Biochimica et Biophysica Acta 1788 (2009) 380–389

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Differential expression of BK channel isoforms and β-subunits in rat neuro-vascular tissues

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ARTICLE INFO

Article history:
Received 29 May 2008
Received in revised form 18 September 2008
Accepted 6 October 2008
Available online 18 October 2008

Keywords:
Isoform
Middle cerebral artery
Basilar artery
Migraine
Slo
BKCa

ABSTRACT

We investigated the expression of splice variants and β-subunits of the BK channel (big conductance Ca^{2+}-activated K^+ channel, Slo1, MaxiK, $K_{Ca}1.1$) in rat cerebral blood vessels, meninges, trigeminal ganglion among other tissues. An α-subunit splice variant $X1_{+24}$ was found expressed (RT-PCR) in nervous tissue only where also the $SS4_{+81}$ variant was dominating with little expression of the short form $SS4_0$. $SS4_{+81}$ was present in some cerebral vessels too. The $SS2_{+174}$ variant (STREX) was found in both blood vessels and in nervous tissue. *In situ* hybridization data supported the finding of $SS4_{+81}$ and $SS2_{+174}$ in vascular smooth muscle and trigeminal ganglion. β-subunits β2 and β4 showed high expression in brain and trigeminal ganglion and some in cerebral vessels while β1 showed highest expression in blood vessels. β3 was found only in testis and possibly brain. A novel splice variant $X2_{+92}$ was found, which generates a stop codon in the intracellular C-terminal part of the protein. This variant appears non-functional as a homomer but may modulate the function of other splice-variants when expressed in *Xenopus* oocytes. In conclusion a great number of splice variant and β-subunit combinations likely exist, being differentially expressed among nervous and vascular tissues.

1. Introduction

In comparison to the large family of voltage-gated potassium channels (>30), the group of calcium-activated potassium channels only includes a handful of genes including the SK, IK and BK channels [1,2]. Of these, the BK channel is expressed in most tissues and shows a unique high unitary conductance and combined activation by intracellular calcium and depolarization, which implies an important role for the channel as a regulator of cell membrane potential in response to calcium signalling e.g. in smooth muscle cells [3,4] and in neurons [5–7]. Although encoded by a single gene, the BK channel appears in different functional forms due to alternative post-transcriptional splicing of the mRNA [8–11] and to co-expression of modulatory β-subunits, both affecting the gating properties of the channel [10,12], its response to second messengers [13] and sensitivity to pharmacological agents [14,15].

Inclusions of intron sequence in the mRNA transcript are the prevalent type of BK channel splice variance, compared to deletion of exons (Table 1). At least ten different splice sites in the vertebrate BK channel α-subunit have been described (see Fig. 1) and further variants can be predicted from GenBank data, including truncated variants where the inserted intron sequence contains a stop codon [11]. In brain tissue [10,16] and in the cochlea [17], different splice variants are apparently expressed simultaneously indicating that splice-variation has implications for the function of the ion channel. In the cochlea, e.g., the spatial distribution of splice variants is suggested to be involved in frequency tuning [17]. The functional implications of the alternative splicing has been studied in some cases and include an inverted response to cAMP, altered response to membrane tension [13,18], hypoxia [19] and steroid hormones [20], impaired membrane expression [21,22], altered response to modulation by β-subunits [6,23] and changed activation kinetics and calcium sensitivity [24,25]. Although identified in various tissues, the tissue distribution of splice variants has not yet been systematically investigated as array-based expression studies do not discriminate between splice variants (e.g. GNF SymAtlas, GEO). Also splice-specific antibodies have not been developed.

The BK β-subunits are a group of small two transmembrane-segment peptides that affects the channel properties. Four such β-subunits are known so far (β1–4) in addition to the identified intracellular $Ca_V\beta1$ [26]. In general, they increase the Ca^{2+} and voltage sensitivity and slow the activation of the BK channel and in addition the β2 and some splice variants of the β3 induces N-terminal dependent "ball" inactivation [27,28]. The β-subunits may also affect the surface expression of BK channels and its acute response to steroid hormones [29,30]. The β1 has

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doi:10.1016/j.bbamem.2008.10.001

been found expressed in a number smooth muscle containing tissues while scarcely expressed in brain and skeletal muscle [15,31]. An essential role of its modulatory effect on BK function has been highlighted in arterial smooth muscle in a mouse knock-out model [3]. β4 is widely expressed but seems to be most abundant in brain tissue and absent in e.g. liver and pancreas [15]. Also β2 is expressed in brain and in addition in heart, while it apparently is absent in aorta, colon, lung, pancreas, bladder [15]. In one study, β3 has been detected in testis, lung, brain and heart but is absent in skeletal muscle, kidney and colon [32], while another study finds β3 in testis only [28].

The BK channel is an important determinator of arterial smooth muscle tone as has been illustrated by the effect of the selective BK-channel inhibitor iberiotoxin, which induces vasoconstriction [33] and attenuates the vasodilatory response of CGRP (calcitonin gene related peptide) [34], acetylcholine [35], NO donor glyceryltrinitrate [36] and PGI_2 [37]. The BK channel thus holds a potential as a possible target for modulation of systemic blood-pressure or local vasomotor-responses (e.g. during migraine or stroke), yet the widespread distribution of BK channels may be a hindrance for a therapeutic strategy due to side effects. An example is the tremorgenic action of the BK-inhibiting mycotoxins paxilline and penitrem [38], which is likely induced by an effect on neuronal firing frequency [39].

The cerebral and meningeal arteries and the trigeminal ganglion are involved in the pathophysiology of migraine headache, which likely includes cranial vasodilation and trigeminal nerve-activity in concert [40]. BK channels are expected to be expressed in these tissues, and may be activated indirectly by NO [36] or CGRP (vascular BK channels) [34], but the molecular composition of these channels in terms of splice variants and β-subunit expression has not been specifically investigated. The aim of the current study is therefore to determine the variants of the BK channels present in the involved tissues (cerebral blood-vessels and trigeminal ganglion). We found that the cerebral vascular and neuronal tissues differ in their expression of two BK splice variants ($SS4_{+81}$, $X1_{+24}$) and three β-subunits (β1, β2 and β4) in particular. Surprisingly, a novel apparently truncated BK isoform ($X2_{+92}$) was found in a preparation of the dura mater containing tissue from the superior sagittal sinus.

2. Methods and materials

2.1. Tissue isolation

Male rats were euthanized with intra-peritoneal injection of pentobarbital. After cessation of motor reflexes, the abdominal cavity was opened. The vascular system was cleared of blood components by infusion of Krebs-buffer (in mM; NaCl 119, $NaHCO_3$ 15, KCl 4.6, $CaCl_2$ 1.5, NaH_2PO_4 1.2, $MgCl_2$ 1.27, and glucose 5.5, pH 7.4) into the left heart ventricle via a syringe needle and drained through an incision in the liver. The perfusion was continued until blood-vessels in the ear pinnae and eye had become transparent. Inner organ tissues were isolated, washed in Krebs-buffer and submerged in RNAlater® fixative (Sigma-Aldrich). For cranial tissues, the brain was exposed by removal of the top of the skull from the vertebral foramen (foramen magnum) and onward through occipital and parietal bones. The brain was removed by gently lifting up the frontal lobe with a blunt spatula, and then retracting it from the cranial cavity. Pieces of the dura mater were scraped off the inside of the skull and samples of the pia mater were peeled off the brain surface upon slight drying. Cerebral arteries (basilar, middle cerebral, anterior cerebral and circle of Willis) were dissected off the brain surface under a microscope. During removal of the brain the trigeminal ganglia were separated from it and were easily isolated from the base of the cranial cavity. Samples used for RT-PCR were rinsed in buffer and either transferred to RNAlater® or frozen on dry ice. Samples in RNAlater® were temporarily stored at 5 °C for 24 h before freezing. For *in situ* hybridization, tissues were fixated in cold formalin (10%) for 24 h and subsequently embedded in paraffin.

