Research Paper

A mutually exclusive alternative exon of slo1 codes for a neuronal BK channel with altered function

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Large-conductance Ca2+- and voltage-activated K+ (BK) channels are comprised of four pore-forming α-subunits (Slo1), whose mRNA is alternatively spliced in a cell-specific manner. Here we report the first case of a correctly spliced mutually exclusive exon in a mammalian (human and mouse) BK channel; an exon coding for the region from S6 to the RCK1 domain is exchanged for an alternative exon of the same length. The slo1 transcript with this novel exon is present in native brain tissues and inclusion of the alternative exon profoundly alters the channel’s gating characteristics: faster activation at low Ca2+ concentrations and greater open probability at resting membrane potential at high Ca2+ concentrations. The novel gating features conferred by the alternative exon are dominant over those of the commonly described Slo1 variant when coexpressed. The evolutionarily preserved splicing of the Slo1 S6-RCK1 linker segment possess great potential to fine-tune neuronal excitability.

Introduction

Large-conductance Ca2+- and voltage-activated K+ channels (BK, also termed BKCa, Slo1, MaxiK or KCa1.1) open in response to depolarization or an increase in intracellular Ca2+ concentration ([Ca2+]i) to mediate K+ efflux. Voltage sensing of the channel involves select residues in the transmembrane segments (S0–S6) and the Ca2+ sensors are located in/near the two cytoplasmic domains termed RCK1 and RCK2.1 Both sensors allosterically control the ion conduction gate located in the transmembrane segment S6.2 This mechanism allows BK channels to participate in a broad range of functions: action potential repolarization, neuronal excitability, neurotransmitter release, hormone secretion, tuning of cochlear hair cells, and modulation of smooth-muscle tone.3,4 The remarkable physiological versatility of BK channels results from post-translational modifications, coassembly with other proteins, and splicing of the channel transcript.

Only one human gene (slo1 or KCNMA1) coding for the pore-forming α-subunit of BK channels exists but the transcript is extensively alternatively spliced. To date, at least nine alternative splicing sites and over 20 individual splice variants have been reported for vertebrate slo15–7 (Fig. 1A). While most variants result from insertions of cassette exons,8 some variants represent exon deletions and intron retention.9,10 However, no usage of mutually exclusive alternative exons to produce functional BK channels has been demonstrated for mammalian slo1.11 In Drosophila slo1,12 one of three mutually exclusive alternative exons can be spliced into a region encoding the C-terminal end of S6 and the beginning of the cytoplasmic RCK1 domain. An alternative exon for this S6–RCK1 linker segment may exist in mice as suggested by one EST clone (BY742482); however, inclusion of this exon results in a frame shift and would consequently lead to expression of truncated non-functional channels.

Here we report the existence of a correctly spliced human slo1 transcript utilizing a mutually exclusive alternative exon targeting the S6–RCK1 linker segment and show that alternative splicing of this exon occurs mainly in brain tissues and results in functional channels with strongly altered gating characteristics.

Results and Discussion

Cloning of novel slo1 splice variants from human LNCaP cells. Searching for the molecular correlate of the BKCa channel found in human LNCaP prostate cancer cells that activates appreciably at negative voltages in the absence of Ca2+,13 we isolated a number of cDNA fragments encoding Slo1 from such cells. From 38 cloned PCR fragments covering the region from S3 to RCK1 we found one novel human slo1 splice variant of particular interest generated by exchange of exon 9 (e9, see Methods) with an alternative exon of the same length (e9alt) starting 104 bp downstream of e9 (Fig. 1A). In addition, we identified four further alternative slo1 splice events as summarized in Supplementary Figure 1. All these splice variants, except for e9alt, produce a frame shift and should result in premature termination of Slo1 protein synthesis in the region between S6 and S9 and thus are expected to lack functional expression.9,14 Therefore, we concentrated on the splicing event characterized by e9alt. Exon 9 is 92 bp long and encodes 31 amino acids comprising the end of the S6 domain, the S6–RCK1 linker and the beginning of the RCK1 domain (Fig. 1C and Suppl. Fig. 2). E9 and e9alt are 61% identical.
on the nucleotide level and 58% on the amino-acid level. Analysis of genomic sequences showed that e9, e9alt, and their flanking intronic sequences are highly conserved in vertebrates (Suppl. Fig. 3), suggesting that splicing of the alternative exon 9 is a widespread phenomenon in a variety of vertebrate species.

**Expression of a novel slo1 variant.** Expression analysis by RT-PCR showed the presence of e9alt-containing transcripts in human cell lines LNCaP, T47D (mammary carcinoma), and G290 (primary glioma). The cell-line specific expression of e9alt is suggested by the lack of RT-PCR signal in neuroblastoma and smooth muscle cells (Fig. 2A, part a).

