Large Conductance Ca\(^{2+}\)-Activated K\(^{+}\) Channel (BK\(_{Ca}\)) \(\alpha\)-Subunit Splice Variants in Resistance Arteries from Rat Cerebral and Skeletal Muscle Vasculature

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Abstract

Previous studies report functional differences in large conductance Ca\(^{2+}\)-activated-K\(^{+}\) channels (BK\(_{Ca}\)) of smooth muscle cells (VSMC) from rat cerebral and cremaster muscle resistance arteries. The present studies aimed to determine if this complexity in BK\(_{Ca}\) activity may, in part, be due to splice variants in the pore-forming \(\alpha\)-subunit. BK\(_{Ca}\) variants in the intracellular C-terminus of the \(\alpha\)-subunit, and their relative expression to total \(\alpha\)-subunit, were examined by qPCR. Sequencing of RT-PCR products showed two \(\alpha\)-subunit variants, ZERO and STREX, to be identical in cremaster and cerebral arteries. Levels of STREX mRNA expression were, however, significantly higher in cremaster VSMCs (28.9±4.2% of total \(\alpha\)-BK\(_{Ca}\)) compared with cerebral vessels (16.5±0.9%). Further, a low level of BK\(_{Ca}\) SS4 \(\alpha\)-subunit variant was seen in cerebral arteries, while undetectable in cremaster arteries. Protein biotinylation assays, in expression systems and arterial preparations, were used to determine whether differences in splice variant mRNA expression affect surface membrane/cytosolic location of the channel. In AD-293 and CHO-K1 cells, rat STREX was more likely to be located at the plasma membrane compared to ZERO, although the great majority of channel protein was in the membrane in both cases. Co-expression of \(\beta1\)-BK\(_{Ca}\) subunit with STREX or ZERO did not influence the dominant membrane expression of \(\alpha\)-BK\(_{Ca}\) subunits, whereas in the absence of \(\beta1\)-BK\(_{Ca}\), a significant proportion of \(\beta1\)-subunit remained cytosolic. Biotinylation assays of cremaster and cerebral arteries showed that differences in STREX/ZERO expression do not alter membrane/cytosolic distribution of the channel under basal conditions. These data, however, revealed that the amount of \(\alpha\)-BK\(_{Ca}\) in cerebral arteries is approximately 20X higher than in cremaster vessels. Thus, the data support the major functional differences in BK\(_{Ca}\) activity in cremaster, as compared to cerebral VSMCs, being related to total \(\alpha\)-BK\(_{Ca}\) expression, regardless of differences in splice variant expression.

Introduction

Potassium channels play an important role in the regulation of VSMC membrane potential and contractile activity. In particular, large conductance Ca\(^{2+}\)-activated, K\(^{+}\) channels (BK\(_{Ca}\)) are activated in response to membrane depolarization and increases in intracellular Ca\(^{2+}\) to affect membrane hyperpolarization [1,2]. While BK\(_{Ca}\) channels are widely expressed in both electrically excitable and non-excitable cells [3,4], they are relatively abundant in smooth muscle and play a key role in the regulation of vascular tone [5,6]. Structurally, the functional BK\(_{Ca}\) channel exists as a tetramer of \(\alpha\)-subunits forming the ion channel pore together with tissue specific auxiliary \(\beta\)-subunits (\(\beta1\)-\(\beta4\)) which are typically present in a 1:1 stoichiometry [7]. The BK\(_{Ca}\) \(\alpha\)-subunit consists of seven transmembrane spanning domains (S0-S6) including the extracellular N-terminus, P-loop between S5 and S6 domains, and a large intracellular C-terminus containing a number of regulatory sites including the regulators of conductance for K\(^{+}\) (RCK 1 and 2) and 2–3 Ca\(^{2+}\) binding sites.