Table 1
List of reported vertebrate BK channel splice variants found at different splice sites

Splice site	Variation a.a.	Tissue origin/reference
A	+36	Extented N-terminal. Mouse, rat and human [11,43].
B	+66	Extented N-terminal. Rat [11,43].
mK44	+44	Human myometrium [12].
SV1	+33	Rat myometrium [21].
X1	+8	Rat cochlea [44].
IsoC	+3	Rat (AY330292.1).
X2	+31	Rat cochlea [17].
	+14*	Rat superior sagittal sinus/dura mater (this study), (BB642344)
SS1	+4	Human hippocampus [52]; human brain [10]; chick hair cells [23]; chick brain [53]; turtle cochlea [24].
	+31	Turtle cochlea [24].
	+40	Human brain [10].
	−96	Rat and human corporal tissue [22].
H	+24	Human (AK128392) [11].
SS2	+58/61	Rat chromaffin cells [43,54]; rat pituitary [55]; rat myometrium
	"STREX"	[56]; rat pancreas and brain [43]; mouse brain [57]; chick hair cells [23]; chick heart [58]; turtle and rat cochlea [17,24].
	+63	Human glioma cells [59].
	+29 "e22"	Human brain [10]; rat skeletal muscle and breast [13]; rat cochlea [60].
	+3	Human brain [10]; mouse [13].
	+4*	Mouse [13].
O	+41	Human (AB113575) [11].
SS3	+8	Human brain [10].
SS4	+27 "Slo_{27}"	Rat brain [25]; human brain [10]; chick hair cells [23]; rat cochlea [17].
SS5	+(8–61)*	Various C-terminal sequences in human, mouse, dog [43], and rat [17].

Numbers denote insert sizes in amino acid residues. Deletion of sequence is indicated by minus sign (−) and stop codon indicated by asterisk (*). The list is non-exhaustive with respect to deletions and truncated variants (see review [11] for details).

2.2. RNA extraction

Total RNA was isolated from tissues with the RNeasy® Mini Kit (QIAGEN). Samples were lysed and mechanically homogenized (Ultra-Turrax). Solid material was cleared by centrifugation and the supernatant was mixed with ethanol and added to RNeasy® mini spin columns. After successive wash and on-column DNase treatment (RNase-Free DNase Set, QIAGEN), the RNA was eluted with RNase-free water and stored at −80 °C. The integrity of the isolated RNA was tested either on a Bioanalyzer RNA chip (Agilent Technologies) or by agarose gel electrophoresis.

2.3. RT-PCR

Potential sites for BK channel transcript variation were found in literature and by alignment of rat BK nucleotide sequences in the NCBI database (Nucleotide). Primers were designed with Vector NTI targeting the rat BK α-subunit (KCNMA1) and β-subunit (KCNMB1–4) mRNA sequences retrieved from the nucleotide database (Table 2). Identical T_m values and a G/C content of 40–60% were aimed for in each primer pair (primer sequences and accession-number for template-sequences are shown in Table 2). Specificity was tested by running a combined BLAST search on both primer sequences. Primer oligo-nucleotides were synthesized by MWG Biotech (Martinsried, Germany).

Reverse-transcription (RT) PCR was performed with the QIAGEN One-Step RT PCR kit combining Omniscript/Sensiscript reverse transcriptase and HotStarTaq DNA polymerase, as described by the manufacturer, except that reaction volume was reduced from 50 to 20 or 10 μl. Primers were used in a concentration of 0.6 μM as default. For simultaneous amplification of α- and β-subunits (multiplex), the concentration of primers for the most dominating transcript was reduced to 0.3 or 0.1 μM to avoid competitive repression, and to yield similar band intensities.

Table 2
List of oligo-DNA primers and probes used in this study

PCR primers	Accession	Sense (forward) 5′–3′	Antisense (reverse) 5′–3′	bp
SV1	NM_031828	GCCAGGCAGATGGTACTCTCAA	CAAGGGCACCAATGCTGAGA	171
X1	"	TGACTGGCAGAGTCCTGGTTGTGTT	GAAGAACACGTTGAAAGCCATGTCG	152
IsoC/X2	"	TGGCTGACTGCAGCTGGATTC	CCCGGTCCTTGTGCAGAAAGT	379
SS1	"	CACAGAATATCTTTCCAGTGCCTTC	ACCTTCTTGGGTCTTAAGGTGGTT	178
SS2	"	TACTGCAAGGCCTGTCATGATGAC	TCATCAGCTTCGGGGATGTGTT	160
SS3/O	"	ACCGCCAACACTGTCACCAAA	ACTCGATGGAGCCCACAAACAC	374
SS4	"	CTCCTGGAATGGACAGATCATCACC	AAGCAAAGGGCTGTGTGAGGTACAG	189
β1	NM_019273	GTATCACACAGAAGACACTCGGGA	AAGAAGGAGAAGAGGAGGATTTGGG	200
β2	NM_176861	ACTGGGAATCACACTGCTGCG	ACTCACAAGGGACATGGACTCCTC	289
β3	XM_227040	CATCGCCATGATGGCCTCCT	TCAGAGCGCCTCCCAGCAAT	485
β4	AB050637	ATGGCGAAGCTCAGGGTGTC	ACTCGAACACCTCGCCGATC	202
ISH probes		Antisense 5′–3′		
$SS2_{+174}$	NM_031828	AACATGCTCGGCTCATTCTCTTGTAGATGGGACA		
$SS4_{+81}$	AF135265	TTGACTGATACCAAAGGCAACTTGCCCGGCTTAGC		
α-actin	NM_031004	GCATAGAGGTCCTTCCTGATGTCAATATCACACTTC		
CGRP	M11597	TAAGGTTGACCTCAAAGGGACTGCCTGGAGGCCGCAT		

PCR primers were designed to amplify transcript regions containing BK channel splice sites (SV1, X1, IsoC/X2, SS1, SS2, SS3/O and SS4), or BK β-subunits (β1–4). For *in situ* hybridization (ISH) probes complementary to BK channel splice insert sequences ($SS2_{+174}$ and $SS4_{+81}$), α-actin and CGRP were designed. Accession number for template sequences are shown together with the expected product size of insert-less variants (bp).

