris–HCl, pH 7.4 + Complete Mini tablet (Roche), calpain I inhibitor, calpain II inhibitor and PMSF. Insoluble material was removed by centrifugation (20K RCF; 10 min, 2×) and supernatants were collected. Rat brain lysates were pre-cleared with glutathione beads for 20 min at 4 °C. GST pulldowns were performed in 500 μl of immunoprecipitation buffer [Tris–HCl (50 mM), pH 7.4, NaCl (50 mM), Triton X-100 (0.25%), Complete Mini tablet, and PMSF] with pre-cleared rat brain lysate (~1 mg) for 3 h at 4 °C. The beads were washed four times with 1 ml RIPA buffer, heated to 95 °C for 6 min in SDS sample buffer, size-fractionated on SDS–PAGE and transferred to nitrocellulose membrane. Membranes were incubated with BKCa (BD Transduction Laboratories 611248) and MAP1A (Chemicon International) antibodies, followed by detection with ECL (Amersham).

2.4. Immunoprecipitations

Immunoprecipitations were performed in 500 μl of Tris–HCl (50 mM), pH 7.4, NaCl (50 mM), Triton X-100 (0.25%), Complete Mini tablet, and PMSF using 9 μg MAP1A antibody or 2 μg BKCa (Alomone Laboratories APC-021) antibody overnight at 4 °C. Immune complexes were collected using protein A (Amersham) or G sepharose (Sigma) for 1 h, followed by extensive washing with IP buffer (7×). All immunoprecipitations included negative controls (preimmune, antibody alone). Blots were developed with the use of either ECL (Amersham) or Supersignal detection (Pierce). Input represents 5% of immunoprecipitation. In all cases, data shown are representatives of three or more similar experiments.

2.5. Immunocytochemistry

Adult mice (Swiss Webster) were perfused with 4% paraformaldehyde (PFA) in 100 mM PBS, pH 7.4. The brains were postfixed in cold 4% PFA overnight, cryoprotected with 100 mM PBS containing 30% sucrose for 24 h at 4 °C and frozen in OCT (Tissue-Tek). Brain sections (40 μm) were prepared on a cryostat microtome and collected on Superfrost slides (Fisher). Sections were blocked in normal donkey serum (5% PBS-T_x) and then incubated with a cocktail of primary antibodies (anti-BKCa antibody (1:500; Alomone Laboratories; APC-107) and anti-MAP1A antibody (1:1000)) for 48 h at 4 °C. After washing, sections were incubated in a cocktail of donkey anti-mouse IgG conjugated with Alexa 488 (1:500, Molecular Probes), donkey anti-rabbit conjugated with Cy3 (1:500, Jackson ImmunoResearch Laboratories) and Toto 3 (1:1000, Molecular Probes) for 2 h at room temperature. Images were acquired using a Carl–Zeiss confocal microscope. To confirm specific binding of antibodies, the binding was blocked with corresponding excess antigen or absence of primary antibody (data not shown).

3. Results

To elucidate novel BKCa regulatory proteins in mammalian cells, we sought to identify proteins that bound to the C-terminal portion of the rat BKCa channel. Part or all of the C-terminal tail of dSlo has been previously used as baits in yeast two-hybrid screens, which identified two novel proteins that interact with and modulate dSlo [22,33]. The C-terminal tail of the rat BKCa cDNA (amino acid 746- C terminus; Fig. 1A) placed in frame within pGBKT7 (Clontech) was used as the bait to screen a human brain cDNA library. We screened more than 6×10^6 clones and obtained five confirmed positive clones, of which one was the C-terminal region of MAP1A (LC2/MAP1A) (Fig. 1B). The clone, representing the complete LC2 coding region, is highly conserved from an evolutionary perspective. MAP1A/LC2 is translated as a polyprotein precursor that is posttranslationally modified to produce the N-terminal MAP1A and the C-terminal LC2 proteins [30]. The specificity of the yeast interaction was confirmed in yeast with a series of mating experiments utilizing the C-terminal BKCa bait and LC2 prey (Fig. 1C). Taken together, the data suggest that the BKCa channel associates directly with LC2.