The BK\(_{Ca}\) \(\alpha\)-subunit is encoded by a single gene (KCNMA1) containing 27 distinct exons, in contrast to each \(\beta\)-subunit, which is encoded by four distinct exons [8]. BK\(_{Ca}\) channels appear to achieve part of their functional diversity through alternative premRNA splicing of the KCNMA1 gene [9,10]. Up to ten alternative splicing sites have been identified for the vertebrate \(\alpha\)-subunit (KCNMA1) [11]. Most variation occurs in the intracellular C-terminal part in the linker region between domains RCK1 and RCK2 and upstream of the “calcium bowl” [12]. Alternative splicing can modify the functional properties of BK\(_{Ca}\) channels, including Ca\(^{2+}\) and voltage sensitivity, cell surface expression, and regulation by diverse intracellular signaling pathways. One of the most thoroughly studied \(\alpha\)-BK\(_{Ca}\) splice variants is the STREX exon (STRes axis regulated EXon), which derives its name from its splicing regulation by stress-axis hormones [13]. It has been shown that the STREX exon (an insertion of 38 amino acids in the C-terminal splice site 2 of the \(\alpha\)-subunit protein) confers distinct functional phenotypes onto BK\(_{Ca}\) channels, such as altered Ca\(^{2+}\) sensitivity and changing responsiveness of channels to CAMP.
signaling from stimulatory to inhibitory, compared with the ZERO variant, lacking this insert [9,14–17]. It has also been shown that BKCa channels containing the SS4 splice variant (an insertion of a 27 amino acid segment upstream to the C-terminal Ca^{2+}-bowl in splice site 4 of the α-subunit) were activated more rapidly than the ZERO variant in the presence of the same voltage stimulus, and the difference in these activation kinetics was dependent on the concentration of intracellular Ca^{2+} [18], also see Figure 1.

Alternatively, it has been reported that intracellular trafficking of α-BKCa may be one of the main post-translational modifications that can regulate the number of ion channels at the cell surface [19]. This mode of regulation can also be modulated by accessory β-subunits. While limited studies have addressed the effects of α-BKCa splice variants on channel trafficking to plasma membrane [20–24], there is some discrepancy in the reported findings. For example, Kim et al (2007) reported the expression of two α-BKCa splice variants, termed VEDEC and QEDRL, in chick ciliary ganglion neurons that differ at the extreme C-terminus. Using HEK293T and NG108-15 cells and a cell surface biotinylation assay, QEERL channels showed markedly higher levels of constitutive expression of α-BKCa at the plasma membrane compared with VEDEC channels, which tend to remain in the cytosol [21]. The same group further showed that co-expression of avian β1-subunits with the VEDEC isoform α-BKCa prevented the inhibitory effect of the VEDEC sequence on cell surface expression [20]. In contrast, studies from Toro and colleagues (2006) showed that co-expression of human β1-subunit with a human pore-forming α-subunit enhanced internalization of the α-subunit [25].

Previous electrophysiological studies from our laboratory have demonstrated that BKCa channel activity differs significantly in VSMCs from cremaster muscle arteries compared with cerebral arteries. In particular, our functional data have revealed a decreased Ca^{2+} sensitivity of cremaster BKCa channels, resulting in more positive levels of Em being required in cremaster VSM cells to generate similar levels of outward K^{+} conductance [26]. Similarly, Jackson and Blair (1995) described cremaster muscle BKCa channels as being normally ‘silent’, but suggested that their activity could be ‘recruited’ during vasoconstriction [27]. Therefore in this study, we first hypothesized that the functional differences between these two resistance vasculatures may be due, in part, to the expression of splice variants of the BKCa α-subunit. We chose to focus on STREX and SS4 splice variants, as splice sites where they are located (i.e. 2 and 4) contain regulatory phosphorylation, palmitoylation and Ca^{2+} interaction sites that could functionally impact channel activity. We further hypothesized that an additional influence of α-subunit splice variation may be on the surface membrane location of the channel and whether this could be affected by the accessory β1-subunit.

**Material and Methods**

**Tissue isolation and vessel RNA purification**

All experiments and protocols were approved by the Animal Care and Use Committee, University of Missouri, USA. Our studies used male Sprague-Dawley rats weighing between 180–280 g. Rats were anesthetized with sodium pentobarbital (Nembutal, 100 mg/kg body weight) given by an intraperitoneal injection. Cremaster muscles were surgically removed, as previously described [28], and placed in a cooled (4°C) dissection chamber. Following sacrifice by anesthetic overdose, a craniotomy was performed and the brain was removed intact and similarly placed in a cooled dissection chamber.

First- and second-order arterioles (1A/2A) from cremaster muscle and mid-cerebral arteries were isolated and rapidly subjected to total RNA purification using a Melt Total Nucleic Acid isolation system kit (Life Technologies, Carlsbad, CA, USA).
following the manufacturer’s instructions. All samples were treated by TURBO DNase digestion (Life Technologies) to minimize contamination with genomic DNA. The concentration and purity of RNA for each sample was determined by UV absorbance using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Rockford, IL, USA) and samples were stored at −80°C until conversion to cDNA. Equal amounts of total vessel RNA extract were then reverse-transcribed into a single strand cDNA using a Superscript III First-Strand synthesis system (Life Technologies) according to the manufacturer’s instructions.

