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Molecular heterogeneity of large-conductance calcium-activated potassium channels in canine intracardiac ganglia

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Large conductance calcium-activated potassium (BK) channels are widely expressed in the nervous system. We have recently shown that principal neurons from canine intracardiac ganglia (ICG) express a paxilline- and TEA-sensitive BK current, which increases neuronal excitability. In the present work, we further explore the molecular constituents of the BK current in canine ICG. We found that the β1 and β4 regulatory subunits are expressed in ICG. Single channel voltage-dependence at different calcium concentrations suggested that association of the BKα with a particular β subunit was not enough to explain the channel activity in this tissue. Indeed, we detected the presence of several splice variants of the BKα subunit. In conclusion, BK channels in canine ICG may result from the arrangement of different BKα splice variants, plus accessory β subunits. The particular combinations expressed in canine IC neurons likely rule the excitatory role of BK current in this tissue.

Keywords: BK channels, alternative splicing, β subunits, intracardiac neurons, autonomic

Abbreviations: BK channel, large conductance calcium-activated potassium channel; IGC, intracardiac ganglia; I_{BK}, BK current; SS, splice site

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Introduction

Large conductance calcium-activated potassium (BK) channels are expressed in a wide variety of cells within the nervous system, including intracardiac (IC) neurons of different species,[1-3] where they contribute to numerous cellular functions, including action potential repolarization and afterhyperpolarization. Diverse properties have been reported for I_{BK} in different tissues, in different cells in a given tissue, in different regions of a single cell, and in the same cell under different stimuli.[4-7] This behavior is accepted to be associated to heterogeneity in the molecular composition of BK channels, which can be achieved, among other mechanisms, by differential expression of BKβ subunits and BKα splice variants. BK channels are usually composed by four BKα pore-forming subunits and auxiliary β subunits. To date, four different BKβ subunits have been ascertained (encoded by KCNMB1 to 4), which display tissue-specific distribution and differentially modulate I_{BK}.[8-13] On the contrary, BKα subunits are encoded by a single gene, KCNMA1, from which a wide diversity of variants can be originated through alternative splicing.[14] At least 13 splice sites have been described for KCNMA1.[6,15] Some of them have been identified in many species and tissues, and thus are considered to be ubiquitous and conserved across species (**Table 1**). However, the particular molecular composition of BK channels and how this identity modulates I_{BK} in different tissues still remains greatly unknown.

We have recently shown that principal neurons from canine intracardiac ganglia (ICG) express a conspicuous BK current

Table 1. Description of the analyzed splice sites

Splice site	Variant (aa)	Named
5'	+66	MANG
	+36	MSS
	0	MDALI
SS1	+4	+4
SS2	+58/61	STREX
	+29	e22
	+3	+3
SS3	+8	+8
SS4	+27	Slo27
3'	+8	VYR
	+61	DEC
	+8	ERL

The table shows the more common variants described for each of the splice sites (SS) analyzed, the number of aminoacids (aa) added to originate each variant and the name by which they are usually known. The Zero variant, which corresponds to the absence of insertion, has been described for SS1 to SS4. The VYR and ERL splice variants have the same number of aa but different sequences.

(I_{BK}), which plays a role in their excitability. We demonstrated that these channels do not present intrinsic inactivation and that the decline of the BK whole cell current was most likely dependent on the intracellular calcium concentration. In the present work, we aimed to further explore the molecular constituents of BK channels in canine IC neurons. Our results suggest that the presence of several splice variants of the BKα subunit, modulated by β4 and/or β1 subunits, may explain the voltage- and calcium-dependence of the BK channel observed in this native preparation.

Results

BK channel β1 and β4 regulatory subunits are expressed in canine ICG. It is well known that BK channel β subunits modulate channel's biophysical properties, including activation voltage-dependence, activation and deactivation kinetics, and inactivation.[16] In particular, the β2 and β3 regulatory subunits are known to be responsible for I_{BK} inactivation.[13] Our recent studies showed that the inactivation of I_{BK} from canine ICG is not due to intrinsic inactivation of BK channels. To provide molecular evidence to this conclusion, we sought to determine which of the four described BK channel β subunits are expressed in IC neurons and would thus confer I_{BK} its particular characteristics. RT-PCR studies evidenced that β1 and β4 subunits were clearly expressed in canine ICG, while a faint expression was observed for β2 and β3 subunits in this tissue (**Fig. 1A**). To further confirm our results, we performed immunofluorescence staining of partially dissociated ICG. IC neurons expressed the β4 subunit (**Fig. 1Ba**), and all those cells that expressed this subunit also expressed the BK channel α subunit (**Fig. 1Bb**). On the contrary, staining for β2 and β3 subunits was only observed in a low percentage of neurons (**Fig. 1Bc and 1Bd**, respectively). These data are in agreement with the lack of single channel inactivation observed in our previous article. In addition, they strongly suggest that β4 subunit (and β1 to a lesser extent) is a likely candidate for modulating I_{BK} in these cells.

