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Cytoplasmic BK$_{Ca}$ channel intron-containing mRNAs contribute to the intrinsic excitability of hippocampal neurons

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**Abstract**
High single-channel conductance $\text{K}^+$ channels, which respond jointly to membrane depolarization and micromolar concentrations of intracellular $\text{Ca}^{2+}$ ions, arise from extensive cell-specific alternative splicing of pore-forming $\alpha$-subunit mRNAs. Here, we report the discovery of an endogenous $\text{BK}_\text{Ca}$ channel $\alpha$-subunit intron-containing mRNA in the cytoplasm of hippocampal neurons. This partially processed mRNA, which comprises $\approx 10\%$ of the total $\text{BK}_\text{Ca}$ channel $\alpha$-subunit mRNAs, is distributed in a gradient throughout the somatodendritic space. We selectively reduced endogenous cytoplasmic levels of this intron-containing transcript by RNA interference without altering levels of the mature splice forms of the $\text{BK}_\text{Ca}$ channel mRNAs. In doing so, we could demonstrate that changes in a unique $\text{BK}_\text{Ca}$ channel $\alpha$-subunit intron-containing splice variant mRNA can greatly impact the distribution of the $\text{BK}_\text{Ca}$ channel protein to dendritic spines and intrinsic firing properties of hippocampal neurons. These data suggest a new regulatory mechanism for modulating the membrane properties and ion channel gradients of hippocampal neurons.

**Keywords**
dendrite, epilepsy, intron-retention, KCNMA1, local splicing

**Comments**

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High single-channel conductance K⁺ channels, which respond jointly to membrane depolarization and micromolar concentrations of intracellular Ca²⁺ ions, arise from extensive cell-specific alternative splicing of pore-forming α-subunit mRNAs. Here, we report the discovery of an endogenous BKCa channel α-subunit intron-containing mRNA in the cytoplasm of hippocampal neurons. This partially processed mRNA, which comprises ~10% of the total BKCa channel α-subunit mRNAs, is distributed in a gradient throughout the somatodendritic space. We selectively reduced endogenous cytoplasmic levels of this intron-containing transcript by RNA interference without altering levels of the mature splice forms of the BKCa channel mRNAs. In doing so, we could demonstrate that changes in a unique BKCa channel α-subunit intron-containing splice variant mRNA can greatly impact the distribution of the BKCa channel protein to dendritic spines and intrinsic firing properties of hippocampal neurons. These data suggest a new regulatory mechanism for modulating the membrane properties and ion channel gradients of hippocampal neurons.

**Results**

**BKCa Channel Intron-Containing mRNAs Are Present in Hippocampal Dendrites.** To determine the repertoire of mRNA splice variants in the postsynaptic compartment, we prepared a cDNA template derived from antisense RNA amplification of polyA mRNA isolated from rat hippocampal dendrites, which were harvested from 14- to 21-day-old cultures *in vitro* (15). Dendrites were carefully harvested individually to ensure that no cell bodies, and thus nuclei, of any neuron or nonneuronal cell were harvested. From this starting material, we detected two separate PCR products using primers specific to intron 16 of KCNMA1 [supporting information (SI) Fig. 5A]. Intron 16 (116 nt long and lies upstream of a previously described “hot spot” for alternative exon usage (16)]. Exons surrounding 116 encode the 3′-end of the first of two highly conserved Ca²⁺-binding RCK (regulators of conductance K⁺ channel) domains and the non-conserved linker region spanning the first and second RCK module (17) (see Fig. 1A). The nucleotide sequence of the PCR products (330 and 390 nt) proved to be identical to the rat genomic BKCa channel genomic DNA sequence deposited in GenBank. We also observed the presence and absence of introns in other mRNAs in amplified polyA mRNA dendrite samples.
containing BKCa channel mRNA (Fig. 1a). The levels of i16-containing transcripts reported here are in agreement with a previous study showing other KCNMA1 splice variants typically represent a small subset (≈10%) of the total KCNMA1 transcript population (19).