Total RNA samples were thawed on ice and added to the master-mix along with primers. Reverse transcription was carried out at 50 °C for 30 min followed by a 15 minute heating step to 95 °C to inactivate reverse transcription enzymes and to activate hot-start polymerase. PCR temperature cycling was executed immediately hereafter consisting of 30 to 33 one-minute cycles (denaturing at 94 °C, annealing at 56 to 64 °C and extension at 72 °C). Cycling was followed by a final extension step at 72 °C for 10 min and cooling to 5 °C. PCR reactions were performed in 0.2 ml tubes on a PTC-200 Peltier thermal cycler (MJ Research). For each PCR run, a negative control without template RNA was included (data not shown). PCR products were analyzed on 2% agarose gels with ethidium-bromide staining (35 min at 80 V) and photographed in an UV illumination box fitted with a digital camera. Gel images were black-white inverted and aligned relative to a DNA ladder for illustration purpose.

2.4. Sequencing

PCR products to be sequenced were cut out of agarose gels guided by UV-light, and purified with QIAquick® Gel Extraction Kit (QIAGEN). In case the DNA yield was insufficient for sequencing, the PCR product was re-amplified with the same primers using diluted purified product as template (1:20000). Air-dried samples were sequenced by MWG Biotech (Martinsried, Germany) using the original sense or anti-sense PCR primers as sequencing primers. Preferably, samples from two or more individual PCR reactions were sequenced in parallel.

2.5. In situ hybridization (ISH)

Oligonucleotide probes complementary to two rat BK channel splice insert sequences ($SS2_{+174}$, $SS4_{+81}$), rat α-actin and calcitonin gene related peptide (CGRP) were designed with Vector NTI and ordered from MWG Biotech (Martinsried, Germany) (Table 2). Oligonucleotides were 3′ labeled with ^{35}S-ATP in a terminal transferase reaction (Amersham) at 37 °C for 1 h. TE buffer (TRIS 10 mM, EDTA 1 mM) and yeast t-RNA (co-precipitant) was added and the product was extracted in phenol/chloroform/isoamyl alcohol (25:24:1), chloroform/isoamyl alcohol (49:1) and precipitated with NaCl and ethanol at −20 °C. The pellet was washed in 80% ethanol, air dried and re-dissolved in TE buffer with dithiothreitol (10 mM).

Paraffin embedded tissues were cut in sections (5 μm) and mounted on Super Frost Plus glass slides. A cat's whisker placed inside the lumen of arteries was used as guidance during section cutting. Slides were immersed twice in toluene (5 min) to remove paraffin and rehydrated in a series of ethanol baths (99%, 96%, 70% and 30%) (3 min). Then washed in PBS (10 min), blocked in methanol with hydrogen peroxide (1%) (20 min), and washed 3 times in PBS (5 min). To reduce background, sections were acetylated for 10 min at room temperature in saline (0.9% NaCl) with triethanolamine (0.1 M) and acetic anhydride (0.25%) (pH 8.0). Then washed in PBS (10 min), SSC (standard saline citrate) (10 min) and PBS (5 min). Finally, sections were dehydrated in ethanol baths (70%, 96%, 99%, 99% and 99%) and air-dried.

Sections were incubated overnight at 37 °C in humidity chamber with 110 μl aliquots of SSC buffer containing labeled oligonucleotide probe (1:100), formamide (45%), 1× Denhardts Solution (0.02% ficoll, polyvinylpyrrolidone and bovine serum albumin) (Sigma), fish sperm DNA (0.5 mg/ml) (Roche), yeast tRNA (0.25 mg/ml), dextran sulfate (10%) and DTT (10 mM). Negative controls were incubated with buffer without probe. Sections were then briefly washed in SSC followed by two washes in SSC with 10 mM DTT (5 min), heated to 55 °C, washed four times in SSC at 55 °C (15 min), twice in SSC (30 min) and twice in deionized water (30 min). Then air dried and dipped in a photographic emulsion coating (Amersham, Denmark) at 42 °C and stored for 3 months at 4 °C in darkness. Afterwards, the slides were developed in Amidol, fixed in thiosulfate (30%), counterstained with Mayer's heamatoxylin, dehydrated in ethanol baths (70%, 96%, 99%, 99% and 99%), washed twice in toluene (5 min) and finally mounted with cover slips.

2.6. Cloning and expression in Xenopus laevis oocytes of BK splice variants

An open reading frame of the rat BK channel was PCR amplified from the conserved third initiation codon (MDALI) to upstream of the C-terminal splice site 5 (ESRDKQstop) introducing an artificial stop-codon. The amplification was therefore independent on known N and C-terminal variations [11,16]. PCR was performed with the Pfu Turbo C_x polymerase (Stratagene) using uracil containing primers, which allow unique 3′ overhangs to be generated though uracil excision [41] (forward primer 5′*GGCTTAAU*ATGGATGCGCTCATCATC, reverse primer 5′ *GGTTTAAU*TCACTGTTTGTCGCGGGACTC). The products were cloned into the pNB1u vector optimized for expression in *Xenopus laevis* oocytes and utilizing a USER cloning cassette [41]. Positive clones were identified with PCR on single colonies and subsequent sequencing. Messenger RNA was transcribed by *in vitro* transcription (T7 polymerase, New England Biolabs) from the cloned DNA template and injected into *Xenopus* laevis oocytes. Activity of cloned BK

channels was recorded by two-electrode voltage clamping in oocytes upon stimulation with a step voltage protocol from −90 to +120 mV from a −50 mV holding potential. See Supplementary material or references for a thorough description of USER cloning [41] and heterologous expression procedures [42].

3. Results

3.1. Identification of potential splice sites

Initially, literature and databases were screened to identify known splice variants of the BK channel α-subunit. The results are summarized in Fig. 1 and Table 1. Most known sites for splice variation observed in vertebrates were included in the study (Fig. 1, Table 1) except from the site mK44 [12], site H [11] and N- or C-terminal sites A, B and SS5 [11,43]. Primer pairs were designed to span a single splice junction except from the IsoC/X2 and SS3/O primers spanning two sites (Fig. 1).

3.2 α-subunit isoforms

BK channel transcripts isolated from rat cerebral arteries, meninges, trigeminal ganglion and brain were amplified with RT-PCR and tested for the presence of splice variants by use of pairs of primers spanning reported splice sites (see Fig. 1 and Table 2), yielding alternate sized RT-PCR products when insert sequences were present. For comparison of transcript levels a number of other tissues were analyzed as well, all showing a profound BK channel expression (Fig. 2). Seven different regions of the BK transcript were amplified with RT-PCR as indicated by primer binding sites in Fig. 1, spanning nine known splice sites (SV1, X1, IsoC, X2, SS1, SS2, SS3, O and SS4).

3.3. SS2

The $SS2_{+174}$ variant containing a 174 bp insert at splice site two (SS2, Fig. 1), also known as the STREX variant (Table 1), was present in small amounts in most tissues compared to the short $SS2_0$ or "zero" form (Fig. 2B). Highest levels were found in sagittal sinus and consistent signals were found in cerebral blood vessels (Fig. 2B) while less expressed in brain and trigeminal ganglion. The identity of the longer PCR-product insert was verified by sequencing of product from the dura mater (Table 4). Also an intermediary form $SS2_{+87}$ was found in thoracic aorta and skeletal muscle (dominant form), with faint signals in common carotid aorta and in heart atrium (Fig. 2B, Table 3). Sequencing showed that this product ($SS2_{+87}$) corresponds to an alternative insert (e22, Table 1) at SS2 previously found in mice [13].