Real-time quantitative PCR
Real-time PCR was performed in triplicate, in 96-well plates, on cDNAs prepared from each sample (n = 4–5) using KAPA SYBER FAST qPCR Kit Master mix (KAPA Biosystems, Woburn, MA, USA). PCR was performed using a Mastercycler EP Realplex2 (Eppendorf-North America, Westbury, NY, USA). Reaction volume/well contained 20 µl of master mix, 1 µl of each sense and antisense primers (5 µM), 1 µl of cDNA template and the remainder DNase-free water. Primers used in this study were designed in regions of transmembrane domains in which no splice variant existed, and shown in Table 1. ZERO variant primers were designed in regions for the template sequences and the expected product sizes are according to the manufacturer’s instructions. The amplification efficiencies between targets and housekeeping genes (i.e. β-actin) were initially verified to be approximately equal (Table 1), allowing the comparative threshold (Ct) method for quantification to be used [30]. The relative expression level (R) was calculated with equations as follows: 

$$R = 2^{\Delta C_{t} \text{calibrator} - \Delta C_{t} \text{sample}}$$

for the target genes in each sample set according to the published 2−ΔΔCt method [30]. Changes in mRNA expression levels were calculated from an average of triplicate measurements and are reported as fold changes relative to the ZERO variant, after normalization to β-actin. Data were analyzed using an unpaired student t-test: a statistically significant difference was assumed at P≤0.05.

Cell surface Biotinylation assay on cultured cells
Plasmid constructs containing cDNA for full-length rat BKCa ZERO variant or BKCa STREX variant (gifts from Dr. Michael J. Shipston) were transiently transfected into AD-293 cells (240085, Agilent Technology, Santa Clara, CA, USA) or CHO-K1 cells (CCL-61, ATCC, Manassas, VA, USA) with FuGENE 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA). A bovine β1-BKCa plasmid DNA was also used in some experiments as its sequence shares high homology (>95%) with rat β1-BKCa channel protein. Cell surface biotinylation assays were performed 24–48 hours post-transfection. In brief, live transfected cells were washed three times with Hanks’ buffered salt solution (HBSS) and then incubated on ice for 2 hours in the presence of a freshly prepared 0.5 mg/ml mixture of biotinylation reagents, EZ Link Sulfo-NHS-Lc-Lc-Biotin (21338, Thermo Scientific) and EZ Link Maleimide-PEG-Biotin (21901, Thermo Scientific). Total protein was determined to allow normalization for Avidin pull-down of biotinylated proteins after quenching of biotinylation process by ice-cold 100 mM glycine in HBSS (3x in 1 min interval incubation). Biotinylated cells were homogenized in RIPA buffer plus 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), incubated on ice (30 min) and sonicated for 45 sec. Cellular debris was removed by centrifugation at 6,000 g for 10 min at 4°C. Total protein concentration was determined using the BCA protein assay kit (Thermo Scientific). Equal amounts of total biotinylated cell lysates were subsequently incubated with MonoSonic Avidin Agarose (20228, Thermo Scientific) overnight at 4°C, followed by washing with cold HBSS (3x) and one time with

| Table 1. Sequence of primers used for end-point and real-time PCR. |
|-----------------------|------------------|------------------|------------------|
| **Accession number**  | **Primer sequence** | **Amplicon length** | **Amplification efficiency** |
|-----------------------|------------------|------------------|------------------|
| Kc6                  | NM_031828        | F: TACTGCAAGGCGCTGTCAATGATG | 342              | 2.063            |
|                       |                  | R: TCATCAGCTTGGGATGTGT    |                  |                  |
| Real-time PCR         |                  |                  |                  |                  |
| STREX                 | NM_031828        | F: TTTGATTGCAGGAGCCTTCTGA | 77               | 2.013            |
|                       |                  | R: TCTCTGACGGGTCTCACGTGAC |                  |                  |
| S54                  | AF_135265        | F: CAACTGGCCTCTGAGAATGTCGTC | 131              | 2.011            |
|                       |                  | R: GGCTGCTATCGGCTCATAC    |                  |                  |
| ZERO                 | NM_031828        | F: AAACAAAGTATCCATCAAGCTGTTG | 137              | 2.006            |
|                       |                  | R: CGTAGTGGCTGTGGGTCGTT    |                  |                  |
| β-actin              | NM_031444        | F: CACTCTAGCACACAGCTGTGCTC | 128              | 1.993            |
|                       |                  | R: GCTCAGGAGGAGAACATGCTTG   |                  |                  |

(F: forward primer, R: reverse primer).