To independently confirm the specificity of the BKCa channel–LC2 interaction in mammalian cells, we performed GST pull-down assays. A GST-C terminal BKCa channel fusion protein was produced in BL21 *Escherchia coli* and bound to glutathione–sepharose beads. Lysates from rat brain were applied to the beads and after extensive washing, the proteins eluted from the beads and size fractionated by SDS–PAGE, transferred to nitrocellose and blotted with a specific LC2 antibody [32,34]. GST-C terminal BKCa channel fusion protein specifically interacted with LC2, whereas GST alone failed to interact with LC2 (Fig. 2A). The interaction was also confirmed in reciprocal experiments, in which a GST fusion protein was produced containing the LC2 clone isolated in yeast two-hybrid screening. The GST-LC2 fusion protein bound to glutathione–sepharose beads was incubated with rat brain lysate followed by extensive washing. The eluted proteins were size-fractionated on SDS–PAGE, transferred to nitrocellulose

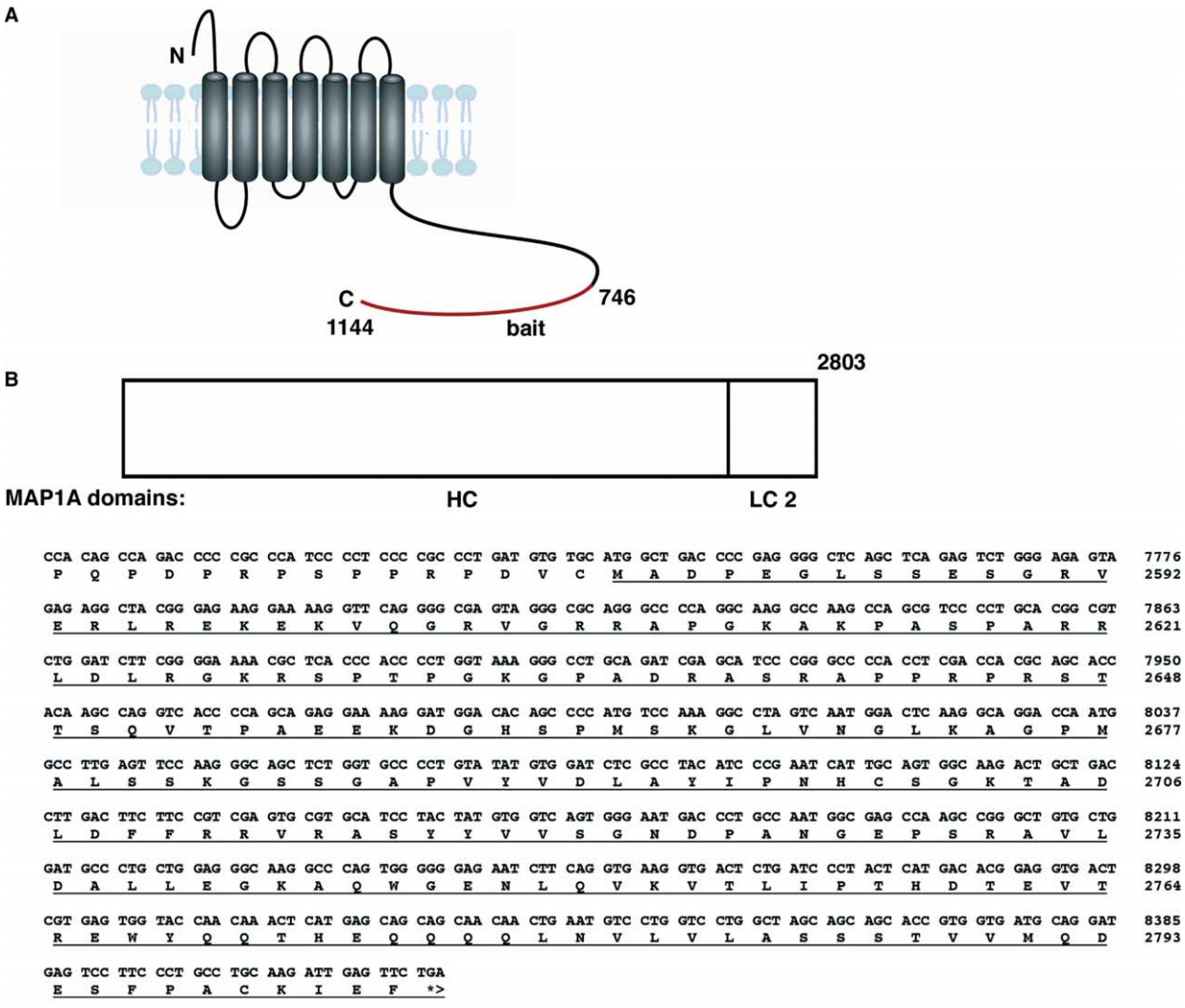

Fig. 1. C-terminus of BKCa channel is associated with LC2 of MAP1A. (A) Schematic representation of BKCa channel demonstrating the region (in red) utilized as bait for yeast two hybrid screening of brain cDNA library. BKCa channel has 7 transmembrane regions, with a large C-terminus. (B) Schematic of MAP1A protein, demonstrating the N-terminal heavy chain (HC) and C-terminal light chain (LC). The DNA sequence and amino acid sequence (human) of prey isolated from yeast two hybrid screen. The underlined region represents the full-length LC2 coding region. (C) Yeast two hybrid analysis of interaction between