3.4. SS4

At splice site four (SS4), an additional PCR product clearly appeared ($SS4_{+81}$) in addition to the shortest transcript form (Fig. 2D). In the trigeminal ganglion and the brain, the longer variant was the dominating form, while it was present to some extent in pia mater, most arteries and in skeletal muscle (Table 3). The longer transcript found in the trigeminal ganglion, was identified (66 out of 81 base-pairs successfully sequenced) by sequencing as the Slo_{27} variant (Table 1) previously found in rat brain [8,25]. Judged by product size and lack of other known inserts, the longer transcripts found in other tissues at this site, most likely contain the Slo_{27} insert.

3.5. IsoC, X2

The IsoC site of variation was identified by alignment of database rat mRNA sequences, where the IsoC transcript (AY330292.1) contains nine additional basepairs at the site. The X2 site has been found to contain an insert sequence in rat cochlea [17].

The primer pair spanning the sites IsoC and X2, yielded a single PCR product in most tissues except from a longer variant found in superior sagittal sinus. Sequencing of this band showed a 92 bp insert at the X2 site ($X2_{+92*}$, Table 4) and no insert at IsoC. The odd number of nucleotides induces a shift in reading frame and a stop codon within the insert. Consequently, the resulting protein becomes truncated 14 amino acids downstream of the X2 splice sites (between the S6 and S7 segment) (Table 4). The insert was isolated from two different animals and sequenced six times from individual PCR reactions with univocal results.

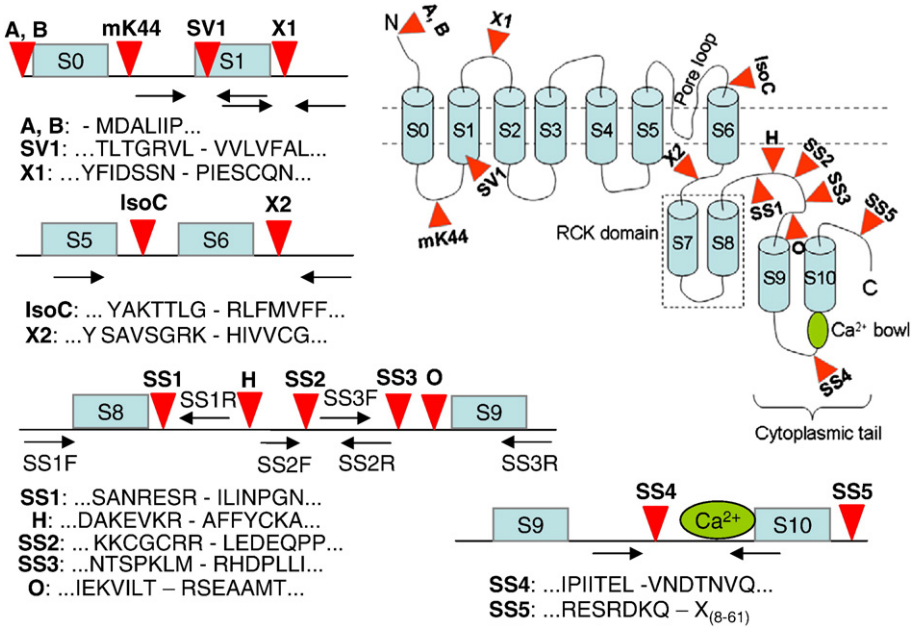

Fig. 1. Conceptual overview of previously reported sites for transcript variation in the vertebrate BK channel α-subunit. (A) Splice sites indicated by hyphens in the rat BK channel peptide sequences, and binding sites for primers, used in the present study, indicated by arrows (not to scale). (B) Splice sites indicated by triangles relative to the ion channel topology (trans-membrane and intracellular segments S0–S10).

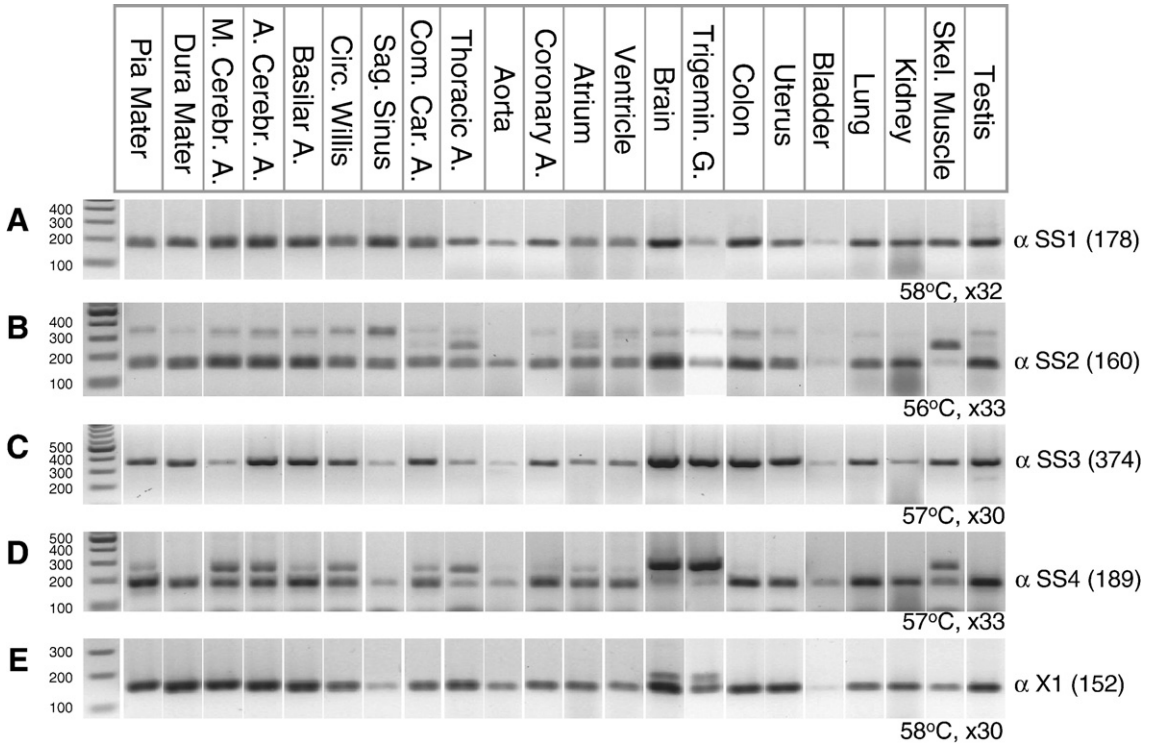

Fig. 2. RT-PCR results showing tissue distribution of transcript variants of rat BK α-subunits, at four different splice sites (SS1–4), using primer pairs amplifying regions of the transcript including these splice sites. RT-PCR products were run on 2% agarose gels and gel images aligned relative to a DNA weight marker (numbers indicate base-pairs). Annealing temperature and PCR cycle number are shown on the right together with the name of the primer-pair and the expected product-size of the shortest transcript variant. (A&C) No apparent variation was found at splice site one (SS1) and three (SS3) where the shortest form was present only. (B) At splice site two (SS2), two longer forms appeared ($SS2_{+87}$, $SS2_{+174}$) which were identified as two previously described 29 and 58 amino acid inserts ("e22" and "STREX"). The 29 amino acid insert was mostly found in thoracic aorta and in skeletal muscle, while the 58 amino acid insert was widely expressed except from in aorta, kidney and skeletal muscle. (D) At splice site four (SS4) a longer form ($SS4_{+81}$) identified as a 27 amino acid insert (Slo_{27}) was found to be dominant in brain and in trigeminal ganglion with some expression in cerebral arteries, arteries and skeletal muscle as well. (E) At splice site X1, a 24 bp longer transcript ($X1_{+24}$) was found in the brain and trigeminal ganglion exclusively.