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water. Finally, the cytosolic fractions of cells transfected with either α-BKCa splice variants or β1-BKCa subunit were separated from biotinylated cell surface proteins by centrifugation (11,000 g/2 min/4°C). The biotinylated membrane proteins were then eluted from the beads by heating at 45°C/15 min in 2× Laemmli protein sample buffer [31]. Isolated cell surface and cytosolic proteins were separated by SDS-PAGE on 4–20% TGX Precast Gels (Bio-Rad, Hercules, CA, USA), transferred onto polyvinylidene difluoride membranes and probed with a mouse monoclonal anti-BKCa channel (clone L6/60, 1:500, NeuroMab, Davis, CA, USA) or an anti-BKCa β subunit antibody (ab3587, 1:500, Abcam, Cambridge, MA, USA). Bound antibody was detected using SuperSignal West Dura ECL Chemiluminescent Substrate (34075, Thermo Scientific). Images were collected using a ChemiDoc XRS+ System (Bio-Rad) and analyzed by Image Lab software. Parallel control biotinylation assays were conducted with mock transfected cells and cells with streptavidin beads in the absence of biotin incubation. In mock transfected cells, no bands were detected related to the α-BKCa splice variants (ZERO, STREX) or the β1 subunit (data not shown). These control studies confirmed the absence of endogenous BKCa channels in CHO-K1 cells and the specificity of the antibodies used in this study.

Cell Surface Biotinylation assay on vessels

Biotinylation of surface proteins in intact cerebral and cremaster arteries was performed to detect the cell surface membrane expression of native α-BKCa channels in these vessel types. To have adequate amounts of total protein, first- and second-order cremaster arterioles from four male Sprague-Dawley rats (180–280 g) were pooled together for each separate experiment. In parallel, the whole Circle of Willis vasculature was isolated from two animals, cleaned of connective tissue and pooled to provide a cerebral artery sample. Arteries were incubated in a freshly prepared 1 mg/ml mixture of Biotin reagents, as above, in whole cell buffer solution (in mM 10 HEPES, 9 Glucose, 6 KCl, 134 NaCl, 2 CaCl2, 2H2O, and 1 MgCl2, 6H2O) for one hour at room temperature while undergoing constant horizontal shaking. The arteries were then incubated at room temperature with quenching solution of 100 mM glycine in PBS for 15 min to remove any unbound biotin. The biotinylated vessels were homogenized to prepare total protein as previously described [32]. Equal amounts of total protein (~50–60 µg) were incubated with Monomeric Avidin Agarose. After one hour of avidin incubation at room temperature [33,34], the non-biotinylated (cytosolic) protein fraction was separated from the biotinylated (cell membrane) protein fraction by centrifugation at 11,000 g/2 min/4°C. Biotinylated surface proteins were eluted from the avidin beads by boiling for 3 min in 2× Laemmli buffer containing β-mercaptoethanol (5% v/v). Western blot analysis of surface and cytosolic proteins was performed using mouse anti-α-BKCa channel (1:500, NeuroMab) or anti-BKCa β subunit (1:500) primary antibodies. Quantification of cell surface and cytosolic protein bands was analyzed using Image Lab software (Bio-Rad) and are expressed as percentage of total protein.

Results

Identification of α-BKCa splice variants by end-point PCR

For initial identification of STREX and ZERO variants, endpoint PCR was performed using primers designed to amplify the alternative splice site 2 (See Table 1 for details). Tests cDNA was used as a positive control [11]. PCR products of three separate experiments from different experimental animals were analyzed by electrophoresis and subsequently verified by sequencing. As shown in Figure 2, two dominant bands were detected in both vasculatures. The lower band with predicted size of 168 bp was determined to be ZERO variant (α-subunit without splice insert) by direct product sequencing and the upper band (~342 bp) was confirmed to be STREX variant (α-subunit with the insertion of 174 bp at splice site 2). The third visible band likely constitutes heteroduplexes between sense and antisense strands of STREX and ZERO products consistent with earlier reports [35].

Quantification of α-BKCa splice variants by qPCR

Identification of the SS4 variant was performed by qPCR together with subsequent quantification of expression levels of STREX variant relative to ZERO using a further set of primers (Table 1). As shown in Figure 3A and B, while a very low level of SS4 was detected in mid-cerebral arteries (0.42±0.1% of total α-BKCa), the variant was undetectable in cremaster vessels. A higher level of expression of the STREX variant was detected in cremaster arteries (28.9±4.2% of total α-BKCa) compared to mid-cerebral (16.5±0.9% of total α-BKCa) arteries (P<0.05). Thus, ZERO variant was calculated to be significantly (P<0.05) greater in mid-cerebral (83.1±0.9% of total α-BKCa) compared to cremaster (71.1±4.2%) arteries (Figure 3A).

