The pore-forming α-subunits of large conductance calcium- and voltage-activated potassium (BK) channels are encoded by a single gene that undergoes extensive alternative pre-mRNA splicing. However, the extent to which differential exon usage at a single site of splicing may confer functionally distinct properties on BK channels is largely unknown. Here we demonstrated that alternative splicing at site of splicing C2 in the mouse BK channel C terminus generates five distinct splice variants: ZERO, e20, e21(STREX), e22, and a novel variant Δe23. Splice variants display distinct patterns of tissue distribution with e21(STREX) expressed at the highest levels in adult endocrine tissues and e22 at embryonic stages of mouse development. Δe23 is not functionally expressed at the cell surface and acts as a dominant negative of cell surface expression by trapping other BK channel splice variant α-subunits in the endoplasmic reticulum and perinuclear compartments. Splice variants display a range of biophysical properties. e21(STREX) and e22 variants display a significant left shift (>(20 mV at 1 μM [Ca2+]i) in half-maximal voltage of activation compared with ZERO and e20 as well as considerably slower rates of deactivation. Splice variants are differentially sensitive to phosphorylation by endogenous cAMP-dependent protein kinase; ZERO, e20, and e22 variants are all activated, whereas e21(STREX) is the only variant that is inhibited. Thus alternative pre-mRNA splicing from a single site of splicing provides a mechanism to generate a physiologically diverse complement of BK channel α-subunits that differ dramatically in their tissue distribution, trafficking, and regulation.

Large conductance calcium- and voltage-activated potassium (BK) channels are uniquely regulated by changes in both transmembrane voltage as well as intracellular free calcium levels (1). They are widely expressed and thus play an important role in the modulation of cellular excitability in many tissues. Hence, they control diverse physiological processes, including regulation of vascular tone (2–4), micturition (5), neuronal excitability (6, 7), neurotransmitter release (8, 9), endocrine function (10–12), innate immunity (13), and hearing (14, 15).

BK channels in native tissues display a physiologically diverse array of phenotypes. Even neighboring cells (16, 17), or compartments within cells (18, 19), may express BK channels with differences in their functional properties. Furthermore, these properties can be modified temporally, for example, during development (20–22) or following a physiological challenge (23–27).

At least two major post-transcriptional mechanisms are involved in generating such functional diversity as follows: alternative pre-mRNA splicing of BK channel pore-forming α-subunits and assembly of α-subunits with a family of transmembrane modulatory β-subunits. Although α-subunits are encoded by a single gene (1, 28–30) (KCNMA1, also referred to as Slo), β-subunits are encoded by four distinct genes (KCNMBI–IV) (31–34).

Several sites of alternative pre-mRNA splicing within the mammalian BK channel α-subunit have been described, the majority of which are located within the intracellular C-terminal domain of the channel α-subunit (35). Analysis of individual alternatively spliced variants generated at distinct splice sites in different species has revealed that alternative pre-mRNA splicing can dramatically modify the functional properties of the BK channel α-subunit, including changes in calcium and voltage sensitivity (36–42), regulation by protein phosphorylation (22, 43, 44), and other intracellular signaling cascades (45, 46) as well as cell surface expression (47, 48).

Although it is known that multiple alternatively spliced variants may be generated from a single site of splicing, the extent of the phenotypic diversity that can be generated by these splicing decisions is largely unknown. In this study we have performed transcript scanning and functional expression to isolate and analyze the properties of five splice variants generated by alternative pre-mRNA splicing at a single site of splicing, the mammalian site C2. This site of splicing is located in the intracellular C terminus of the α-subunit within the linker region between the two putative regulators of potassium conductance domains (Fig. 1a) (49, 50) and likely represents an important region in the control of BK channel function. We demonstrate that a functionally diverse array of splice variants with a range of calcium and voltage sensitivities, distinct tissue distribution, differential sensitivity to protein phosphorylation, and subcellular localization are generated from a single site of splicing. The control and coordination of these splicing decisions at a single site of splicing provide a powerful mechanism to generate a physiologically diverse complement of BK channel α-subunits.
Experimental Procedures

HEK293 Cell Culture and Transfection

HEK293 cells were subcultured essentially as described (44, 51). Briefly, cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum in a humidified atmosphere of 95% air, 5% CO₂, at 37 °C. Cells were passaged every 3–7 days using 0.25% trypsin in Hank’s buffered salt solution containing 0.1% EDTA. For biochemical studies, cells were grown to 70–80% confluence in 25- or 75-cm² flasks. For electrophysiological or imaging assays, cells were plated on glass coverslips. Twenty-four hours prior to the experiment, cells were washed, and medium was replaced with Dulbecco’s modified Eagle’s medium containing insulin-transferrin-sodium selenite (ITS) serum replacement (Invitrogen). Cells were transiently transfected at 40–60% confluence with the respective cDNA using Lipofectamine 2000 (Invitrogen), essentially as described by the manufacturer. Medium was replaced after 24 h, and assays were performed 48–72 h post-transfection. Stable cell lines were also created by selection and maintenance by using 0.8 mg/ml genetin (Invitrogen).