Calcium- and voltage-dependence of single BK channels in IC neurons. Our previous whole cell current studies could not provide detailed information on BK channel calcium- and voltage-dependence. To cover this aspect, we studied the single channel voltage-dependence at different calcium concentrations in excised patch experiments from our native preparation. **Figure 2** depicts examples of single channel recordings at increasing calcium concentrations and the open probability (Po) *vs.* voltage relationship for the calcium concentrations assayed. The analysis of voltage- and calcium-dependence led to $V_{1/2}$ values of –35.7 ± 11.1 mV, 29.8 ± 2.2 mV and 70.0 ± 15.1 mV; at 10, 3 and 0.3 μM Ca^{2+}, respectively. These results deviate from similar analysis using heterologous expression of BKα and either β1 or β4 subunits.[8,9,17,18] These deviations could be due to the possibility that in our native preparation, more than one β subunit modulates the BKα subunit. Alternatively, or in addition, a BKα isoform different from that used in the heterologous expression studies could be expressed in canine IC neurons.

A combination of BK channel α-subunit variants is expressed in canine ICG. Multiple splice sites (SS) have been described for the BK channel α subunit.[6] Some of the resulting splice variants have been reported to modulate BK channel electrophysiological properties. In the present study, we have focused on six particular SS (**Table 1**) to determine which of the associated splice variants were expressed in canine ICG. A summary of the results obtained is shown in **Table 2**. Of note, PCR amplification of the SS1 region originated two amplicons (**Fig. 3A**, lane 2). Their molecular weights were compatible with the SS1-insertless variant (lower band) and the variant bearing an insertion of 4aa at the SS1 site (upper band). Sequencing of both PCR products confirmed their anticipated identities. When the SS2 region was amplified in ICG, we observed a band compatible with the shorter (58aa) STREX splice variant (**Fig. 3A**, lane 3, upper band). Additionally, two bands with lower mobility were also observed in this tissue. Their molecular weight was compatible with the Zero and +4 variants. Their identities were confirmed by sequencing. PCR analysis of the SS3 region and sequencing of the obtained band evidenced the expression of the SS3-Zero variant. Regarding the SS4 region, a band compatible with the slo27 variant was detected (**Fig. 3A**, lane 5, middle band). Two additional bands were also observed. Sequencing of these 3 amplicons confirmed the expression of the slo27 variant and the SS4-Zero variant (lower band). The upper band corresponded to a heteroduplex of Zero and slo27, a phenomenon previously reported by Lai and McCobb respect to SS2.[19]

We also determined the splicing variants at the N- and C-terminus of the BK channel α subunit in canine ICG. Sequencing of the PCR products obtained upon amplification of the N-terminal region evidenced the presence of transcripts bearing the MSS translational start site. As this start site is in frame with the 3' start site beginning MDALI, we cannot ensure the presence of transcripts starting with the latter start site in ICG. Regarding the C-terminus, we were able to detect the presence of the 3 most common variants at this site, ending VYR, DEC and ERL. A schematic