Cell Surface expression of BKCa ZERO or STREX variants in expression systems

To investigate the cell surface location of α-BKCa splice variants in a cell culture system, equal amounts of full-length cDNAs of rat ZERO or STREX variants were initially transfected into AD-293 cells (Figure 4A). As shown in Figure 4B, although both variants were predominantly targeted to the cell surface, the STREX variant of α-BKCa shows a significantly higher level of cell surface expression (P = 0.02, unpaired t-test) than the ZERO variant. Conversely at the cytosolic level, the ZERO variant shows a significantly higher level of expression as compared with STREX (P = 0.02, unpaired t-test). The ratio of membrane to cytosol expression was also significantly higher for the STREX variant than ZERO (Figure 4C).

To confirm that this trafficking pattern was not cell dependent, similar experiments were conducted using CHO-K1 cells (Figure 5A). As illustrated in Figure 5B, the STREX variant again shows increased distribution at the cell membrane compared with ZERO (P = 0.002, unpaired t-test). The cytosolic expression of STREX in CHO-K1 cells shows significantly lower expression than the ZERO variant, similar to that observed in the AD-293 cell culture system. As shown in Figure 5C, the ZERO variant shows a significantly lower ratio of membrane to cytosolic expression in CHO-K1 cells. Since these two α-BKCa subunit splice variant exhibit the same expression pattern in AD-293 and CHO-K1 cells, we used CHO-K1 cells as our cell system model for future experiments.

Cell Surface location of co-transfected BKCa ZERO or STREX variants with β1-subunit in CHO-K1 cells

As holo-BKCa channels in resistance arteries contain both α- and β1-BKCa subunits, we hypothesized that co-expression of β1-subunit in CHO-K1 cells may equalize the cell surface levels of the two variants. To investigate the impact of β1-BKCa subunit on cell surface expression of α-BKCa splice variants ZERO and STREX, β1-BKCa subunit was co-transfected with either the ZERO or STREX variant (Figure 6A). In the presence of β1-BKCa subunit, the α-subunit variants continued to be predominantly located in the plasma membrane with the STREX variant present at higher levels than ZERO (Figure 6B). This resulted in the same relative
**Figure 2. Detection of zero and STREX variants in rat cerebral and cremaster arteries.** End-point PCR products generated from cDNA derived from rat total RNA transcripts, with and without the STREX exon. Size markers are shown in the lane marked as M. Additional lanes display PCR products detected in testis (lane 1, included as a positive control), mid-cerebral arteries (lane 2) and cremaster arterioles (lane 3). Results are representative of n = 3 separate experiments.
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**Figure 3. QPCR of BKCa α-subunit splice variants in rat cerebral and cremaster arteries.** (A) Relative mRNA expression levels for STREX, SS4 and ZERO variants calculated as a percentage of total α-BKCa mRNA detected in mid-cerebral arteries and cremaster arterioles. (B) QPCR products of SS4, STREX and ZERO variants of α-BKCa subunit as separated on a 2% agarose gel. Size markers shown in the lane denoted M. QPCR products generated from cremaster arterioles are depicted in lanes 1, 3, 5 and products from mid-cerebral artery are shown in lanes 2, 4, and 6. Real-time PCR results are shown for n = 4–5 samples of each vascular bed performed in triplicate.
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expression pattern (Figure 6C) as seen when the α-BKCₐ splice variants were expressed without the β₁-BKCₐ subunit (Figure 5C). These data could indicate a dominant cell surface expression pattern of α-BKCₐ splice variants of ZERO and STREX in cell culture systems.

To determine the effect of ZERO or STREX variants on the cellular distribution of β₁-BKCₐ subunits, biotinylated β₁-BKCₐ subunit proteins were also probed in cells co-transfected with β₁-BKCₐ and either the ZERO or STREX variant. As shown in Figure 7A, a high level of cell surface expression of β₁-BKCₐ subunit (84.7±4.4%) was observed when co-transfected with either the ZERO or STREX variant. To assess whether overexpression of β₁-BKCₐ subunit, alone, is sufficient to stimulate its surface trafficking, CHO-K1 cells were transfected with only β₁-BKCₐ cDNA and β₁-BKCₐ subunit surface location assessed by the cell-surface biotinylation assay. Under these conditions, as shown in Figure 7B, the cell surface labeling of β₁-BKCₐ subunit showed a significant decrease (P = 0.01) to approximately 60.2±5.2%, which was accompanied by a significant increase in cytosolic levels of β₁-BKCₐ subunit from approximately 15.3±4.5% to 39.8±5.2%. These data appear to indicate a stimulatory effect of α-BKCₐ splice variants on the surface trafficking of the regulatory subunit β₁-BKCₐ while there was no apparent impact on the surface membrane location of either ZERO or STREX by β₁-BKCₐ subunit.