3.6. X1

At the site X1, located in the extra-cellular loop between S1 and S2 in the protein [44], a 24 bp longer transcript variant ($X1_{+24}$) was found in the brain and trigeminal ganglion exclusively (Fig. 2E) representing an eight amino acid insert previously found in rat cochlea [44]. The splice site is located between the second and third nucleotide in the asparagine codon upstream of the insert (see Fig. 1). Asparagine is therefore changed into lysine (see Table 3).

Table 3
Summary of α-subunit transcript variation and β-subunit expression observed with RT-PCR

RT-PCR signals	α-subunit splice variants					β-subunits			
Tissue	$X1_{+24}$	$X2_{+92*}$	$SS2_{+87}$	$SS2_{+174}$	$SS4_{+81}$	β1	β2	β3	β4
Pia mater	−	−	−	+	+	+	+	−	+
Dura mater	−	−	−	(+)	−	+	+	−	+
Middle cerebral artery	−	−	−	+	+	+	+	−	+
Anterior cerebral artery	−	−	−	+	+	+	+	(+)	+
Basilar artery	−	−	−	+	(+)	+	+	−	+
Arterial circle of willis	−	−	−	+	+	+	+	−	+
Superior sagittal sinus	−	+	−	+	−	(+)	−	−	(+)
Common carotid artery	−	−	(+)	(+)	+	+	−	−	+
Thoracic aorta	−	−	+	(+)	+	+	−	−	+
Aorta	−	−	−	−	−	(+)	(+)	−	+
Coronary artery	−	−	−	(+)	−	++	(+)	−	+
Heart atrium	−	−	(+)	(+)	(+)	+	++	−	++
Heart ventricle	−	−	−	(+)	−	++	++	(+)	++
Brain	+	−	−	+	++	(+)	++	−	++
Trigeminal ganglion	+	−	−	+	++	(+)	++	−	++
Colon	−	−	−	+	−	+	+	−	+
Uterus	−	−	−	(+)	−	+	+	−	(+)
Bladder	−	−	−	(+)	−	+	+	−	+
Lung	−	−	−	(+)	−	++	++	−	++
Kidney	−	−	−	−	−	+	(+)	−	++
Skeletal muscle	−	−	++	−	+	−	+	−	−
Testis	−	−	−	+	−	+	++	+	++

−: No apparent signal. (+): Weak positive signal. +: Clear positive signal. ++: Strong positive signal.

3.7. SV1, SS1, SS3, O

At splice sites SS1, SS3/O and SV1, a single PCR product appeared (Figs. 2A, C and 3B) with a size corresponding to the shortest form of the transcript at these sites (178, 374 and 171 bp respectively). The SV1 product was co-amplified with the β2 or β3 subunits (Fig. 3B, C). At SV1, a 99 bp insert has been described in rat [21,45], at SS3 a 24 bp insert have been observed in humans [10] and at O, a 123 bp insert may be found in humans [11]. None of these were found in the tissues

Table 4
Translated sequences of the inserts identified at splice sites X1, X2, SS2 and SS4 (insert sequences in italics)

Insert	Sequence
$X1_{+24}$	-SSK-*SRTADSLI*-PIE-TCA AA G AGC CGC ACA GCA GAC TCT TTG AT C CCA ATA
$X2_{+92*}$	-AVSGRK-*QCLPATCPKLLLSS***stop** -GCG GTT AGT GGA AGA AAG CAA TGT TTG CCC GCT ACG TGC CCG AAA TTG CTG CTC TCA TCC **TGA** ATC GGA ATA AAT TCG GCG GGA CTT TTA ACA AAC ATG GAG GCA GAA AG CAC ATT-
$SS2_{+87}$ "e2"	-RRL-*KVEARARYHKDPFMHKNATPNSQHMPKPV*-EDE-
$SS2_{+174}$ "STREX"	-RRP-*KMSIYKRMRRACCFDCGRSERDCSCMSGRVRGNVDTLERTFPLSSVSVNDCSTSFRAF*-EDE-
$SS4_{+81}$ "Slo_{27}"	-TEL-*AKPGKLPLVSVNQEKNSGTHILMITEL*-VND-

The nucleotide sequence is shown for the inserts found at X1 and X2 (underscored), which at the X2 site induces a shift in reading frame and a stop-codon (**TGA**).

investigated as indicated by PCR product size. At SS1, inserts of 4, 31 or 40 amino acids have been described in humans and in turtles [10,24] (Table 1). The two longer forms were not observed as seen on gels, while the shortest form, potentially giving an increase in PCR product size of merely 12 bps, cannot be easily excluded. Sequencing of the product from middle cerebral artery showed that this was of the shortest form (RESR-ILIN). The uniformity of product size and equivalence with the product from middle cerebral artery, suggests that the shortest form is dominating through-out the tissues investigated.

3.8. β-subunits β1–4

The β1, β2 and the β4 subunits were expressed in cerebral vessels, meninges, brain and trigeminal ganglion (Fig. 3, Table 3). The β1 showed a somewhat higher expression in blood vessels compared with brain and trigeminal ganglion (Fig. 3A), while the β2 and β4 showed higher expression in brain, trigeminal ganglion and heart compared to the cerebral arteries and meninges (Fig. 3B and D). Co-amplification (multiplex) of the α-subunit transcript served as internal positive controls in these experiments.

An unexpected longer band appeared at 300–400 bp with the β2 primers (Fig. 3B). Sequencing identified this band as non-specific amplification of RAB3 GTPase-activating protein. The β3 subunit was found expressed in testis only, except from very weak signals in the anterior cerebral artery and heart ventricle (Fig. 3C, Table 3). A shorter product at app. 250 bp, and a long product at app. 1000 bp, was found as well in pia mater, brain and testis respectively (Fig. 3C). This could be due to alternative splicing of the β3 subunits as the primer pair spanned two splice junctions, yet the known splice variation of β3, is generated at a splice-site outside (upstream) of the region covered by the primer-pair [27]. The α-subunit primers used (SV1) generated no extra bands of such sizes when tested alone (data not shown).