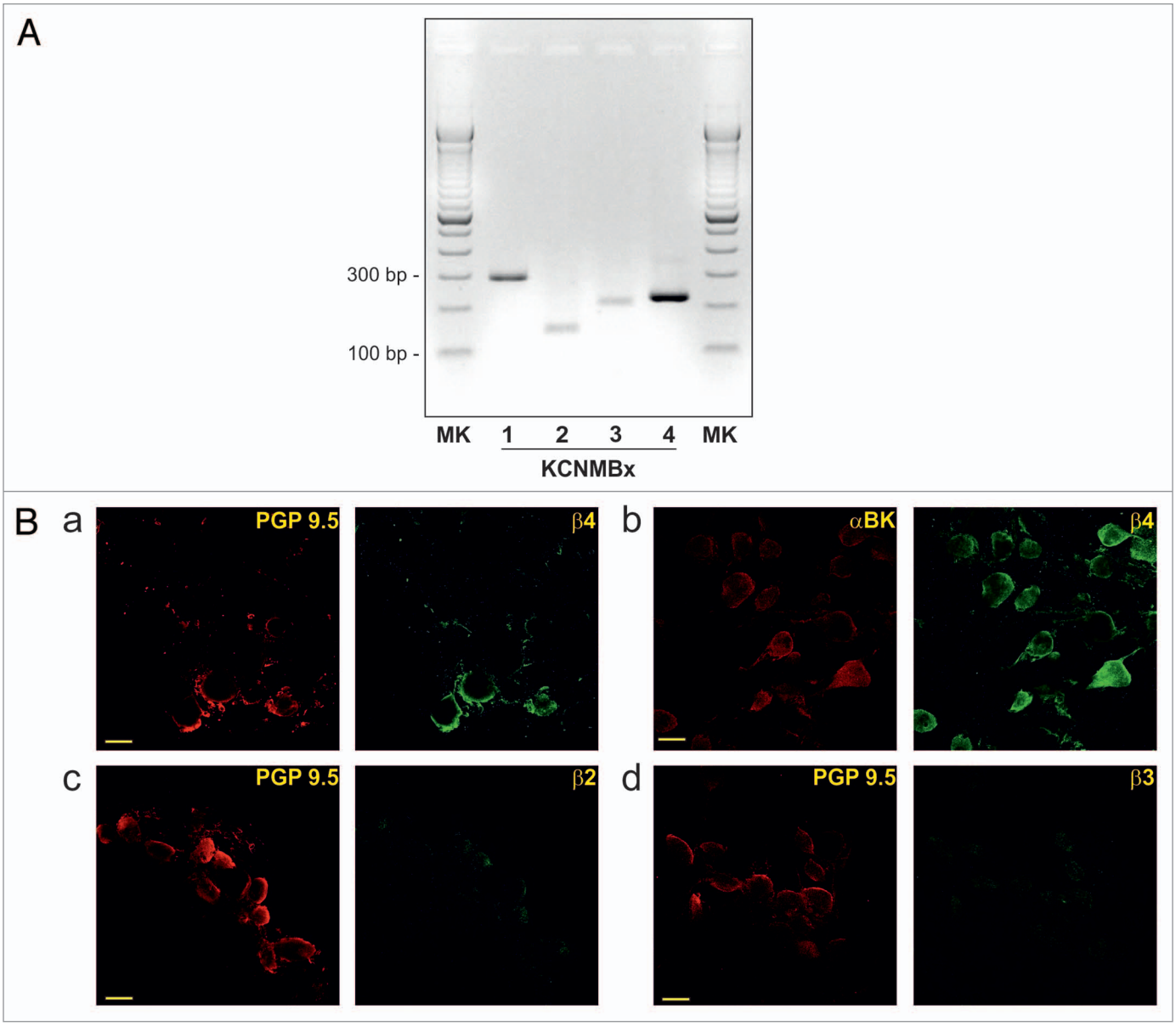

Figure 1. BK channel β1 and β4 regulatory subunits are expressed in canine ICG. (**A**) Picture depicts the results of RT-PCRs performed as described in Methods using specific primers for BK channel β regulatory subunits. PCR products were resolved in 2% agarose gels. The bands correspond to the expected amplicons (KCNMB1, 278 bp; KCNMB2, 137 bp; KCNMB3, 201 bp; KCNMB4, 216 bp). A molecular marker (MK) was run side by side with the samples, and the corresponding base pair (bp) sizes of two of its bands are indicated on the left. n = 1–3. (**B**) Pictures show representative images of individual confocal sections obtained from partially dissociated ganglia stained with the standard immunohistochemical technique described in Methods. The antibody used is indicated at the top right corner of each image, and the scale bar was 40 μm. Percentage of positive cells with respect to PGP 9.5 was 47.6 for β2 (n = 63 and 30 cells, PGP 9.5 and β2, respectively), 6.3 for β3 (n = 32 and 2 cells, PGP 9.5 and β3, respectively) and 129.2 for β4 (n = 24 and 31 cells, PGP 9.5 and β2, respectively). Percentage of β4 positive cells with respect to BKα was 116 (n = 263 and 305 cells, BKα and β4, respectively).

representation of the genomic organization leading to these 3' ends is presented in **Figure 3B**. Note that only one of the canine previously described assemblies (**Fig. 3B**, d) was present in ICG. The other two assemblies identified (**Fig. 3B**, b and c) had not been formerly reported in canine tissue.