fusion proteins consisting of DNA binding domain and C-terminus of BKCa channel, Lam, or p53, and DNA activation domain fusion protein and LC2 or T antigen. C-terminal BKCa channel mated with LC2 demonstrated growth on drop-out media and +Gal staining, whereas mating with T antigen failed to elicit growth or Gal staining. LC-2 mating with Lam or p53 also yielded no growth or Gal staining.

and blotted with a specific BKCa antibody. GST-LC2 fusion protein specifically interacted with the BKCa channel, whereas GST alone failed to interact with the BKCa channel (Fig. 2B). Taken together, these data confirm the interaction between LC2 and the BKCa channel, first identified in yeast two-hybrid screening.

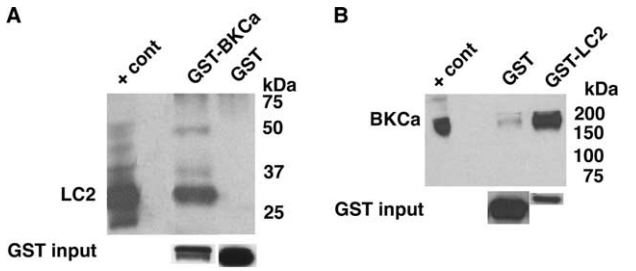

Fig. 2. Specific interaction of C-terminal of BKCa channel and LC2. GST fusion proteins were prepared for the C-terminus of BKCa channel and LC-2 region of MAP1A recovered from yeast two hybrid screen. Rat brain extracts were incubated with GST fusion proteins, followed by extensive washing. (A) Immunoblot using LC2 specific antibody demonstrates that GST-C terminal BKCa fusion protein can specifically interact with LC2, whereas GST alone failed to co-precipitate LC2. Lower panel demonstrates immunoblot using GST antibody, showing the amount of GST fusion protein used in pull-down experiments. (B) Immunoblot (upper) using BKCa antibody demonstrates that GST-LC2 can co-precipitate BKCa channel from rat brain extract, whereas GST alone failed to co-precipitate LC2. Lower panel demonstrates immunoblot using GST antibody, showing the amount of GST fusion protein used in pulldown experiments.