3.9. Localization of $SS2_{+174}$ and $SS4_{+81}$, by in situ hybridization

As RNA was isolated from crude tissue homogenates, the splice variants $SS2_{+174}$ and $SS4_{+81}$ identified in cerebral vessels and trigeminal ganglion with RT-PCR could originate from different cell types within the tissue sample. To investigate the localization of the splice variants, tissue sections of basilar and middle cerebral artery and the trigeminal ganglion were hybridized with oligo-nucleotide probes complementary to the splice variant sequences (Table 2). Positive signals were observed within the arterial smooth muscle layer (Fig. 4, TM) in basilar (BA) and middle cerebral arteries (MCA) for both probes and consistent signals were seen in the trigeminal ganglion (TGG) as well. Signal levels within tissue sections were markedly higher than background levels on slides outside tissues and in negative control tissues (Fig. 4). As positive controls, the basilar and middle cerebral artery was hybridized with a probe against α-actin and trigeminal ganglion with a probe against CGRP yielding strong signals (Fig. 4). The finding of hybridization signals within smooth muscle indicates that splice variants, identified with RT-PCR, are indeed expressed in smooth muscle cells.

3.10. Activity of splice variants $X1_{+24}$ and $X2_{+92}$. Expression in Xenopus oocytes

The splice variants of the vertebrate BK channel containing inserts at splice X1 and X2 have until now only been identified on transcript level by RT-PCR [17,44]. To investigate if these transcript variants results in functional channels we cloned the coding sequences from cDNA prepared from the middle cerebral artery or dura mater. An open reading frame was chosen starting at the third known initiation codon (MDALI) and ending an artificial stop codon (ESRDKQstop) introduced upstream of the C-terminal splice site (SS5). By this, amplification was independent on potential N and C-terminal

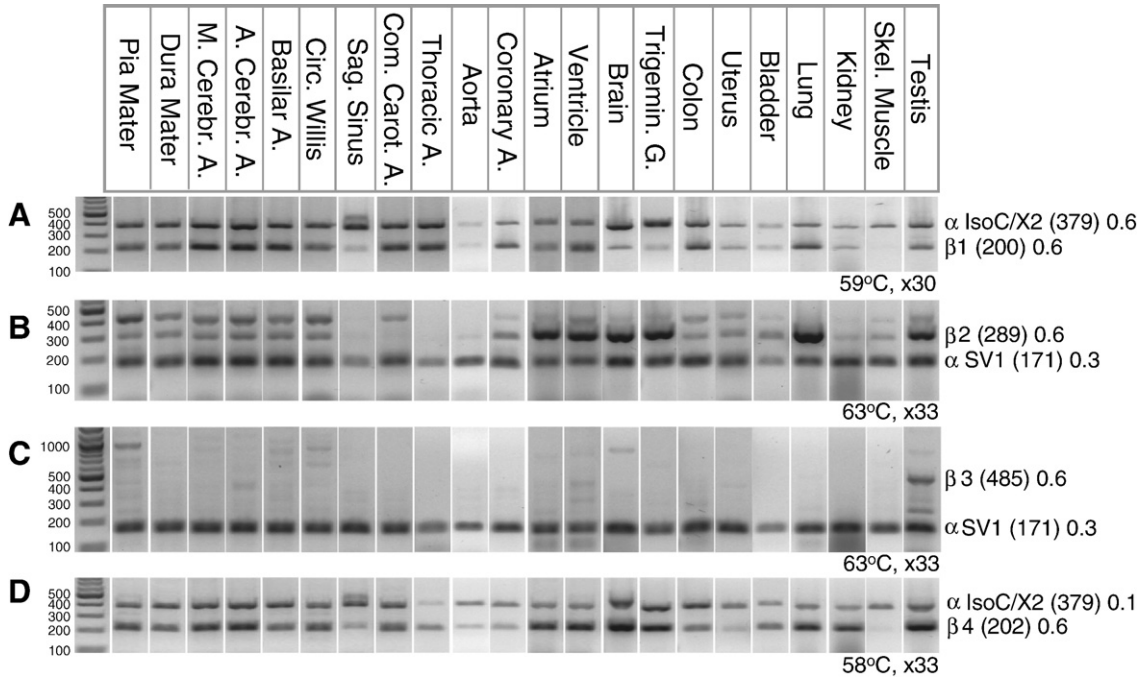

Fig. 3. RT-PCR results showing expression of rat BK β-subunits (β1–4), co-amplified with BK α-transcript (multiplex PCR). Two different regions of the α-subunit were co-amplified as internal controls (IsoC/X2 or SV1), and to investigate the splice-compositions at these sites. RT-PCR products were run on 2% agarose gels and gel images aligned relative to a DNA weight marker (numbers indicate base-pairs). Annealing temperature and PCR cycle number are shown on the right with name of the primer-pair, primer concentrations (in μM) and the expected base-pair product-size. No additional longer bands appeared with the primers spanning the splice sites IsoC/X2 (A) or SV1 (C), except from in sagittal sinus, where a novel 92 bp insert was found at the X2 site ($X2_{+92*}$: Band between 450 and 500 bp in A and D). β1 and β4 were found in most tissues except from skeletal muscle (A, D), and β2 was predominantly expressed in heart, nerve tissue, lung and testis, while absent in sagittal sinus, common carotid artery and thoracic aorta (bands at 300 bp in B). The longer bands in B at app. 400 bp were a non-specific amplification by the β2 primers of rat RAB3 GTPase-activating protein. The β3 subunit was found expressed in testis only with some weak additional bands of longer size seen in pia mater and brain (C).