Cell surface expression of total α-BKCₐ protein in cerebral vs cremaster arteries

Previous electrophysiological studies from our laboratory have demonstrated that BKₐ channel activity differs significantly in VSMCs from cremaster muscle arteries compared with cerebral arteries [26]. Our functional data show a decreased Ca²⁺ sensitivity of cremaster VSMC BKₐ channels compared with those of cerebral arteries, resulting in more positive levels of Em being required for cremaster VSMCs cells to generate similar levels of outward K⁺ current. As a result, we hypothesized that these functional differences in channel activity could arise from differences in the molecular configuration of the channel in the two VSMC types affecting channel properties such as cell surface trafficking of α-BKCₐ protein. Although the lack of specific available antibodies to distinguish ZERO from STREX variants is a technical limitation to detect α-BKCₐ splice variants at the protein level, the biotinylation assay was used to determine the extent of α-BKCₐ protein at the cell surface compared with the cytosolic fraction. Experiments were performed using homogenates of whole cerebral and cremaster arteries. As shown in Figure 8A and B, in both vessel types more than 90% of total α-BKCₐ channels were located at the cell membrane. However, when cell surface expression of α-BKCₐ was normalized to equal amounts of α-smooth muscle actin in each preparation, cerebral arteries show approximately 20 times higher level of total amount of α-BKCₐ protein at the cell membrane compared with cremaster arteries (Figure 9).
Discussion

In previous studies, we reported functional differences in BKCa channels in VSMCs between cerebral arteries and those from cremaster muscle [26,32]. In those studies, we showed that BKCa from cremaster VSMCs exhibit a decreased Ca\(^{2+}\) sensitivity and suggested that this may, in part, be due to a decrease in the amount of the \(\beta_1\) regulatory subunit. Regulation of BKCa is, however, complex involving mechanisms at the levels of expression and post-translational modification, as well as its physical relationship to cellular organelles such as the sarcoplasmic reticulum [5,6,8]. On the basis of this, the aims of the present study were to examine whether differences exist in the expression of splice variants of the \(\alpha\)-subunit of BKCa and if membrane location of the channel differed between the two vascular beds.

The major findings of the present studies were as follows: Firstly, qPCR studies demonstrate a significantly higher mRNA expression for the BKCa \(\alpha\)-subunit STREX splice variant in rat cremaster arteries compared with that in cerebral arteries. Secondly, we detected the predominant cell surface expression of both the \(\alpha\)-BKCa splice variants ZERO and STREX in cell expression systems, with no apparent impact of \(\beta_1\)-subunit co-expression on the degree of cell surface localization. Thirdly, although the \(\beta_1\)-subunit expressed alone is able to reach the cell membrane, a significant proportion remains cytosolic compared with its predominant cell surface localization in the presence of either the ZERO or STREX variant. Finally, cell surface labeling revealed that the vast majority of \(\alpha\)-BKCa channel (>90%) in both cremaster and cerebral arteries is located in the cell membrane fraction under basal conditions. However, a major difference between the two vascular beds was that the total amount of plasma membrane \(\alpha\)-BKCa is approximately 20 times lower in cremaster arterioles as compared with small cerebral arteries.