We next determined the endogenous KCNMA1 exonic combination between i16 to e18 in both hippocampal tissue and isolated dendrites. This region consists of four downstream exons: two constitutive exons (e17 and e18) and two alternatively spliced exons (e17a and e17b). Here we amplified cDNA with two sets of PCR primers: (i) e16 forward and e18 reverse to capture “all BKCa channel transcripts” or (ii) i16–1 forward and e18 reverse to capture “only i16-containing transcripts” (Fig. 1a). The PCR products were simultaneously assayed with the two different extension primers (XP2 and XP3) to capture all four exonic combinations (Fig. 1a). In the “all BKCa channel transcripts” population, every exonic combination was detected, but two highly abundant variants, e17–e18 and e17–e17a–e18, made up the bulk of the BKCa channel transcript population (Fig. 1a).

The Subcellular Localization of BKCa Channel and the i16-Containing Splice Variant mRNAs in Hippocampal Neurons. In situ hybridization (ISH) is one method for assessing the dendritic localization of mRNA transcripts in neurons, but the methodology as standardly practiced is not particularly robust. For our studies, we developed a novel, highly sensitive Quantum Dot (Qdot)-based ISH protocol with i16-containing BKCa channel mRNA-specific probes to visualize its endogenous subcellular localization throughout the somatodendritic compartment (Fig. 1b and SI Fig. 6). This procedure allows detection of low abundance signals because of a prephotobleaching step eliminates any endogenous background autofluorescence (SI Fig. 6). For the i16-containing BKCa channel mRNA, a series of puncta are detectable in the cell soma that extends into the proximal and distal dendrite. Signal intensity is strongest in the first 50-μm proximal segment of the dendrite and diminishes as a function of distance from the cell soma (Fig. 1b and SI Fig. 7). A comparison with theISH signal of the KCNMA1 e22–e25 exon probe of the mature BKCa channel mRNA shows a similar pattern of distribution (Fig. 1b and SI Fig. 7). This pattern is coincident with where dendritic BKCa channel activity is most predominant in the most proximal portions of the dendrite while decaying with distance from the cell body (12). The importance of these data are twofold. First, they provide independent corroboration of the somatodendritic presence of the i16-containing BKCa channel mRNA. Second, they represent, to our knowledge, the first report of an endogenous intron-containing mRNA that is exported from the nucleus and transported to the somatodendritic cytoplasm. Furthermore, these phenomena are not restricted to cultured hippocampal neurons. Similar levels of i16-containing BKCa channel mRNA were detected by our quantitative PCR approach in fetal and adult brain tissues (data not shown). We followed this observation by using ISH to show the presence of i16-containing BKCa channel mRNA in the somatodendritic products. The i16-containing transcript represented 10.3% ± 2.3% of the total BKCa channel transcript population (Fig. 1a).
Reducing BK<sub>Ca</sub> Channel i16-Containing Splice Variant mRNA Levels Alters the Distribution of BK<sub>Ca</sub> Channel Protein in Dendrites. Given the subcellular distribution and localization of i16-containing BK<sub>Ca</sub> channel mRNA, we next determined whether i16-containing BK<sub>Ca</sub> channel mRNAs contribute to the abundance and localization patterns of BK<sub>Ca</sub> channel protein. Two non-overlapping siRNAs specific for the i16 sequence (si16) were synthesized and transfected into primary hippocampal neurons. The siRNA-treated hippocampal neurons maintained normal cellular morphology (Fig. 2). Using ISH, we next show i16-specific siRNA treatment depleted the pools of i16-containing BK<sub>Ca</sub> channel mRNA in the cytoplasm of hippocampal neurons (Fig. 2a). Control sense i16–1 probes do not show any significant signal in untreated cultures (Fig. 2b). As a control for off-target effects, each of the two nonoverlapping i16–1 siRNAs were transfected individually, yielding the same phenotype (data not shown). The mature BK<sub>Ca</sub> channels α-subunit mRNAs expressing backbone exons (e22–e25) were unchanged in abundance and subcellular distribution in si16-treated neurons (Fig. 2c) as expected because siRNAs modulate their target mRNA effects through cytoplasmic reduction of the target RNA. For these photomicrographs, we chose to use used ISH with alkaline phosphatase and NBT/BCIP (Nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate) detection with differential interference contrast optics to visually highlight the cellular morphology of the siRNA-treated neurons.