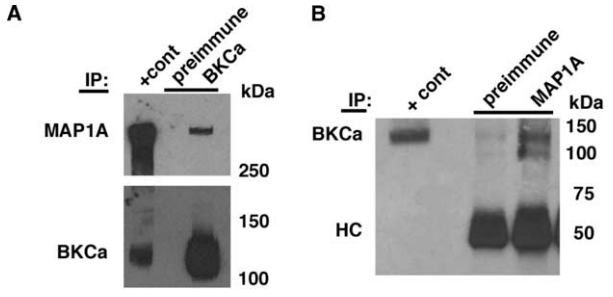

Fig. 3. BKCa channel is associated with MAP1A in rat brain. (A) MAP1A (upper) and BKCa (lower) immunoblot of BKCa and pre-immune immunoprecipitations from rat brain. BKCa specifically associates with MAP1A. (B) BKCa immunoprecipitates of MAP1A and pre-immune immunoprecipitations from rat brain. MAP1A specifically associates with BKCa channel.

We next addressed whether LC2 binding to BKCa channel in native tissue can recruit MAP1A heavy chain into the macromolecular complex. BKCa channel was immunoprecipitated from rat brain lysate using a specific anti-BKCa antibody (Fig. 3A; lower panel), whereas pre-immune serum failed to immunoprecipitate BKCa channel. MAP1A heavy chain was co-immunoprecipitated with BKCa channel (Fig. 3A; upper panel). In reciprocal experiments, MAP1A was immunoprecipitated from rat brain lysate using an anti-MAP1A antibody, and the immunoprecipitate was analyzed for bound BKCa channel. BKCa channel co-immunoprecipitated with MAP1A, whereas BKCa channel was not detected in pre-immune serum precipitates. Taken together, the data confirm the association of the BKCa channel with the MAP1A macromolecular complex in vivo.

Cerebellar Purkinje cells demonstrate high levels of the BKCa channel [6], although the precise functional role of the channels in these neurons has not been clearly identified [35]. However, it has been proposed that BKCa channels regulate the excitability of Purkinje cells by contributing to afterhyperpolarizations and perhaps by shaping individual action potentials [35]. To confirm the interaction of the BKCa channel and MAP1A in vivo, we performed immunohistochemistry in the cerebellum of adult mouse brain with specific anti BKCa channel and MAP1A antibodies. We demonstrated significant co-distribution of MAP1A and BKCa channels in cerebellar Purkinje cells (Fig. 4). Taken together with the yeast two hybrid, GST pull-down, co-immunoprecipitation results, these findings suggest that the BKCa channel and MAP1A/LC2 form a macromolecular complex in vivo. Furthermore, the findings provide a biochemical basis for the recent reports [28] suggesting that the actin cytoskeleton serves to functionally regulate the BKCa channels in brain.

4. Discussion

In the present study, we show that the BKCa channel is part of a macromolecular complex that includes the light chain and heavy chain of MAP1A. We initially identified this novel interaction utilizing yeast two-hybrid screening of a human brain cDNA library. The interaction was confirmed utilizing GST fusion protein co-precipitation assays and in vivo utilizing co-immunoprecipitation and co-localization with immunohistochemistry. This finding is consistent with recent work demonstrating the critical role of ion channels/transporters C-terminus in mediating interactions with proteins that regulate trafficking, and interactions with the cytoskeleton and regulatory proteins.

MAP1A binds to microtubules [36,37] and microfilaments [31,38,39]. In the brain, light chains can be found either associated with or not associated with the heavy chain of MAP1 [40]. MAP1 has been proposed to be involved in regulating the interaction between axonal microtubules and actin filaments, which is believed to be essential for neuronal morphogenesis and function. Association of MAP1B with microtubules is mediated by two unique microtubule binding domains located on the heavy chain [41] and light chain [42,43]. Several regions located in the heavy chain of MAP1A have also been suggested to be responsible for the MAP1A association with microtubules [44,45]. Despite the lack of homology between LC2 and the microtubule binding domain of LC1, the N-terminus of