Although a single gene, KCNMA1, encodes the pore forming BKCa \(\alpha\)-subunit in vertebrates, there is considerable phenotypic diversity of these channels in different tissues. Several factors are known to contribute to this diversity including, alternative splicing [36], the co-expression of regulatory \(\beta\)-subunits [37,38], and post-translational modifications including protein phosphorylation [39]. Modulation of BKCa channels by a complex network of signal transduction pathways such as reversible protein phosphorylation has been studied extensively [39–41]. Importantly, alternate splicing of pre-mRNA leading to channel variants with differing degrees of modulation by reversible protein phosphorylation represents a potential mechanism to generate functional diversity of ion channels. In studies of cloned mouse BKCa variants, expressed in HEK293 cells, Tian et al. (2001) demonstrated that cAMP-dependent protein kinase (PKA)-mediated phosphorylation activates BKCa ZERO variant, but inhibits the STREX variant, which could thus impact channel function including Ca\(^{2+}\) sensitivity [17]. The level of STREX expression also has important modulatory consequences as it has been previously shown that only one subunit within the tetrameric holo-channel needs to be of the STREX type to alter channel function [42]. It has also been shown that protein palmitoylation (a post-translational modifica-
tion affecting multiple ion channels) can regulate the activity and surface expression of BK$_{Ca}$ channel a-subunits in native tissues and cultured cells [19]. Specifically, Tian and colleagues described palmitoylated BK$_{Ca}$ channels that include plasma membrane associated STREX variants that are inhibited by PKA-dependent phosphorylation, whereas ZERO channels are activated by PKA [43]. Therefore, the finding of lower expression of the ZERO variant and higher expression of STREX in cremaster arteries compared with mid-cerebral arteries could conceivably relate to functional alterations in BK$_{Ca}$ Ca$^{2+}$ sensitivity, as we have previously observed for cremaster vascular smooth muscle cells [32].

However, in the present studies the cremaster vessels have been shown to express a higher level of STREX as compared to the cerebral arteries which may have been expected to convey a higher Ca$^{2+}$ sensitivity [9] rather than the decreased Ca$^{2+}$ sensitivity we previously reported [26]. This apparent discrepancy may relate to a number of factors including the dominant effects of differences in overall expression levels and also that the cremaster vessels were previously shown to have a lower ratio of b$_1$:a subunit [9]. Importantly, the b$_1$ subunit has previously been shown to contribute to the Ca$^{2+}$ sensitivity of the channel [44]. An additional consideration is that our measurements of STREX and ZERO expression were limited to the mRNA levels. In this previous study, we also reported a decrease in the ratio of BK$_{Ca}$ a:b subunits in the crude membrane fraction of cremaster vessels compared with small cerebral arteries [26]. The data from the present study suggest it is unlikely that a lower level of b$_1$-BK$_{Ca}$ would negatively influence insertion of the a-subunit into the membrane, as our data in cell expression systems showed similar levels of surface location for a-BK$_{Ca}$ in the presence and absence of the b$_1$-subunit. Interestingly, and in contrast, the presence of the a-subunit in the expression system resulted in an increased proportion of b$_1$-subunit being located at the cell surface.

In earlier studies, Jackson and Blair (1998) suggested that BK$_{Ca}$ is 'silent' in cremaster muscle vasculature under basal conditions, but may be 'recruited' under stimulated conditions. Such stimulation was suggested to include vasoconstriction evoked by catecholamines and high tissue PO$_2$ levels [27]. Whether such recruitment involves differences in splice variant expression, translocation of channel subunit proteins to the plasma membrane or post-translational modifications such as phosphorylation has not been fully elucidated. Given the high levels of a-subunit protein found at the membrane in both vessel types, it is unlikely that a simple difference in membrane vs. cytosolic pools explains the differences observed between cremaster and cerebral vessels. This does not, however, exclude the possibility that a dynamic...
alteration in channel protein trafficking occurs under other conditions.

In the present study, we also found a very low level of expression of the SS4 α-subunit variant relative to the ZERO variant in mid-cerebral, with no detectable expression in cremaster arteries. Although expression of the SS4 variant in vasculature (identified by RT-PCR) has been previously reported in cerebral and coronary arteries [11], its functional importance, particularly in native tissues such as small arteries, is unknown. Similarly, the functional significance of a lack of SS4 variant expression (as we report for cremaster muscle arterioles) is unclear. Using a Xenopus oocytes expression system, previous studies have suggested that ZERO and SS4 variants exhibit identical BKCa channel characteristics, including single-channel conductance and voltage dependent activation [18]. Those authors did, however, show that the activation rates of SS4 channels were more rapid at a similar voltage compared with the ZERO form when [Ca2+]i was higher than 5 μM. In addition to a comparative lack of information as to any functional significance of very low expression levels of the SS4 variant (approximately 0.4% of total α-BKCa mRNA) in cerebral arteries, it should also be considered that non-smooth muscle cell contamination (for example from neurons or adventitial cells) in whole vessel preparations could contribute to this signal.