Transcript Scanning for Site C2 Splice Variants

Transcript scanning was initially performed on total cDNA generated from 19-day-old whole mouse embryo RNA (embryo 19-day cDNA, mouse tissue rapid-scan cDNA pool; OriGene) by PCR amplification between exons 15 and 25 (see Fig. 1b) using forward (fwd), 5’-GTC CTT CCC TAC TGT TTG-3’, and reverse (rev), 5’-GTC TTT GAG CTC ATG ATA GT-3’ primers. PCR amplicons were cloned directly into the pCR2.1 TOPO vector (Invitrogen) according to the manufacturer’s instructions. Clones were characterized by agarose gel electrophoresis and sequenced on both strands by automated sequencing (MWG Biotech, Germany).

BK Channel Splice Variant Expression Constructs

The cloning and subcloning of C-terminal hemagglutinin (-HA) tag constructs of the mouse BK channel splice variants ZERO and e21(STREX) in the mammalian expression vector pcDNA3 (Invitrogen) have been described previously (44, 51). Full-length, -HA epitope-tagged, constructs (in pcDNA3) of the e20, e22, and e23 constructs, used in this study, were generated as described in the supplemental SD1.

qRT-PCR TaqMan™ Analysis

Primers and probes for TaqMan™ quantitative real-time PCR (qRT-PCR) assays, specific for each respective splice variant plasmid DNA.

Total RNA and cDNA Preparation for qRT-PCR TaqMan™ Analysis

Total RNA from selected tissues was prepared using the Qiagen RNeasy mini kit according to the manufacturer’s instructions. RNA was treated with RNase-free DNase, and reverse transcription was performed in 20-μl reactions containing 1× reverse transcriptase buffer (Qiagen), 0.5 mM of each dNTP, 1 μM oligo(dT) primer or random hexamers (Amersham Biosciences), 10 units of RNasin (Promega), 4 units of Omniscript reverse transcriptase (Qiagen), and 2 μg of total RNA. Reactions were incubated for 60 min at 37 °C, and then cDNA products were stored at −20 °C before TaqMan™ analysis. Control reactions were performed in parallel to exclude contamination from genomic DNA by exclusion of reverse transcriptase, or primers, from the reverse transcriptase reaction.

Patch Clamp Electrophysiology

Macropatch current recordings were performed in the inside-out configuration of the patch clamp technique, at room temperature (20–24 °C), using equimolar potassium gradients essentially as described (44, 51). The pipette solution (extracellular) contained the following: 150 KCl, 5 NaCl, 2 MgCl₂, 20 glucose, 10 HEPES, pH 7.4. The bath solution (intracellular) contained: 140 KCl, 5 NaCl, 0.1 CaCl₂, 2 MgCl₂, 20 glucose, 10 HEPES, pH 7.4. The bath solution contained 1 mM BaCl₂ and 1 mM BaCl₂−30 μM ATP, pH 7.3. Intracellular free calcium ([Ca²⁺]) was buffered using 1 mM BAPTA or dibromo-BAPTA as appropriate. Patches were maintained at holding potential of −50 mV and stepped to the voltages indicated in the figure legends for 100 ms. Peak tail current amplitudes were analyzed to determine conductance-voltage curves.

For analysis of BK channel variant regulation by endogenous protein kinase A phosphorylation, all experiments were performed in the inside-out configuration of the patch clamp technique at room temperature (20–24 °C) using physiological potassium gradients (44, 51). The pipette solution (extracellular) contained (in mM) the following: 140 KCl, 5 NaCl, 0.1 CaCl₂, 2 MgCl₂, 20 glucose, 10 HEPES, pH 7.4. The bath solution (intracellular) contained (in mM) the following: 140 KCl, 5 NaCl, 2 MgCl₂, 30 glucose, 10 HEPES, pH 7.3. Intracellular free calcium ([Ca²⁺]) was buffered using 1 mM BAPTA or dibromo-BAPTA as appropriate. Patches were maintained at a holding potential of −50 mV and stepped to the voltages indicated in the figure legends for 100 ms. Peak tail current amplitudes were analyzed to determine conductance-voltage curves.
rate of 1–2 ml/min). For some experiments cAMP was added directly to the bath.

Data acquisition and voltage protocols were controlled by an Axopatch 200 A or B amplifier and pCLAMP6 or pCLAMP9 software (Axon Instruments Inc., Foster City, CA). All recordings were sampled at 10 kHz and filtered at 2 kHz. Channel activity was allowed to stabilize for at least 10 min after patch excision. BK channel activity was stable for >1 h under the recording conditions used (data not shown), in the absence of channel modulators.