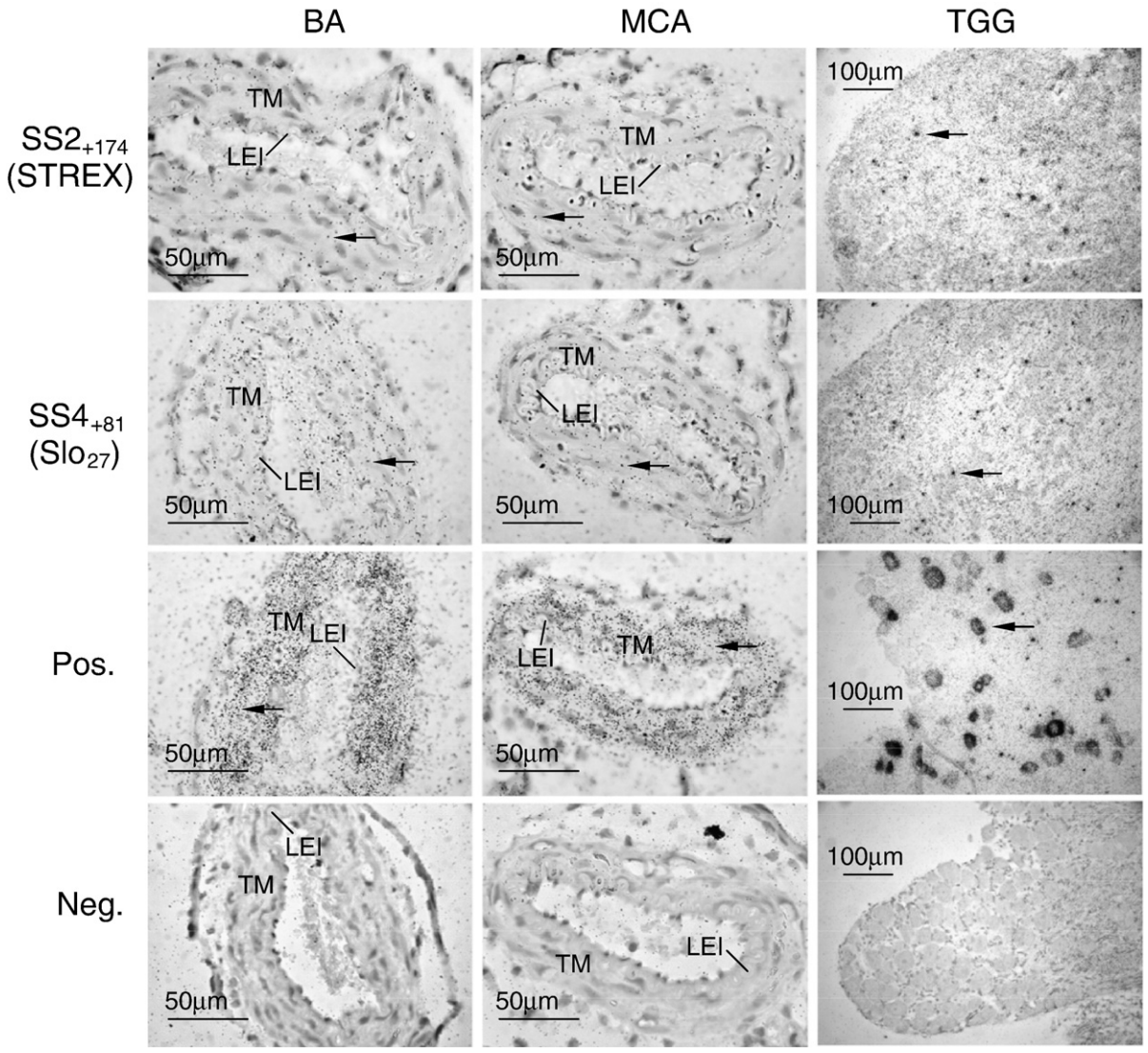

Fig. 4. *In situ* hybridization showing expression of two BK channel splice variants in basilar artery (BA), middle cerebral artery (MCA) and trigeminal ganglion (TGG). Tissues sections were hybridized with ^{35}S-labeled oligonucleotide probes complementary to the splice inserts $SS2_{+174}$ (STREX) and $SS4_{+81}$ (Slo_{27}). Positive signals (black dots exemplified by arrows) were found within the smooth muscle layer (TM) in BA and MCA and widespread in TGG for both probes. As positive controls (Pos.), BA and MCA were hybridized against α-actin, and TGG against calcitonin gene-related peptide. Negative controls (Neg.) were incubated with buffer without probe. Magnification: BA and MCA ×63; TGG ×20. TM: tunica media. LEI: lamina elastica interna.

transcript variation [11,43]. The amplified sequences were cloned into a vector optimized for expression in *Xenopus* oocytes. Clones were obtained containing the $X1_{+24}$ in combination with the $SS4_{+81}$ insert isolated from middle cerebral artery, or the $X2_{+92}$ insert cloned from the dura mater. The $X1_{+24}$ variant without $SS4_{+81}$ seems to be rare as no clones were obtained containing this combination (out of 30–40 clones). Upon injection of *in vitro* transcribed mRNA into *Xenopus* oocytes, we found expression of the $X1_{+24}$ variant (combined with $SS4_{+81}$) (Fig. 5A,B), which showed a faster activation than the Zero variant (observed as higher relative current level after capacitance peak) (Fig. 5B) and a slight change in the voltage–current relation (Fig. 5C). The variant containing only the $SS4_{+81}$ did not show the change in activation speed (not shown). The $X2_{+92}$ variant yielded no detectable macroscopic current in oocytes indicating that the premature stop codon and truncation after segment S6 hampers the formation of functional channels. However, when co-expressed with the Zero variant it appeared that $X2_{+92}$ induces a slowing of activation (lowered current level after capacitance peak, Fig. 5B) and as well a change in the voltage–current relation (Fig. 5C) suggesting that the $X2_{+92}$ forms heteromeric complexes with the Zero subunits. Thus, the $X1_{+24}$ (with $SS4_{+81}$) variant is a functional channel while the $X2_{+92}$ is a silent but possibly modulatory BK variant.

4. Discussion

4.1. Multiple isoforms, possible co-expressions, novel truncated isoform

The finding of different splice variants ($X1_{+24}$, $X2_{+92}$, $SS2_{+174}$ and $SS4_{+81}$) expressed in cerebral arteries, meninges, trigeminal ganglion and brain in combination with the β-subunits β1, β2 and β4 reveals a multitude of possible constellations of functional BK channels in these tissues. It is known that the STREX ($SS2_{+174}$) and Zero ($SS2_0$) splice variants respond similarly or invertedly to co-expression with the β4 subunit at low versus high calcium concentrations [6], The interaction of STREX with β1 and β2, which were found in cerebral vessels, meninges and trigeminal ganglion, has not been investigated. Other α- and β-subunit interactions that are functionally relevant are those between the $SS4_{+81}$ (Slo_{27}) or $X1_{+24}$ variants and β1, β2 and β4 subunits, which are expressed in brain and trigeminal ganglion. The Slo_{27} variant is known to show a slightly altered Ca^{2+} response, but its