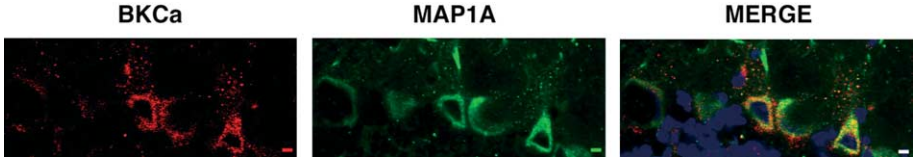

Fig. 4. BKCa channel and MAP1A are co-localized in adult mouse cerebellar Purkinje cells. Representative confocal images of immunostaining of adult mouse cerebellum, for BKCa (red) and MAP1A (green). BKCa and MAP1A are co-localized/distributed (merged image) on the soma of the Purkinje cells. Nuclear staining was Toto3 (blue). Scale bar, 5 μM.

LC2 also bound to microtubules in vivo [40]. LC2 not only binds to cellular microtubules, but also stabilizes them in the presence of colchicine. The LC2 N-terminus was necessary and sufficient for microtubule binding and polymerization. The C-terminus of LC1 can undergo dimerization/oligomerization, which can facilitate microtubule polymerization. The LC2 C-terminus is also capable of binding to microfilaments and actin. The association of MAP with actin may be regulated by post-translational modification, as the binding of MAP1B heavy chain/light chain complex to actin requires prior treatment with alkaline phosphatase [38], whereas phosphorylation of MAP1B enhances binding to microtubules [46]. Similar regulation of MAP1A association with actin and microtubules has not been established, but may be important for the regulation of the BKCa channel complex. The leucine-rich acidic nuclear protein (LANP) interacts with the light chain of MAP1B and modulates its effects on neurite extension [47]. Similarly, caldendrin, a neuronal Ca^{2+}-sensor protein, associates with the microtubule cytoskeleton by binding to LC3 of MAP1A/B [48].

Actin, the main component of the intracellular cytoskeleton, is believed to contribute to neurite growth and synapse formation. Alteration in actin filaments has been shown to regulate ion channel activity, such as the *N*-methyl-D-aspartate channels [26], Na^+ channels [49,50] and K^+ channels [27,51]. Recent reports have also indicated that the actin cytoskeleton can have profound effects on the BKCa channel in hippocampal neurons. For instance, disruption of the cytoskeleton after brief treatment with cytochalasin D markedly decreased BKCa channel activity, characterized by shortened open time and reduced open frequency. These findings suggest that rat hippocampal CA1 pyridamidal neuron BKCa channels can be directly modulated by dynamic assembly/disassembly of submembranous actin cytoskeleton [28]. In contrast, cytochalasin D increased BKCa channel activity in rabbit coronary artery smooth muscle cells [28,29]. Our findings demonstrate that the BKCa channel may associate with the actin cytoskeleton through its interaction with the light chain of MAP1A. This finding has important implications, as the actin cytoskeleton plays a critical role in the regulation of cell shape, motility and participates in the targeting of proteins to various cellular compartments. The differential effects of cytoskeleton modification upon BKCa channel activity in brain and smooth muscle [29] may be due to differences in species, binding partners and/or β subunit expression. Moreover, it has been shown that it plays an important role in postsynaptic anchoring and the maintenance of long-term potentiation. In addition to brain, MAP1A is expressed in muscle and heart, which also express BKCa channels [30]. Therefore, the BKCa-MAP1A association found in brain may also be present in skeletal and cardiac muscle. Whereas both actin filaments and microtubules may mediate inhibition of smooth muscle BKCa channel activity, only actin filaments are involved in the stretch sensitivity of the BKCa channel [29]. Taken together, our findings that the BKCa channel can physically interact with the actin cytoskeleton through direct binding with MAP1A, along with electrophysiologic evidence that disruption of the actin cytoskeleton leads to significant changes in biophysical properties of the channel [28,29] suggests a newly identified intracellular signaling pathway impinging on the BKCa channel.

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