Single-channel open probability ($P_o$) was derived from single-channel analysis using pSTAT (Axon Instruments) for patches with <4 channels. In the case of patches with >4 channels, an integration-over-base-line algorithm was used, using Igor Pro 4.1 (WaveMetrics, Lake Oswego, OR). In the latter case, $N \times P_o$ (number of functional channels × open probability of channel) values were determined as follows. All-point histograms were plotted to obtain the “offset,” i.e. leak current, as well as the single-channel current amplitude from the peak intervals. After subtraction of the offset from the traces, these were integrated over 0.5–60-s segments. The integral divided by integration time and single-channel current amplitude gives $N \times P_o$.

To determine the mean percent (%) change in channel activity after a treatment, in patches with low to moderate levels of channel expression, mean $P_o$, or $N \times P_o$, was averaged from 3–5 min of recording at +40 mV immediately before and 10 min after the respective drug treatment. Mean change in activity was expressed as a percentage (%) of the pretreatment control ± S.E. In the respective figure legends and text a positive percentage (%) change in activity reflects activation, whereas a negative percentage (%) change reflects channel inhibition.

**Immunohistochemistry and Imaging**

**Cell Permeabilization and Fixation**—Transfected HEK293 cells were washed briefly in phosphate-buffered saline (PBS). Cells were fixed and permeabilized with 2% paraformaldehyde plus 0.3% Triton X-100 overnight at 4 °C. Cells were fixed for a further 10 min at room temperature in 4% paraformaldehyde. Cells were then blocked (3% bovine serum albumin in PBS plus 0.05% Tween 20) for 1 h at room temperature. The HA epitope tag was probed using a rabbit polyclonal anti-HA antibody (0.5 μg/ml, Zymed Laboratories Inc.). After washing three times with PBS, at room temperature, cells were incubated with Alexa-488-conjugated secondary anti-rabbit antibody (1:1000, Molecular Probes). Following three washes in PBS, coverslips were mounted on microscope slides using Mowiol.

**Nonpermeabilized Fluorescent Labeling**—For cell surface (N-terminal FLAG epitope) labeling prior to fixation or permeabilization, a 1:100 (20 μg/ml) dilution of mouse monoclonal anti-FLAG antibody (M2, Sigma) was applied to cells for 1–2 h on ice. Alexa-594-conjugated anti-mouse IgG (1:1000, Molecular Probes) was used as the secondary antibody.

Confocal images were acquired on a Zeiss LSM510 laser scanning microscope, using a 63x oil Plan Apochromat (NA = 1.4) objective lens, in multi-tracking mode to minimize channel cross-talk. All figure images are unprocessed single sections taken through the middle of the cell.

**Immunoprecipitation and Western Blotting**

Cells were lysed at 4 °C in buffer containing 150 mM NaCl; 50 mM HEPES, pH 7.5; 1.5 mM MgCl₂; 10 mM sodium pyrophosphate; 20 mM NaF; 1 mM EDTA; 5 mM EGTA; 10% v/v Glycerol; 1% Triton-X-100, and protease inhibitor mixture (Roche Applied Science). Cell lysates were spun and supernatants pre-cleared for 30 min with protein-G beads (Sigma). Lysates were then incubated overnight at 4 °C with 10 μl (4.6 μg/μl) of anti-FLAG M2 antibody (Sigma) and 40 μl of protein-G beads (1:1 in lysis buffer). Beads were washed a minimum of five times with lysis buffer. Bound complexes were eluted with SDS sample buffer, separated through a 10% SDS-polyacrylamide gel, and electroblotted to polyvinylidene difluoride membrane. Blots were probed overnight with 0.25 μg/ml rabbit anti-HA polyclonal antibody (Zymed Laboratories Inc.) at 4 °C before incubation with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:5000, Diagnostics Scotland, UK) for 1 h at room temperature. Signals were detected using enhanced chemiluminescence, and images were captured on a Syngene Genegnome camera system using Genesnap software (Syngene).

**RESULTS**

**Multiple Splice Variants Expressed at Site of Splicing C2**—Transcript scanning of cDNA generated from 19-day-old whole mouse embryonic RNA, using primers designed to amplify across alternative site of splicing C1 and C2 (Fig. 1, a and b), resulted in a family of alternatively spliced transcripts with distinct inserts at site of alternative splicing C1 and C2 (Fig. 1c). Size and sequence analysis of >400 transcripts revealed expression of two distinct variants at site of splicing C1 (Fig. 1, b and c). The insertless “null” variant and the four amino acid insert resulting from inclusion of exon 17 has been reported previously. At site C2, five distinct variants were isolated (Fig. 1, b and c), of which four have been reported previously in the mouse as follows: the “ZERO” variant resulting from splicing of exon 19 to exon 23; the ε20 variant resulting in a three-amino acid (IYF) insert from inclusion of exon 20 between exon 19 and exon 23; the ε21 variant resulting in the 59-amino acid, cysteine-rich, stress-regulated exon (STREX) insert resulting from inclusion of exon 21 between exon 19 and exon 23; and the ε22 variant resulting in a 29-amino acid insert resulting from inclusion of exon 22 between exon 19 and exon 23. We also isolated a novel variant (Δε23) resulting from skipping of exon 23. This results in splicing of exon 19 to exon 24 leading to a frameshift that introduces a premature stop codon within exon 24. This generates a truncated channel terminating in the amino acids . . . RRRMTPC*. Analysis of >400 transcripts revealed that either of the null or ε17 site C1 splice variants may be expressed in the same transcript with any of the site C2 splice variants (Fig. 1d).