To verify usage of e9alt in native tissues, we performed e9/ e9alt specific RT-PCRs with RNA isolated from various mouse tissues. For both variants, PCR products were of the expected size (Fig. 2A, part b). Products obtained from cerebellum were sequenced and revealed the expected DNA sequence. In 90-days old mice, the signal corresponding to e9alt was obtained exclusively from brain samples. Quantitative real-time PCR showed that the ratio of slo1 e9alt to e9 in LNCaP cells and mouse cerebellum was 1:117 and 1:24, respectively (Fig. 2B). Brain samples of 21-days old mice also showed e9alt signals (Fig. 2A, part c) while expression of e9alt was not detected in day-18 embryonal tissues (not shown), indicating a developmental regulation of e9 splicing.

We verified the brain expression pattern of e9alt transcripts by in situ hybridization analysis. Whereas the corresponding sense probes did not produce any specific labeling above background, hybridization of the e9-specific riboprobe revealed an mRNA distribution pattern with highest transcript levels throughout the cerebral cortex, in pyramidal neurons, and in the olfactory bulb (Fig. 2C), as observed by Sausbier et al. and granule cells of the hippocampus, in cerebellar Purkinje cells, and in the striatum (Fig. 2C, red line). Arrows designate sites of alternative splicing identified in mammalian slo1 mRNAs. Region corresponding to exon 9 is highlighted in red. Lower: clones used to identify transcripts with e9alt (red), e9 (black), and e9alt (blue).

**Regulation of the mutually exclusive e9/e9alt splicing remains to be investigated.** A weaker splicing 5'-acceptor site in e9alt compared to e9, as predicted by NetGene2 (www.cbs.dtu.dk/services/NetGene2), may explain the low abundance of e9alt containing transcripts. However, further experiments are necessary to clarify why e9alt usage is restricted to brain tissue and certain cancer cells.

**Electrophysiological properties of the novel slo1 e9alt variant.** Patch-clamp analysis revealed clear functional differences between the Slo1 e9 and e9alt channels expressed in HEK 293 cells. In the virtual absence of intracellular Ca$^{2+}$, Slo1 e9alt channels (Fig. 3A and B, middle) activated with much smaller depolarization (≥50 mV) than Slo1 e9 channels (≥100 mV) (Fig. 3A and B, left). Voltage and Ca$^{2+}$ dependence of channel activation was assayed by measuring peak tail currents following 10-ms pulses to varying voltages in varying [Ca$^{2+}$]. Such data (Fig. 3B) revealed that Slo1 e9alt channels exhibit a much shallower activation curve and a reduced impact of Ca$^{2+}$ on the half-maximal activation voltage. Moreover, Slo1 e9alt channels were open at resting voltages in high [Ca$^{2+}$] (Fig. 3B, middle; for detailed analysis of the voltage dependence see Suppl. Fig. 5). The greater baseline channel activity of Slo1 e9alt is also manifested in the small but significant inward currents at -150 mV (Fig. 3A, arrows). Under physiological conditions, Slo1 e9alt channels are expected to be more effective in stabilizing cell-resting potentials.

**Activation kinetics of Slo1 e9alt was markedly faster than that of Slo1 e9 in 5 nM Ca$^{2+}$.** Increasing [Ca$^{2+}$], accelerated activation of both variants and at 100 μM, the difference became indistinguishable (Fig. 3C). Marked difference in deactivation was also observed between the two variants but the Ca$^{2+}$ dependence was opposite in that deactivation time courses at -150 mV were indistinguishable in 5 nM Ca$^{2+}$, while in 100 μM Ca$^{2+}$ Slo1 e9alt deactivated about 3-fold faster than Slo1 e9 (Fig. 3D).

**Recordings from cells expressing both variants** (Fig. 3A, B, and D). This resembles the Slo1 e9alt behavior. Voltage dependence of activation was intermediate to results obtained from homomeric channels (Suppl. Fig. 5). Activation kinetics was similar to that of Slo1 e9, whereas...
deactivation was indistinguishable from Slo1 e9alt (Fig. 3C and D). Thus, e9alt alters channel gating and both Slo1 variant subunits are able to form hetero-

cmeric channels.

Assaying the ion selectivity of Slo1 e9alt channels under biionic conditions revealed an about 30-fold higher permeability for K+ over Na+ and Li+ indicating that K+ selectivity is preserved. In addition, both variants were completely blocked by 2 mM extracellular tetraethylammonium (not shown).