BK<sub>Ca</sub> Channel i16-Containing mRNAs Contribute Significantly to the Populations of BK<sub>Ca</sub> Channel in Dendritic Spines. Having established an effective siRNA treatment protocol to selectively reduce i16-containing BK<sub>Ca</sub> channel mRNA levels, we next assessed the subcellular distribution of BK<sub>Ca</sub> channel protein in the dendritic spines of i16-specific siRNA-treated and untreated neurons by using triple label fluorescence. MAP2 (microtubule-associated protein 2) staining was used to identify dendritic processes in hippocampal neurons (Fig. 3a Left). Phallotoxins bind with high affinity to the filamentous actin (F-actin) and are frequently used to identify and quantify the levels of the cytoskeletal protein in dendritic spines. To quantify the distribution of BK<sub>Ca</sub> channels in dendritic spines, we determined how frequently BK<sub>Ca</sub> channel puncta were colocalized with F-actin in spine structures by using volume measurement analysis. We then compared the fluorescence intensity signal observed from BK<sub>Ca</sub> channels and phallolidin in dendritic spines of untreated and si16-treated neurons (Fig. 3). In Fig. 3a, we have taken one plane in a z-stack to show the representative signal obtained with MAP2 and BK<sub>Ca</sub> channel antibodies. Outlined next to the photomicrograph is the boundary of Alexa 488-phalloidin staining within the same optical section (Fig. 3a Center). For the initial analysis, a line scan across dendritic spines (as represented between the two arrows) highlights the presence of BK<sub>Ca</sub> channels and phallolidin in dendritic spines of si16-treated neurons was dramatically reduced compared with control samples: 88.33% (41.67%, si16 treated culture; n = 60; P < 0.001). Data are mean ± SEM.
head. Importantly, there was no difference in the intensity of phalloidin staining between untreated and si16-treated neurons (Fig. 3b; control; 419.09 ± 43.01, n = 60 and si16; 415.20 ± 32.46, n = 60). There was, in contrast, a quantifiable difference in BKCa channel protein distribution (Fig. 3b; control; 274.47 ± 14.97 and si16; 179.44 ± 4.74, n = 60, P < 0.001). To further refine this difference, we transformed the BKCa channel fluorescence intensity differences relative to phalloidin signal in Fig. 3b to reflect a measure of their incidence of colocalization (Fig. 3c). The observed BK channel fluorescence ranged from ~50% to 75% of the overall phalloidin signal in the spine heads of untreated neurons. Therefore, to be more rigorous we selected a lower value (50%) as our minimum parameter to establish the incidence of BKCa channel and phalloidin colocalization. The spine heads showing ~50% BKCa channel fluorescence intensity relative to phalloidin intensity were annotated as normal. By using these parameters of colocalization within a region of interest in a spine head, untreated cells are about two times more likely to have BKCa channel protein in their spine heads above our 50% threshold as compared with si16-treated cells (Fig. 3c).

In untreated neurons, the BKCa channel puncta predominately showed normal colocalization with phalloidin (~85%; Fig. 3c). We see a dramatic change in si16-treated neurons in the colocalization pattern of BKCa channel puncta in dendritic spines. Here, the colocalization incidence was significantly reduced (~42%; Fig. 3c). Collectively, these experiments highlight the local contribution i16-containing mRNAs make to the BKCa channel protein distribution in hippocampal dendritic spines.