The ε21(STREX) splice variant is highly conserved, at the amino acid level, from fish to man. Nine of 59 amino acid substitutions exist between the murine and Zebrafish sequences suggestive of a common function across vertebrate evolution (Fig. 2). In contrast, the ε22 splice variant is poorly conserved, with 11 of 29 amino acid substitutions between man and mouse. Thus, it is likely that distinct selection pressure and function of this variant is manifest across species.

**Tissue Distribution of Splice Variant mRNA**—To address the tissue distribution of each site C2 splice variant mRNA, across different mouse tissues, we developed splice variant-specific quantitative real time PCR TaqMan™ assays (see supplemental SD2). Total BK channel mRNA expression between different tissues (Fig. 3) varied over 3 orders of magnitude. Total BK channel mRNA levels were highest in adult brain, prostate, and 19-day-old whole mouse embryo. The lowest levels were observed in heart and liver. Expression of total BK channel mRNA in these tissues was less than 3% of that observed in 19-day-old whole mouse embryo. Comparison of splice variant mRNA expression across tissues, normalized to expression in 19-day-old whole mouse embryo, demonstrated tissue heterogeneity in the relative levels of each variant. Adult brain, adrenal gland, and prostate had highest levels of the ZERO variant mRNA. The ε21(STREX) variant mRNA was predominantly expressed in endocrine tissues such as the adrenal gland, pituitary, and prostate. Splice variant ε22 mRNA was predominantly expressed in embryonic tissue, whereas Δε23 mRNA levels varied by 2-fold across...
different tissues. In our assays, splice variant e20 was consistently below the level of detection in all tissues suggesting that this variant is expressed at less than 1% of total BK mRNA levels.

Comparison of the mRNA expression of each variant, as a proportion of total BK channel mRNA levels in individual tissues, further demonstrated that different tissues express a distinct complement of site C2 splice variants (Fig. 4). For example, in adult brain, which has one of the highest levels of total BK channel mRNA, the ZERO variant constitutes 90% of all variant mRNAs expressed. In contrast, in tissues with very low total BK mRNA levels, such as liver, the predominant splice variant mRNA is Δe23.

Distinct Biophysical Properties of Site C2 Splice Variants—Previous functional studies of the e21(STREX) splice variant has demonstrated a dramatic shift in the apparent calcium sensitivity of the channel compared with the ZERO variant (40, 41, 45). To address whether phenotypic variation in intrinsic biophysical properties is also introduced by other site C2 splice variants, we analyzed the calcium and voltage sensitivity of BK channels in macropatches from HEK293 cells expressing
the respective site C2 splice variant (Fig. 5). Each site C2 splice variant was expressed as a C-terminal -HA-tagged channel (Fig. 5). As reported previously, the -HA tag does not affect properties of ZERO or e21(STREX) channels, and no significant differences in channel properties compared with the un-tagged variants were observed in these studies (not shown).

Expression of ZERO, e20, e21(STREX), and e22 variants in HEK293 cells resulted in robust voltage- and calcium-sensitive outward macropatch currents in HEK293 cells (Fig. 5a). In contrast, no detectable macropatch currents were observed in cells transfected with the Δe23 splice variant, even at voltages greater than +100 mV in the presence of 100 μM [Ca\(^{2+}\)]. This suggests that channels formed by Δe23 are either nonfunctional or not expressed at the plasma membrane.

The e20 splice variant displayed a calcium and voltage sensitivity similar to that previously reported for the murine ZERO variant. The
was 71.2 \pm 6.8 \text{ and } 68.3 \pm 5.6 \text{ mV for the e20 and ZERO variants, respectively (Fig. 5, b and c). As shown previously, inclusion of e21(STREX) results in a large left shift in the conductance-voltage relationship, compared with ZERO, with a $V_{0.5(max)}$ at 1 \mu M [Ca$^{2+}$] of 34.3 \pm 8.4 \text{ mV (Fig. 5, b and c). This value is intermediate to the $V_{0.5(max)}$ for ZERO and e21(STREX) under these recording conditions. Furthermore, both e21(STREX) and e22 variants had considerably slower deactivation kinetics compared with ZERO or e20 (Fig. 5a).