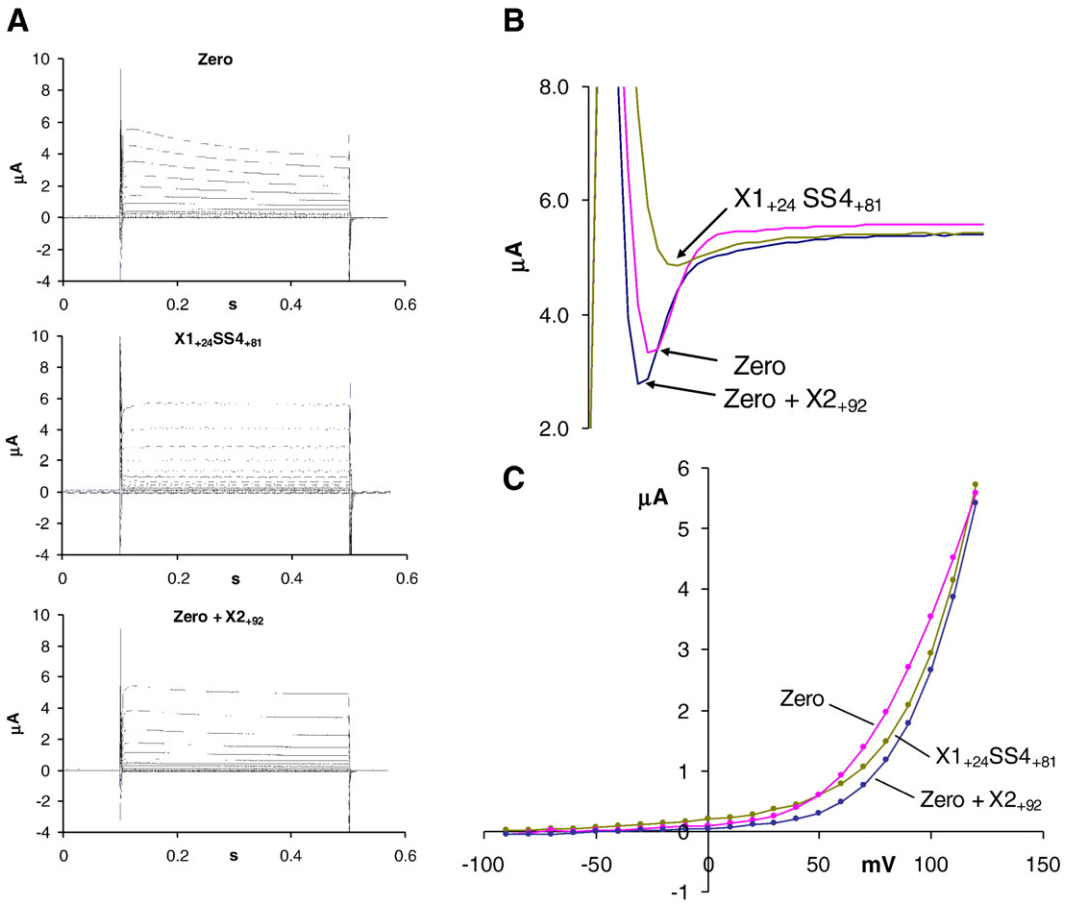

Fig. 5. Expression of BK splice variants in *Xenopus* oocytes. (A) Current recordings from oocytes expressing the variant containing the $X1_{+24}$ insert ($X1_{+24}SS4_{+81}$), the insert-less variant (Zero), or insert-less co-expressed with the $X2_{+92}$ variant (Zero +$X2_{+92}$). Membrane potential was clamped in steps to −90 to +120 mV for 400 ms from a holding potential of −50 mV. (B) First 20 ms of upper current traces shown in A (+120 mV). The $X1_{+24}$-containing variant channels apparently show faster current activation than the Zero variant as indicated by current level after the capacitance peak (arrowheads). The $X2_{+92}$ variant yields no detectable current when expressed alone (not shown) but appears to affect the properties of the Zero variant when co-expressed. (C) Peak current levels from traces shown in A, plotted against step voltage, suggesting differences in the voltage-activation among the Zero, $X1_{+24}SS4_{+81}$ and Zero +$X2_{+92}$ variants.

interaction with β-subunits is unknown [25]. Not least, the $X2_{+92}$ variant found in the superior sagittal sinus or dura mater may exert a modulatory effect on other BK variants (Zero) while being a non-functional ion channel itself. Obviously, this variant needs to be studied further. The $X2_{+92}$ insert sequence translates into 14 amino acids followed by a stop codon (Table 3) resulting in a truncated protein isoform lacking the intracellular segments S7 to S10. The sequence can be found in an EST clone isolated from mouse retina (100% similarity) (accession BB642344) [11]. Previous studies with artificial truncated constructs has shown that such channel may show little expression due to mis-trafficking [46] or impaired tetramerization [47]. Yet, it has been argued that minute but significant activity, can be observed in *Xenopus* oocytes [48].

An insert very similar to $X2_{+92}$ was previously found in rat cochlea [17] (reported as peptide sequence only), which contains no stop codon due to an additional single nucleotide in the first codon in the insert (93 instead of 92 basepairs). It would be a remarkable finding if splice inserts differ among tissues on single nucleotide level.

The co-existence of multiple splice variants in most tissues raises the question regarding dominant-recessive behavior as the BK channel protein consists of a tetramer of α-subunits. The STREX splice variant ($SS2_{+174}$), which was found in several tissues, is known to exert a dominant effect on BK channel characteristics when co-expressed with the Zero (insert-less) isoform, inducing a reversal of the ion channel response to cAMP (becomes inhibitory) [49]. It appears that if just one of four α-subunits contains the STREX insert, the whole channel phenotype is changed.

4.2. Splicing pattern of the BK α-subunit

According to the predicted structure of the rat BK channel gene (KCNMA1) on chromosome 15 (Celera assembly, NCBI Mapview, Celera Genomics WGS), all splice sites identified so far represents exon–exon boundaries except from the IsoC variant. The listed potential IsoC variant (Table 1), which was not observed in this study and found in a sequence database only, may be of minor importance. The insert sequences $X1_{+24}$, $X2_{+92}$, $SS2_{+87}$ and $SS4_{+81}$ are all retrieved from introns in between the splice site-flanking exons and constitutes only a small part of the intron. This indicates that the splice variants are in fact generated by specific splicing of mRNA and not due to biasing amplification of genomic DNA or intermediately spliced pre-mRNA. The $SS2_{+87}$ insert sequence is placed in an intron upstream of the $SS2_{+174}$ (STREX) insert which is recognized by the Celera assembly as an exon. The shortest form at splice site two is thus generated by removal of the intron sequence and the adjacent downstream exon. In contrast, the Reference assembly (RGSC, Human Genome Sequencing Center, NCBI Mapview) of the rat BK gene shows a conflicting exon structure with little congruence between known splice sites, origin of splice variant insert sequences and predicted exon–exon boundaries.

4.3. Differential expression of functional forms and pharmacotherapy

Pharmacological modulation of BK channels in artery smooth muscle cells is a potential strategy for treating hypertension or vascular

headache, yet selective targeting is a concern due to the widespread distribution of the channel. The functional diversity based on splice variants and β-subunit co-expression is interesting due to the large number of potential functional forms, which may have different physiological roles and a differentiated tissue expression pattern.

Regarding pharmacology, the β-subunits are interesting in that β1 and β2 reduces charybdotoxin sensitivity [50,51] and is necessary for the activation of BK by DHS-1 [14,51], while β4 renders the channel insensitive to iberiotoxin and charybdotoxin [15]. On the other hand, very little is known about the pharmacology of the α-subunit splice variants. A study has shown a difference in the response to amphipaths (membrane modulating agents) relating to the presence of the STREX exon [18].

4.4. Potential implication for headache

When comparing the cerebral arteries and the trigeminal ganglion, both tissues contain BK channels, but they vary greatly in their content of the $SS4_{+81}$ and $X1_{+24}$ variants which were found mostly in the nerve. The β2 and β4 showed a somewhat stronger expression in the nerve, while β1 was more expressed in the cerebral arteries. These findings may be of interest for the development of pharmacological strategies against migraine headache, which is a neuro-vascular disorder involving cerebral vasodilation and activation of nociceptive trigeminal nerves. Selective targeting of BK channels in either of these tissues may be of value as treatment. An inhibition of smooth muscle BK channels would precipitate increased vascular constriction, which is known to give acute relief during migraine, while modulation of BK channels in the nerve may affect excitability.

Although the impact of the newly identified $X2_{+92}$ BK variant found in dural tissue is not known, this variant may be of particular therapeutic interest as it showed a highly restricted expression.

Acknowledgements

We thank Dr. H.H. Nour-Eldin, Dr. M.H. Nørholm and as well the Plant Biochemistry Laboratory, Faculty of Life Sciences, University of Copenhagen, for their invaluable assistance and for kindly providing the USER-optimized pNB1u cloning vector.

The study was supported by the Lundbeck Foundation as part of the Lundbeck Foundation Center for Neurovascular Signalling (LUCENS), The Danish Medical Research Council, and the Region of Copenhagen.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2008.10.001.

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