In parallel control experiments, we examined the differential distribution of another member of the K+ channel family, Kv2.1. As a prevalent component of the somatodendritic delayed-rectifier potassium currents in mammalian neurons, Kv2.1 plays a prominent role in regulating Ca2+ influx and suppressing neuronal excitability (20). We assessed the colocalization of Kv2.1 channels with the phalloidin signature of individual dendritic spines. In contrast to the changes observed in BKCa channel differential distribution, the localization of Kv2.1 was unaltered between control and si16-treated cultures (n = 15; control, 85.0 ± 7.2; si16, 83.3 ± 8.0; Student’s t test, P = 0.88; image shown in SI Fig. 8). As an additional control, we assessed the spine colocalization of the NR1 subunit of the NMDA receptor (21) with and without i16 siRNA treatment. Again, no discernible difference was found (n = 13; control, 80.8 ± 6.4, si16-treated, 75.0 ± 9.4; Student’s t test, P = 0.66). These controls show that the siRNA-induced difference in BKCa channel spine localization is selective. Given this striking change in the pattern of BKCa channel protein distribution in dendritic spines, we next screened for associated functional consequences.

**BKCa Channel Intron-Containing mRNAs Contribute to the Excitability of Hippocampal Neurons.** Hippocampal cells typically fire a burst of action potentials characterized by spike accommodation in which subsequent action potentials broaden often leading to spike failure (22). In hippocampal neurons, BKCa channels play a role in both action potential repolarization (12) as well as spike broadening during repetitive firing (23). Complex spike bursts of this sort are thought to underlie some adaptive processes during the acquisition of learning and memory (24). Abnormally large BKCa channel currents are the primary cause for changes in the patterns of complex spikes in some forms of epilepsy and dyskinesia (25). Having established an effective siRNA treatment protocol to selectively reduce i16-containing BKCa channel splice variant mRNA and protein levels, we next recorded from hippocampal neurons and analyzed their firing patterns and action potential profiles. Mock-treated, non-KCNMA1 siRNA-treated (Ambion negative controls), or si16-treated neurons were not detectably different from the control sample in either the shape of the evoked actions potentials, action potential generation threshold, resting membrane potential, or input resistance (Fig. 4). However, the maximum number of evoked action potentials was significantly reduced in the si16-treated neurons compared with control, mock-treated, and non-KCNMA1 siRNA-treated neurons (Fig. 4c). These findings show that the i16-containing mRNA is integral to the regulation of functional BKCa channel expression mediated excitability.

**Spike Accommodation in Hippocampal Neurons Requires the Presence of BKCa Channel Intron-Containing mRNAs.** To further characterize the functional significance of the i16-containing BKCa channel transcript, we compared the input–output function of mock-treated and i16-specific siRNA-treated neurons. With small current injections, the control, mock-treated or non-KCNMA1
siRNA-treated neurons showed brisk spiking activity. However, as the size of the current injections increased, spike accommodation became more apparent and reduced the number of spikes fired (Fig. 4f). In contrast, the si16-treated cells exhibited marked spike accommodation even for small current injections, essentially leaving the neurons unable to encode different stimulus levels (Fig. 4f). These results show that a reduction in BK<sub>Ca</sub> channel <i>α</i>-subunit-intron-containing mRNA levels alters membrane excitability of hippocampal neurons. These data are consistent with previous studies demonstrating that changes in BK<sub>Ca</sub> channel activity alter the membrane properties (2).

**SK Channel Inhibition Increases the Excitability of i16-Specific siRNA-Treated Neurons.** Reduction of BK<sub>Ca</sub> channel intron-containing mRNA levels mimics the proposed role for the β4 subunit in the regulation of the <i>K<sub>CNMA1</sub></i> α-subunit, which has been reported to play the functional role of inhibiting BK<sub>Ca</sub> channels from contributing to membrane repolarization (26). BK<sub>Ca</sub> channels are negative feedback regulators of calcium influx in hippocampal and many other neurons; therefore, the down-regulation of BK<sub>Ca</sub> channel activity by si16-treated or β4 subunit regulation should result in sustained calcium influx. This indeed could reduce the firing properties of si16-treated neurons by enhancing the activation of calcium-mediated after-hyperpolarization currents via SK channels. One apamin-sensitive isoform, SK2, is localized throughout the postsynaptic compartment in both the shaft and spines of dendrites (27). Thus, to test the notion that the reduction of i16-containing splice variant levels is reducing functional BK<sub>Ca</sub> channel activity and increasing SK channel activity, we analyzed the firing patterns of si16-treated neurons in the presence of SK channel blocker apamin. Here, we show the maximum number of evoked action potentials is significantly increased in si16-treated neurons in the presence of apamin (Fig. 4c). Collectively, these results are consistent with previous studies, which suggest that BK<sub>Ca</sub> channel activity is an intrinsic determinant of membrane properties. Moreover, they offer the first evidence for a functional role of a cytoplasmically localized, endogenous intron-containing mRNA in altering the membrane excitability of hippocampal neurons.