**Δe23 Splice Variant Is a Dominant Negative of Plasma Membrane Expression**—The lack of functional expression of the novel Δe23 splice variant suggested that the channel is nonfunctional or is not expressed
at the plasma membrane. A conserved motif within the intracellular C terminus of mammalian BK channels is required for efficient export of channels from the endoplasmic reticulum (47), although the precise role of the intracellular C terminus in plasma membrane targeting is controversial (52). The C-terminal truncation of the Δe23 splice variant would result in loss of this motif. Thus, Δe23 channels would be predicted to reside largely in the endoplasmic reticulum. In support of this, confocal imaging of HEK293 cells, transiently or stably expressing the FLAG- and/or -HA-tagged Δe23 variant, revealed a predominant intracellular localization of the channel (Fig. 6a). This is in contrast to the robust plasma membrane targeting of either the FLAG- or HA-tagged ZERO, e21(STREX), or e22 variants (Figs. 6a and 7a). The Δe23 splice variant was largely expressed in a diffuse perinuclear distribution and co-localized with an endoplasmic reticulum marker (not shown). The lack of membrane expression of the Δe23 splice variant was not a result of reduced protein expression. Indeed, both single cell image analysis (Fig. 6a) and Western blotting of cell lysates (Fig. 6b) revealed robust expression of this C-terminally truncated protein. Furthermore, inefficient membrane targeting was unlikely to be due to incorrect assembly of homomeric Δe23 channels as Δe23 splice variant α-subunits were able to homotetramerize (Fig. 6c). This would be in accordance with the truncation in this variant occurring after the first regulator of potassium conductance domain, which is required for α-subunit tetramerization (49, 50).

The Δe23 splice variant may act as a dominant negative of cell surface expression. Conversely, Δe23 trafficking to the plasma membrane may be rescued upon co-assembly with a trafficking “normal” variant. To address these issues, we co-expressed the C-terminal -HA epitope-tagged Δe23 variant (Δe23-HA) with a C-terminal -GFP-tagged e21(STREX) variant, e21(STREX)-GFP, and we analyzed plasma membrane and perinuclear localization in HEK293 cells. Expression of Δe23-HA alone resulted in less than 4% of transfected cells with any visible staining at the cell periphery (Fig. 7, a and d). This is in agreement with the lack of cell surface staining observed for N-terminal FLAG-tagged constructs (FLAG-Δe23 (not shown) or FLAG-Δe23-HA (Fig. 6a)) and the lack of functional expression of outward potassium currents in the electrophysiological assays (Fig. 5). The very low level of plasma membrane localization of the Δe23-HA splice variant may reflect saturation of the endoplasmic reticulum export control mechanisms. Alternatively, it may reflect an intrinsically low probability of membrane trafficking for this C-terminally truncated variant. In contrast, greater than 70% of transfected cells showed robust e21(STREX)-GFP expression at the cell periphery (Fig. 7, a and d) as for FLAG- or -HA epitope-tagged ZERO (Figs. 6a and 7d), e21 (not shown), or e22 splice variants. More importantly, co-expression of the Δe23-HA variant with the e21(STREX)-GFP variant completely blocked plasma membrane expression of e21(STREX)-GFP in HEK293 cells. Co-expression resulted in co-localization of e21(STREX)-GFP with Δe23-HA in a perinuclear-endoplasmic reticulum localization identical to that of Δe23-HA alone (Fig. 7, a and d). Identical intracellular trapping was also observed on co-expression of the Δe23 variant with ZERO or e22 splice variants (not shown). This strongly suggests that the Δe23 splice variant is a dominant negative of cell surface expression.

The enhanced intracellular trapping of e21(STREX), in the presence of Δe23, is most likely because of co-assembly of the Δe23 and e21(STREX) splice variant α-subunits as heteromultimers in the endoplasmic reticulum. In support of this conclusion, epitope-tagged Δe23 and e21(STREX) variants could be reciprocally co-immunoprecipitated from HEK293 cells (Fig. 7, b and c). Trafficking of the e21(STREX)-GFP splice variant to the plasma membrane was not affected by co-expression with the ZERO-HA variant (Fig. 7, a and d). Thus, the lack of plasma membrane targeting, and enhanced perinuclear localization, of the e21(STREX)-GFP variant in the presence of Δe23-HA was not simply an artifact of overexpression of the two variants.