Apart from differences in splice variant expression, it would be expected that the marked difference in total BKCa channel protein expression would be of functional significance. This is despite the large conductance (approx. 240 pS) in VSMCs from both vascular beds. Specifically, an ~20-fold higher level of α-BKCa protein was detected in cerebral arteries compared with cremaster arteries. Importantly, this would be reflected at the plasma membrane because a similar proportion of total BKCa was surface located in both cerebral and cremaster arteries, as shown by our biotinylation assay. While measurements were performed on homogenates of whole vessels, the majority of signal would be expected to derive from the VSMC layers. Endothelial cells of healthy arteries are thought to be devoid of BKCa channels, although this point has been somewhat controversial [45]. Cellular capacitance measurements performed in our previous studies indicate that VSMC size in the two vessel types is similar, suggesting that functional effects of the expression difference would not be compensated by differences in size alone [26]. The earlier study also demonstrated, in crude membrane fractions, increased α-BKCa protein (approx. 3x) in cerebral vessels as compared to cremaster arterioles. Despite these measurements of marked differences in expression at the

![Figure 7. Cell surface expression of β1-BKCa subunit in the presence and absence of α-BKCa splice variants.](image-url)
protein level actual K+ currents only differed by approximately 2x (at 5 mM Ca²⁺). Conceivably the seemingly disparate findings may relate to the functional status of the channels including as determined by post-translational modifications such as phosphorylation or possibly the influence of splice variants not directly examined in this study [46]. Another factor perhaps affecting the differences in absolute protein levels between cerebral vessels and cremaster arterioles relates to differences in adventitial structure that we have previously demonstrated [47]. It could be argued that these differences impact the access of the biotinylation reagents.

Figure 8. Membrane and cytosolic expression of α-BKCa subunits in cerebral vs cremaster arteries. α-BKCa channels are predominantly located at the cell surface of VSMCs in both rat cerebral and cremaster arteries under basal conditions. (A) Cell surface α-BKCa proteins were determined using the biotinylation assay from whole cerebral (Circle of Willis pooled from 2 animals in each experiment) and cremaster arteries (first- and second-order cremaster arterioles pooled from 4 animals in each experiment). Results are shown for n = 6 separate experiments for cerebral and n = 5 for cremaster arteries. Results are shown as mean ± SEM. (B) Membrane to cytosolic ratio of α-BKCa channels in cerebral vs cremaster arteries (the corresponding Western blot showing cell surface and cytosolic levels of α-BKCa protein is shown in Figure 9A). doi:10.1371/journal.pone.0098863.g008

Figure 9. Quantification of cell surface expressed α-BKCa channels in cerebral vs cremaster arteries. Total amount of membrane α-BKCa channel in cerebral arteries is approximately 20 times higher than that in cremaster arteries. The Western blot in panel (A) shows a representative experiment depicting the distribution of α-BKCa protein in membrane and cytosolic fractions prepared from cerebral arteries and cremaster arterioles. The scan beneath the blot quantifies the intensity of the α-BKCa protein immunoreactive band in each sample, as detected by densitometry. The Western blot in panel (B) shows a loading control in which 2 μg of total protein for both cerebral arteries (CA) and cremaster arterioles (CMA) was probed with α-actin SMC antibody. Panel (C) shows group data for n = 5 experiments. Results are shown as mean ± SEM (*P<0.05, unpaired t-test). doi:10.1371/journal.pone.0098863.g009
We believe this to be unlikely, however, as the biotinylation reagents are small in regard to molecular weight and it was previously shown that the molecules easily penetrate the vascular wall [33]. Further, while this could theoretically effect the magnitude of the protein expression levels it would not impact the ratio to β-subunit ratios nor the distribution between the plasma membrane and cytosol. As both the cell surface biotinylation approach and measurements performed in crude membrane fractions showed qualitatively similar same results, the conclusion that protein expression levels are greater in cerebral vessels appears robust.

In summary, significant differences exist with respect to the BKCa splice variants expressed in cremaster muscle arterioles compared with small arteries from the cerebral vasculature. Specifically, a higher expression level of the STREX variant of the α-subunit was observed in arterioles from cremaster muscle. While functional significance of this finding is yet to be fully demonstrated, it appears that it does not affect the plasma membrane location of the channels as >95% of α-subunit was found to be at the cell surface in both vessel preparations. In contrast, a marked difference in the detectable expression level was observed, with cerebral arteries expressing α-subunit protein at a level 20 times greater than that of cremaster arterioles.

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Author Contributions

Conceived and designed the experiments: ZN JHJ APB MAH. Performed the experiments: ZN ML MDL. Analyzed the data: ZN ML MDL JHJ APB MAH. Wrote the paper: ZN MAH. Editing of the manuscript: ZN ML MDL JHJ APB MAH.

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