**Discussion**

Under normal cellular conditions, unspaced or incompletely spliced intron-containing mRNAs are routinely sequestered within the nucleus (28) and when transported to the cytoplasm they are subject to cellular nonsense-mediated degradation (29). Although intron retention is not an entirely unique phenomenon to neurons, having been described for cyclooxygenase 3 (30) and EAAT2 variants (31), these events are rarely observed in higher eukaryotes (32). In fact, database entries annotating intron-containing mRNAs often represent them as artifacts with little likelihood of influencing cellular physiology. Yet, several reports have begun to pinpoint a mechanism for promoting the nuclear export of incompletely spliced intron-containing mRNAs (33, 34).

At least two posttranscriptional regulatory mechanisms are used to generate BK<sub>Ca</sub> channel heterogeneity: (i) extensive alternative splicing of BK<sub>Ca</sub> mRNA and (ii) differential use of <i>α</i>-subunits. We have previously speculated that by coupling the extranuclear splicing capability of the somatodendritic compartment with local protein synthesis (15) or simply locally translating (35, 36) intron-containing mRNAs as readthrough products, there may be another novel layer of posttranscriptional regulation supplementing the functional complexity of individual synapses (15). As recent reports suggest, splicing activity residing outside the nucleus exists as both stimulus-dependent and constitutive mechanisms for regulating gene expression (33, 37). With particular reference to the i16-containing BK<sub>Ca</sub> channel mRNA, the position of the intron is notable. For maximal activation, BK<sub>Ca</sub> channels require a source of intracellular Ca<sup>2+</sup>. Often, these spikes of Ca<sup>2+</sup> are generated by selectively coupling the opening of Cav channels subtypes in close proximity to BK<sub>Ca</sub> channels (38) and perhaps as part of large macromolecular signaling complexes (39). Intron 16 immediately precedes an exon that codes for the nonconserved region linking the Ca<sup>2+</sup>-sensing RCK1 and RCK2 domains. Within the proline-rich sequence encoded in this exon, a noncanonical Src homology domain 3 (SH3) is expressed which allows BK<sub>Ca</sub> channels to coordinate their spatial distribution within the actin cytoskeleton through the actin adaptator protein cortactin (40). In the i16-containing mRNA knockout experiments, we observed a radically different distribution where the BK<sub>Ca</sub> channel protein was far less likely to colocalize with filamentous actin in dendritic spines. Changes in BK<sub>Ca</sub> channels firing properties are synchronized with alterations in actin cytoskeletal dynamics (41) in some forms of stroke and epilepsy (40). One obvious question is what extent neurons use an i16-containing mRNA to generate a functional BK<sub>Ca</sub> channel that can properly coordinate with the actin cytoskeleton. The anti-BK<sub>Ca</sub> channel antibody used here recognizes an epitope encoded within final intracellular C-terminal residues (e.g., exons 24 and 25). In the context of our BK<sub>Ca</sub> channel immunofluorescence data, the most likely scenario is the cytoplasmic intron-retained channel mRNA transcript yielding a protein with C-terminal sequence intact. Such a cytoplasmic pre-mRNA splicing regulatory checkpoint by spliceosome-competent ribonucleoprotein complexes has recently been described in anucleate platelets (33). It is unclear if such a cellular process in the nervous system may create any novel, alternatively spliced mRNAs encoding a protein which generates a gain-of-function characteristic that alters the excitability of hippocampal neurons.