As a further functional test of whether heteromultimerization of Δe23 with other splice variants may generate functional channels at the plasma membrane, we analyzed single channel events in isolated inside-out patches of HEK293 cells co-expressing the Δe23 variant with either ZERO or e21(STREX) variants. For these assays, we exploited ZERO and e21(STREX) splice variant mutants (Y294V-HA and STREX-Y294V-HA, respectively) that contain a single point mutation (Y294V) in the channel pore to reduce sensitivity to extracellular tetraethylammonium (TEA) (51). If the TEA-sensitive Δe23 α-subunits assemble with α-subunits of another (TEA-insensitive) splice variant, and form functional channels at the plasma membrane, we would predict that
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FIGURE 7. Δe23 is a dominant negative inhibitor of splice variant cell surface expression. a, representative single confocal sections of a permeabilized HEK293 cell co-expressing -HA epitope-tagged ZERO variant and -GFP-tagged e21(STREX) variant (upper panels), or -HA epitope-tagged Δe23 variant and -GFP-tagged e21(STREX) variant (lower panels). Separate images for the respective -HA (red) and -GFP (green) channels are shown, with the merged image on the right. Note robust peripheral membrane expression of ZERO-HA and e21(STREX)-GFP in the upper panels but intracellular perinuclear retention of both Δe23-HA and e21(STREX)-GFP BK channels in the lower panels. b and c, dominant negative effect of Δe23 is because of heteromultimer formation with other splice variants. Representative co-immunoprecipitation experiment from HEK293 cells co-transfected with the constructs as indicated. BK channel immunoprecipitation (IP) was performed using the α-FLAG antibody. Blots were probed with α-HA antibody and detected by enhanced chemiluminescence. Control (cont) immunoprecipitations were performed using nonimmune serum with transfected cells as shown. d, summary of membrane and perinuclear distribution of Δe23, e21(STREX), and ZERO splice variants expressed alone or in co-expression studies, as indicated. Data are presented as the percentage of the total number of transfected cells (n = 4 – 14 experiments per group, n = > 96 cells in each group), with the membrane or perinuclear distribution of the variant indicated in boldface presented. Note complete suppression of e21(STREX) variant membrane expression on co-transfection with Δe23, with concomitant increase in e21(STREX) perinuclear localization. Data are mean ± S.E., * p < 0.01 ANOVA compared with Δe23 variant expression alone.

single channel events, with a reduced single channel amplitude in the presence of 2 mM extracellular TEA, would be observed (51). The single channel amplitude under these conditions is an indication of the stoichiometry of the TEA-sensitive (Δe23 variant) to the TEA-insensitive (Y294V-HA or STREX-Y294V-HA variant) α-subunits in the tetramer. We only observed one patch (out of more than 120 patches exposed to greater than +80 mV and 100 μM [Ca^{2+}]) in which a large conductance channel was observed, when the Δe23 variant was expressed alone in HEK293 cells. This is in agreement with the lack of Δe23 splice variant protein expression at the plasma membrane (Figs. 6 and 7). In no patch (out of more than 50 tested) were any BK channels observed that were sensitive to 2 mM external TEA when Δe23 was co-expressed with another, TEA-insensitive, splice variant (not shown). Thus, all single channel events recorded in these co-expression assays were the result of homotetramers of the respective TEA-insensitive ZERO, or e21(STREX), variants.

Taken together, these data support the hypothesis that the novel Δe23 splice variant acts as a dominant negative of cell surface expression.

Regulation of Splice Variants by PKA-dependent Phosphorylation—The single channel conductance of each variant was determined in excised inside-out patches exposed to physiological potassium gradients in which less than four channels were present. There was no significant difference in single channel conductance between the ZERO, e21(STREX), and e22 splice variants (121.7 ± 8.9, 123.8 ± 5.4, and 125.2 ± 7.4 pS, respectively). In contrast, the e20 splice variant had a significantly increased single channel conductance, under identical recording conditions, of 143.3 ± 7.2 pS. In only one patch (out of more than 120 patches exposed to greater than 100 μM [Ca^{2+}] and depolarized to greater than +80 mV) did we observe a single channel event in HEK293 cells transfected with the Δe23 splice variant. Although full characterization was not possible, the single channel conductance was similar to that of the other variants (117 pS). We have never recorded such a channel in our native or mock-transfected HEK293 cells under these recording conditions. However, this channel was only active at voltages greater than +60 mV in the presence 100 μM [Ca^{2+}].

Alternative pre-mRNA splicing is an important determinant of BK channel regulation by PKA phosphorylation (22, 43, 44). As we have reported previously, ZERO splice variants are activated (mean activation was 110.1 ± 9.5%, n = 8 of control; Fig. 8, a and b) by closely associated PKA, in excised inside-out patches, upon application of 0.1 mM cAMP to the intracellular face of the patch. In contrast, under identical recording conditions, the e21(STREX) variant is inhibited by endogenous PKA (mean inhibition was −60.6 ± 10.3%, n = 5 of control; Fig. 8, a and b). The effect of cAMP on both ZERO and e21(STREX) channel activity is completely abolished in the presence of the specific PKA inhibitor peptide, PKI-(5–24), or in the absence of ATP. The mean cAMP-induced change in activity in the presence of PKI-(5–24) was −21.1 ± 4.5%, n = 4, and 1.3 ± 2.4%, n = 4, for ZERO and e21(STREX), respectively. In the absence of ATP, the mean change in activity was 1.1 ± 6.2%, n = 4, and 2.1 ± 4.0%, n = 4, respectively.