Discussion

In the present work, we have explored the molecular identity of BK channels in canine ICG. Our results point at two different levels of the BK channel macromolecular complexity: (1) The principal β subunit expressed in ICG is β4. This subunit has been reported to be mainly expressed in brain and has complex Ca^{2+}-dependent effects.[9] However, the $V_{1/2}$ values obtained at different Ca^{2+} concentrations in canine IC neurons do not completely match those previously reported in similar conditions in heterologous expression systems. (2) Multiple BKα splice

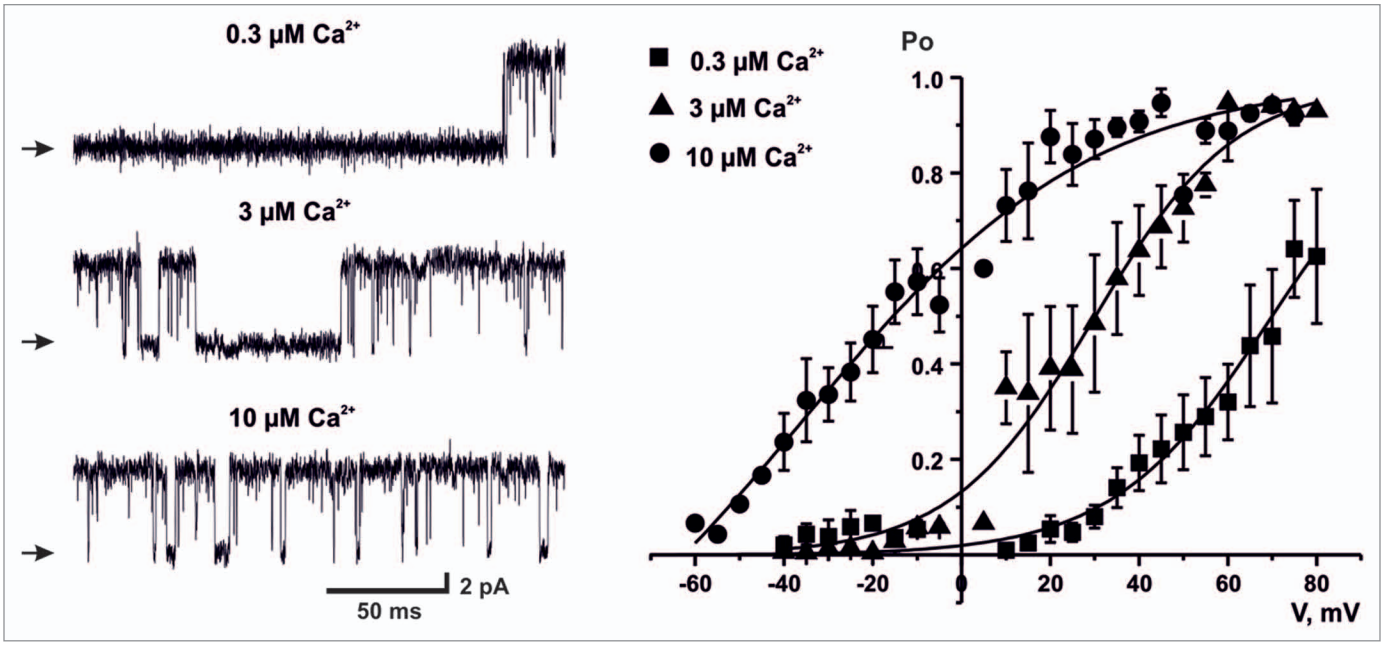

Figure 2. Calcium- and voltage-dependence of single BK channels in IC neurons. *Left*, representative single channel traces elicited by voltage steps to +40 mV at the indicated calcium concentrations. Arrows indicate the closed state of the channel. *Right*, plot of the open probability (Po) as a function of voltage at increasing calcium concentrations. Data are displayed as the mean ± SE (n = 1–10). $V_{1/2}$ are −35.7 ± 11.1 mV, 29.8 ± 2.2 mV and 70.0 ± 15.1 mV (10, 3 and 0.3 μM Ca^{2+}, respectively).