Slo1 e9 and e9alt channels were further compared on the single-channel level. All-points histograms for Slo1 e9 channels at +50 mV allowed distinguishing between closed and open-channel current levels (Fig. 4A). By contrast, Slo1 e9alt channel openings were much less stable compromising the detection of discrete current levels (Fig. 4B). At negative voltages, both variants exhibited short openings that were subjected to single-channel dwell-time analysis (Fig. 4C), excluding events shorter than 40 μs. In symmetrical (140 mM) K+, the single-channel conductance of Slo1 e9 channels of 204 ± 5 pS (n = 4) significantly (p = 0.007) exceeded the 135 ± 9 pS (n = 3) of Slo1 e9alt channels. Mean open lifetime, derived from single-exponential fits to lifetime histograms (Fig. 4C), was about twice as long (p = 0.008) for Slo1 e9 compared with Slo1 e9alt channels (e9: 134 ± 15 μs, n = 8; e9alt: 58 ± 9 μs, n = 6). In accordance to macroscopic data, increasing [Ca2+]i strongly enhanced activity of Slo1 e9alt channels (not shown). Open probability of Slo1 e9alt channels at -50 mV (>0.01%, 3 patches) was substantially higher than for Slo1 e9 channels (<0.0015%, 4 patches). Note that data shown in Figure 4A and B are derived from patches containing more Slo1 e9 (~200) than e9alt channels (~10).

Since neuronal BK channels can harbor β4 subunits, which can alter channel function,16 we also coexpressed e9alt with β4 and evaluated the impact of this subunit on the resulting ion currents. β4 slowed down channel activation kinetics and affected the channel’s voltage dependence in a calcium-dependent manner, but the hall-

Figure 2. Expression of slo1 e9 and e9alt splice variants in cell lines and native tissues. (A) Slo1 transcripts containing e9alt are predominantly expressed in native brain tissues. Representative results of RTPCRs performed on mRNA isolated from human cell lines (part a) and tissues from postnatal day 90 (part b) and 21 (part c) mice. T47D—mammary carcinoma cell line; G290—glioma primary cell line; SMC—vascular smooth muscle primary cell line. (B) Quantification of expression of slo1 transcripts containing e9 and e9alt in LNCaP cells and mouse cerebellum. Real-time RTPCR data are normalized to β-actin expression levels and represented as average ± SD of 3 (LNCaP) or 2 (mouse cerebellum) parallel experiments. (C) E9 and e9alt transcripts exhibit similar distribution patterns in mouse brain. In situ hybridization of mouse brain slices from postnatal days 12 (P12, left) and 21 (P21, right) with probes against e9 (upper) and e9alt (lower). Films were exposed for 1 day (e9) or 3 days (e9alt).

Figure 3. Voltage- and Ca2+-dependence of hSlo1 e9 and e9alt channels. (A) Macroscopic inside-out patch recordings from hSlo1 e9 and/or e9alt expressing HEK 293 cells in 5 nM (top) and 100 μM Ca2+ (bottom). Currents were elicited by 10-ms depolarizations to -50, 50, 150 and 250 mV from -150 mV. The dashed lines indicate zero current level. Arrows highlight the steady-state channel activity at -150 mV. (B) Normalized conductance-voltage plots obtained from the analysis of tail currents (as in A) with superimposed Boltzmann fits (see Suppl. Fig. 5). The fit curves of the Slo1 e9 channels are shown as dotted grey lines for reference in the e9alt and the coexpression panels. Time courses of activation at +150 mV and deactivation at -150 mV were fitted with mono-exponential functions (superimposed in A), yielding time constants plotted against [Ca2+], in (C and D), respectively.
mark of the e9alt behavior, channel opening at resting voltages in high [Ca^{2+}], was unaffected by β4 (Suppl. Fig. 6).

In summary we conclude that the sequence of the S6/RCK linker affects BK gating in a qualitatively different manner than the linker length. Whereas the latter produces shifts in the half-maximal activation voltage only, changes observed for Slo1 e9alt additionally concern the steepness of the voltage dependence, the ability of the channel to close at negative voltages, and the single-channel characteristics. These features also exclude this variant to underlie the BK/LNCaP phenotype originally initiating this study.

The reduced single-channel conductance and the flickery gating likely result from the amino-acid changes I323A + E324A in Slo1 e9alt channels. A Drosophila A3 splice variant (corresponding I323 + D324) behaves similarly. Guo et al. proposed that reduced hydrophobicity at position 323, contributing to the S6 gate, promotes flickery gating and sub-conductance states. However, those channels did not activate like the Slo1 e9alt variant at resting voltages. We, thus, assume that flickery gating and lower single-channel conductance of Slo1 e9alt channels result from changes within the S6 gate, whereas the inability to close at low potentials, in particular in high [Ca^{2+}], must result from changes in the linker and/or the start of RCK1. The functional effects of specific amino-acid exchanges suggest that the S6/RCK linker possess defined stable structures. Since in the crystal structure of MthK the corresponding region was disordered, we speculate that the linker segment switches between different conformations during channel gating. A loss of contacts necessary to keep the channel closed at negative potentials may account for the high voltage-independent activity of Slo1 e9alt channels.