To examine whether introduction of other alternatively spliced inserts, at site of splicing C2, also modifies BK channel regulation by PKA-dependent protein phosphorylation, we assayed the e20 and e22 splice variants under identical conditions. Both the e20 and e22 splice variants displayed activation by endogenous PKA similar to that observed with the ZERO variant. Mean activation in the presence of cAMP was 128.6 ± 25.4%, n = 10, and 76.8 ± 12.7%, n = 6, for the e20 and e22 splice variants, respectively. More importantly, the effect of cAMP on both variants was completely abolished in the presence of PKI-(5–24) (mean change in cAMP-induced activity was −7.3 ± 8.2%, n = 6, and −5.2 ± 5.6%, n = 6, for e20 and e22, respectively). Similarly,
the effect of cAMP was completely abolished in the absence of ATP (mean change in activity was 2.9 ± 5.6%, n = 4, and −0.8 ± 11.8%, n = 4, for the e20 and e22 splice variants, respectively). Thus, the e20 and e22 splice variants are activated in a similar manner to the ZERO variant by PKA phosphorylation. The e21(STREX) splice variant, which introduces an additional PKA consensus motif (44) within the STREX insert, is the only site C2 splice variant to be inhibited by PKA.

**DISCUSSION**

The pore-forming α-subunits of BK channels are encoded by a single gene (KCNMA1) (28–30). Considerable functional variation may be generated by alternative pre-mRNA splicing in species as diverse as worms and humans. Inclusion of specific exons is known to modify physiologically relevant properties of the expressed channel protein. However, the extent to which exon inclusion, or exclusion, at a single site of splicing is important in generated functional diversity of BK channels is poorly understood.

In this report, we have isolated and systematically characterized the functional properties of five, alternatively spliced, murine BK channel variants that are generated by splicing at site C2 (see Fig. 1a). Four of the splice variants have been isolated previously (ZERO, e20, e21(STREX), and e22), although only the ZERO and e21(STREX) splice variants have been characterized functionally. We have also identified a novel, C-terminally truncated, splice variant (Δe23) that results from skipping of
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exon 23. This exon was previously thought to be a constitutively expressed exon.

Individual splice variants show distinct patterns of tissue distribution, cell surface expression, biophysical properties, and regulation by cAMP-dependent protein phosphorylation. Taken together, alternative splicing at site of splicing C2 would provide a powerful mechanism to specify BK channels with a spectrum of properties. These channels may be individually tailored to the properties of individual cell types, perhaps at different developmental stages or in response to a physiological challenge.

Differential mRNA Expression of Functionally Diverse Splice Variants in Murine Tissues—We have developed high sensitivity and specificity TaqMan™ real time PCR assays to quantitate, for the first time, the mRNA expression of each site C2 splice variant in different murine tissues. By using these assays, total BK channel mRNA levels were found to be highest in adult brain and a number of endocrine tissues but with extremely low mRNA expression in heart and liver. These total BK channel mRNA expression patterns largely agree with previous Northern blots, as well as protein and functional expression studies in other mammalian species.

Considerable diversity in splice variant mRNA expression profile, across different tissues, was observed. This may provide fine tuning of BK channel properties in different tissues according to physiological need. We cannot address directly whether the mRNA expression profiles determined for each splice variant directly translates into levels of protein (or functional) channel in each tissue. However, the mRNA expression profile likely provides an important correlate of splice variant protein expression. For example, e21(STREX) variant mRNA is highly expressed in endocrine tissues such as the adrenal gland and pituitary, as described previously (40, 41), whereas ZERO is the predominant brain variant. Heterologously expressed e21(STREX) variant channels display a significant left shift in half-maximal voltage of activation ($V_{0.5(max)}$) compared with ZERO (40, 41, 45, 53). Furthermore, e21(STREX) variants are also potently inhibited by cAMP-dependent protein kinase phosphorylation (44, 45), whereas ZERO is activated.

In many anterior pituitary cell types, native BK channels display a left-shifted $V_{0.5(max)}$ and are potently inhibited by PKA phosphorylation (10, 45, 54), properties that correlate with the STREX phenotype. In contrast, many neuronal BK channels reveal properties similar to the ZERO variant: low calcium sensitivity and activation by protein kinase A. The native BK channels in brain largely display a lower calcium sensitivity and are activated by PKA, properties consistent with a ZERO phenotype. Further detailed expression and functional analysis is required to address the spatial and temporal expression of BK channel splice variants at the mRNA, protein, and functional channel level in different tissues.