Functionally, most K<sup>+</sup> channels tend to hyperpolarize the cell and moderate the effects of excitatory input (22). In contrast, BK<sub>Ca</sub> channels cause rapid spike repolarization and a post-burst fast after hyperpolarization to facilitate repetitive firing in hippocampal neurons (2). The facilitory effect of BK<sub>Ca</sub> channels is most conspicuous in the form of gain-of-function phenotypes that greatly enhance neuronal excitability to great detriment. A single point mutation in the first RCK domain underlies an enhanced rate of repolarization in the human syndrome of coexistent generalized epilepsy and paroxysmal dyskinesia (25). A similar gain-of-function phenotype that sharpens action potentials, supports higher firing rates with the loss of SK channel recruitment, and shows distinctive temporal lobe seizures was revealed when the auxiliary BK<sub>Ca</sub> channel β4 subunit was deleted in β4-null mice (26). In the absence of BK<sub>Ca</sub> channel activity whether genetically (42) or pharmacologically induced (2), the characteristic bursting properties of neurons are attenuated with increasing current injection as spike accommodation becomes more apparent. BK<sub>Ca</sub> channels are potential drug targets for several clinical disorders such as autism (43) and epilepsy (25). Therefore, the functional significance of cytoplasmic intron-containing BK<sub>Ca</sub> channel mRNAs suggests that intron-retained channel mRNAs and their locally translated proteins may be a novel class of therapeutic targets for disorders linked to this channel.

Although small changes in an individual ion channel conductance or localization can greatly impact both action potential generation and the intrinsic firing properties of a neuron, this role has never been assigned to an endogenous cytoplasmic intron-containing mRNA. Furthermore, these data highlight the influence BK<sub>Ca</sub> channel α-subunit intron-containing mRNAs and mRNA splice variant expression will have on heterogeneity of BK<sub>Ca</sub> currents at the subcellular level in hippocampal neurons. The interrelationship of these distinct subclasses may account, in part, for flexibility in the creation of functional phenotypes for the encoded protein.
Experimental Procedures

Hippocampal Cultures. Primary cultures were plated as previously described (15).

Dendrite RT-PCR Analysis. The pools of dendrites were harvested, subjected to two rounds of antisense RNA amplification procedure as previously described (15), and used as a template for PCR.

MALDI-TOF MS and Quantitative KCNM1 Splice Variant Detection. MALDI-TOF MS analysis of PCR amplicons of dendrite and hippocampus KCNM1 cDNA was performed by using PCR primers selecting for either all transcripts or MS analysis of PCR amplicons of dendrite and hippocampus.

ISH Using Cultured Neurons. Antisense digoxigenin-labeled KCNM1 RNA probes were generated by in vitro transcription. Anti-digoxigenin Fab fragments conjugated to Odot S65 were used for detection (Invitrogen). The samples were subjected to photobleaching, and the Odot signal was detected by using the Olympus Fluoview 1000 confocal scan head attached on inverted microscope. See SI Experimental Procedures.

ISH Using Adult Rat Brain Sections. Fresh frozen adult rat brains were sectioned at 15-μm thickness. Anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Roche) were used for detection with NBT/BCIP (Nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indoly phosphate; Roche) for visualization under brightfield optics.

Immunocytochemistry. Primary neurons were fixed 10–14 days after plating and as described in SI Experimental Procedures. For each cell, five randomly placed line scans were taken from three separate regions of interest for each dendritic segment and analyzed with Metamorph image processing software.

siRNA Treatments. Cultured primary rat hippocampal neurons were transfected in DharmaFect 3 (Dharmacon) 7–9 days after plating (see SI Experimental Procedures). The cultures were maintained at 37°C with 5% CO2 for 72 hr.

Whole-Cell Recordings. Bathing solution consisted of 140 mM NaCl, 3 mM KC1, 1 mM CaCl2, 1 mM MgCl2, and 10 mM Hepes, adjusted to a pH of 7.4 with NaOH. The internal solution consisted of 120 mM potassium gluconate, 20 mM KC1, 10 mM Hepes, 0.1 mM EGTA, 2 mM MgCl2, 2 mM ATP, and 0.25 mM GTP, adjusted to a pH of 7.4 with KOH.

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