Because Slo1 e9 and e9alt proteins can form heteromeric channels, we suggest that a small population of BK channels exhibits enhanced activity at low [Ca^{2+}], and with smaller depolarizations, in fact even at resting voltages. These features could enhance the established role of BK channels in promoting proliferation in various cancer cells. Moreover, the unusual gating properties may enable BK-expressing cells, such as cerebellar or hippocampal neurons, to maintain high-frequency activity. Thus, mutually exclusive Slo1 e9/e9alt splicing may serve as a fine-tuning mechanism of neuronal firing.

**Methods**

**Cell culture and molecular biology.** HEK 293 cells (DSMZ), LNCaP cells (DSMZ), and T74D cells (ATCC) were cultivated as described previously. hKCNMA1 (hSlo1, U11058) was subcloned into pCI-neo (Promega). PCR clones were ligated into pGEM-T vector (Promega). Slo1 e9alt expression clone was obtained by PCR using two clones with overlapping e9alt sequences. Resulting fragment comprising nt 550–1750 of Slo1 was cloned into pCIneo KCNMA1 vector using Padi/Pml restriction sites. All constructs were verified by sequencing. Human and mouse KCNMA1 exon nomenclature was according to: human (Homo sapiens) ENST00000358063; mouse (Mus musculus) ENSBTAT0000017701 (www.ensembl.org).

Human smooth muscle cells were prepared from Vena saphena, kindly provided by Dr. E. Bretschneider, FSU Jena. cDNAs from neuroblastoma cell line SH-SY5Y and human primary glioma cell line G290 were kindly provided by Dr. K. Schönherr and Dr. L. Pusch, FSU Jena. Sv129 mice tissues were kindly provided by C. König, FSU Jena. Total RNA extraction from cultured cells or tissues was performed with Qiagen RNA Easy Kit. First-strand cDNA was synthesized with SuperScript II or III cDNA synthesis Kit (Invitrogen). cDNAs were subjected to PCR analysis with Expand High Fidelity PCR Kit (Roche) (see Suppl. Tables 1 and 2 for primer sequences and pairs here and below). Quantitative RT-PCR was performed on Mastercycler® ep realplex (Eppendorf) using Light Cycler RT-PCR Kit (Roche). Cycler conditions were as follows: 10 min initial denaturation; 36 cycles of 10 s denaturation at 95°C, 10 s annealing at 59°C, 20 s extension at 72°C. Specificity of oligos for detection of e9 and e9alt was validated prior to RT-PCR and quantitative RT-PCR experiments (Suppl. Fig. 4 and Suppl. Table 3).

**In situ hybridization (ISH).** Radiolabeled riboprobes were generated by in vitro transcription using e9- and e9alt-specific cDNA fragments from mouse cerebellum cloned in pGEMT vector as templates. ISH histochemistry was carried out on frozen sagittal
mouse brain sections as previously described (Heuer et al., 2003, Suppl. Methods). After hybridization, the sections were exposed to X-ray film (BioMax MR, Kodak) for one or three days.

**Electrophysiology.** Electrophysiological recordings were performed as described previously. The external solution contained (in mM) 140 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES, pH 7.4. For biionic measurements, NaCl or LiCl replaced KCl. The internal solution contained (in mM) 140 KCl, 10 HEPES, pH 7.4. Free [Ca²⁺] was adjusted by addition of (in mM): 10 EGTA (nominally calcium free; calculated ca. 5 nM); 10 EGTA + 5.9 CaCl₂ (100 nM); 10 HEDTA + 3.3 CaCl₂ (1 μM); 10 HEDTA + 8.3 CaCl₂ (10 μM); 10 HEDTA + 9.9 CaCl₂ (100 μM). For analysis of the voltage dependence of channel activation see Supplementary Figure 5. For single-channel analysis we used the 50% threshold method. The number of channels per patch was estimated based on the open probability as determined from macroscopic data, the single-channel conductance, and the current amplitude at +250 mV. Data are given as mean ± S.E.M. (n), with n = number of independent experiments. Statistical significant differences between groups of data were tested using two-sided Student’s t-test and ANOVA with Bonferroni correction for multiple comparisons.

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**Note**

Supplementary materials can be found at: www.landesbioscience.com/supplement/SoomCHAN2-4-Sup.pdf

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