The e21(STREX) insert is highly conserved across vertebrates, at the amino acid level, suggesting it plays a common functional role, perhaps in the maintenance of high frequency or duration of action potentials (11, 20). To date, a homologous e21(STREX) variant in invertebrate species (such as Drosophila or Caenorhabditis elegans) has not been identified; thus e21(STREX) likely represents a vertebrate specialization. In contrast to the e21(STREX) splice variant, the e22 variant is poorly conserved between mouse and human (11 of 29 amino acid substitutions), indicating that the properties, and function, of this variant may be distinct between species. Indeed, inclusion of the human e22 variant results in no significant shift in the conductance-voltage relationship (55). This is in contrast to the significant (greater than 20 mV) left shift of the $V_{0.5(max)}$ conferred by the murine e22 variant in this study. The rate of channel deactivation was intermediate to that of the ZERO and e21(STREX) splice variants. Furthermore, although the e22 splice variant displays biophysical characteristics intermediate to that of e21(STREX) and ZERO, the response to protein kinase A phosphorylation is identical to that of ZERO channels. As the ZERO, e20, and e22 variants retain the conserved C-terminal PKA consensus phosphorylation motif, it is likely that this site is important for mediating PKA-dependent activation in all three variants as is the case for ZERO (44, 51). Transcripts containing exon 22 have been described previously in cDNA libraries from murine brain and skeletal muscle (29). The homologous exon is expressed in human (55) and rat (56) brain, and a long variant is up-regulated in human glioma tissue (39). However, in the murine tissues examined, expression of the e22 splice variant mRNA is relatively low in adult tissues apart from breast. In contrast, the e22 splice variant mRNA is expressed at high levels at embryonic stages in the mouse. Whether this represents a developmentally restricted pattern of expression or, alternatively, expression in tissues not sampled in the adult in our assays remains to be determined. Taken together, expression of these distinct variants would provide an array of BK channels whose functional impact would be optimal across a wider dynamic range of cellular voltages, intracellular free calcium levels, and in response to modulation of intracellular signaling cascades.

A Novel C-terminal Splice Variant Dominant Negative α-Subunit of Cell Surface Expression—We have isolated and characterized a novel splice variant (Δe23) that results from skipping of exon 23. The Δe23 splice variant is deficient in trafficking to the cell surface and acts as a dominant negative inhibitor of cell surface expression of other splice variants. This phenotype is similar to a C-terminally truncated variant in rabbit, rbSlo2 (47), generated as result of splicing at a site 3' to site of splicing C2. The intracellular trapping of the Δe23 splice variant may thus be because of the lack of the recently identified endoplasmic reticulum exit signal, located between residues 1105 and 1110, in full-length channels (47). As for rbSlo2, the murine Δe23 splice variant can assemble as tetramers. However, we observed no significant decrease in total protein expression of the Δe23 variant, compared with full-length channels, as reported for rbSlo channels that are truncated N-terminal to amino acid Asn-1061 (47). In humans, insertion of an alternatively spliced exon at the N terminus also generates BK channels with defective trafficking to the cell surface. These α-subunits also act as a dominant negative of cell surface expression (48). Thus, exon inclusion (48) as well as exon exclusion (this study and Ref. 47) can generate BK channel α-subunits with deficient cell surface trafficking.

Is there a physiological function for the Δe23 and other trafficking-deficient splice variants? In the mouse myometrium, total BK channel protein expression increases toward term. Paradoxically, the BK channel current decreases (25, 57). In this system, cell surface clustering of BK channel α-subunits is reduced with redistribution to a perinuclear/ endoplasmic reticulum localization (57). Whether up-regulation of the Δe23 splice variant, in late pregnancy, underlies such down-regulation of cell surface expression remains to be determined. Furthermore, in tissues with low total levels of BK channel mRNA expression, the Δe23 variant is the predominant splice variant expressed. This suggests that transcriptional as well as post-translational mechanisms are used, in these tissues, to suppress functional BK channel expression.

How the splicing decisions, for the alternatively spliced exons at site of splicing C2, are controlled and coordinated are poorly understood (27, 58). Alternative splicing of the e21(STREX) exon can be dynamically regulated, for example, in response to circulating hormones (24, 27), during stress (23), pregnancy (25), and upon cellular depolarization (58). However, factors that may regulate splicing decisions for the e20, e22, or Δe23 splice variants are essentially unknown. An intrinsic calcium-calmodulin regulatory response element has been identified as an
important factor in control of e21(STEREX) exon splicing. However, similar elements do not control splicing of exon 22 (58).

Taken together, alternative splicing at site of splicing C2 would provide a powerful mechanism to specify BK channels with a spectrum of properties. This would include differences in calcium sensitivity, kinetics, cell surface expression, and regulation by protein phosphorylation at the plasma membrane. As the splicing decision can be dynamically regulated, this would allow channel properties to be individually tailored to the demands of individual cell types. This may be manifest at different developmental stages or in response to a physiological challenge. Such the demands of individual cell types. This may be manifest at different developmental stages or in response to a physiological challenge. Such functional diversity, and plasticity, generated by splicing at a single site

Importantly, our data reveal new insights into the diversity, and plasticity, of the functional properties of BK channels that can be generated by alternative pre-mRNA splicing from a single site of splicing.

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Functionally Diverse Complement of Large Conductance Calcium- and Voltage-activated Potassium Channel (BK) α-Subunits Generated from a Single Site of Splicing

Lie Chen, Lijun Tian, Stephen H.-F. MacDonald, Heather McClafferty, Martin S. L. Hammond, Jean-Marc Huibant, Peter Ruth, Hans-Guenther Knaus and Michael J. Shipston

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