variants are expressed in canine ICG. We have characterized, for the first time, the BKα splice variants expressed in this native preparation. However, we cannot address to which extent each of them will effectively contribute to BK current in IC neurons. It is notable that the specific splice variants' combination identified in canine ICG is somewhat different from that detected in canine brain (**Table 2**). This observation supports the accepted idea of the occurrence of BKα tissue-specific hallmarks given by a particular set of splice variants, which confer unique tissue-specific identity to I_{BK}. Interestingly, we have identified the presence of STREX at SS2 and of slo27 at SS4. These insertions result in the inclusion of 58 and 27 extra aminoacids, respectively, and thus are suggested to cause bigger effects on I_{BK} than the shorter insertions that we identified in canine tissues. In fact, several studies have been published that show major effects of the independent inclusion of either variant on I_{BK}.[4,5,18,20] Also, some attempts have been made to study the effects of the simultaneous presence of multiple variants.[21,22] Again, our $V_{1/2}$ values obtained in canine IC neurons deviate from published results. Our observations suggest that the MSS N-terminus may be expressed in canine ICG, and that the original transcripts will carry one or more insertions. Although at present we cannot define the exact combination of BKα splice variants in ICG, the anticipated portrait is quite different from the expression vectors used in heterologous transfection experiments. Thus, the mismatching between the results presented here and those published elsewhere may not be unexpected. Moreover, one has to bear in mind that the heterologous expression studies are often performed with a single variant, which excludes the possibility of heterotetramers, a channel arrangement likely to occur in our native preparation. Additionally, up to four BKβ can be assembled with the BKα tetramer to render functional channels. Both the number of β subunits and its identity are unfixed and influence BK channel properties.[23] Little is known about the interplay among alternatively spliced α subunit variants and β subunits. Petrik and Brenner performed an elegant characterization of the effects of β4 on STREX BK channels.[18] These studies were performed using a mouse zero BKα subunit, a framework that does not resemble much the BKα in our native preparation. Also, Erxleben and collaborators studied the effects of STREX inclusion and β1 modulation on I_{BK}.[20] Although their assays were performed in the BKα N-terminus MSS background, our results present differences with their observations in terms of calcium- and voltage-dependence, probably due to the predominant expression of β4 in IC neurons. Comparisons among native and heterologous expression preparations are difficult to interpret. Still, our molecular results suggest that BK channels expressed in canine ICG are probably composed of BKα bearing more than one splice variant plus β4 and/or β1. In this line, we recently demonstrated that 100 nM Iberiotoxin blocked BK channels in IC neurons. However, this block occurred at times between 3 and 14 min (data not shown). This behavior perhaps reflects the heterogeneous subunit arrangements of BK channels in ICG, in agreement with the molecular evidence presented here. This heterogeneous expression anticipated in canine ICG for both α and β subunits adds further complexity to the biophysical response of the channel, and warrants further investigation. For example, experiments showing inhibition

of BK current by Protein Kinase A, or an apparent sensitivity to inhibition by oxidation would be indicative of the predominance of BKα-STREX channels[20] in IC neurons.

In summary, in the present addendum we provide molecular evidence for the presence, in canine ICG, of BK channel components known to affect channel function. Although our study does not cover all the possible splice variants reported for this type of channel, it is reasonable to postulate that the splice variants we identified collectively contribute to the functional effect of the BK channel on membrane excitability observed in this native tissue.

Materials and Methods

Canine IC ganglia and neuron isolation. Parasympathetic ganglia and neurons from the atrial ganglionated plexi of the dog were dissociated as previously described.[3] Briefly, canine hearts were obtained, and the fat pads on the ventral, lateral and dorsal aspects of the atrium were quickly removed and placed in a normal Krebs (NK) solution in ice. Individual ganglia were removed from the fat pads and cleaned under a dissection scope. The ganglia were either flash-frozen in liquid N_2 for subsequent RNA extraction or dissociated with 0.1% collagenase-elastase and 0.2% trypsin. Individual cells were obtained by triturating the remaining tissue with a Pasteur pipette. Cells were resuspended in Dulbeco's modified eagle media (DMEM), supplemented with 1% fetal bovine serum, 100 μg/ml penicillin-streptomycin, 2 mM glutamax, 10 μg/ml S7 and 0.11 g/ml piruvic acid; and plated on collagen coated bottom glass Petri dishes (MatTek Coorp., Ashland, MA). Cells were placed overnight in a CO_2 incubator at 37°C. To obtain partially dissociated ganglia, the trituration step was minimized.

Immunohistochemistry. Partially dissociated ganglia were obtained as described above. Ganglia were mounted on glass slides and stained with a standard immunohistochemical technique. Briefly, after fixation with a 4% paraformaldehyde-0.2% picric acid solution and permeabilization with 0.2% Triton X-100, tissue was incubated overnight at 4°C with 1:100/200 dilutions of primary rabbit polyclonal antibodies against the BK channel α subunit or the neuronal marker PGP9.5 and a monoclonal antibody against the BK channel β subunits 2–4 (β2–4). Immunostaining of the β1 subunit was not performed due to the lack of availability of a reliable antibody. Tissue was then incubated with a 1:1,000 dilution of anti-rabbit Alexa-594 and/or anti-mouse Alexa-488-conjugated secondary antibodies for 2 h at room temperature. Specificity of polyclonal primary antibodies was assessed by preincubation with a control peptide antigen. Ganglia were mounted using Pro-Long antifade mounting media and visualized under a Fluoview Olympus laser scanning confocal microscope (40 oil immersion objective) equipped with argon and He/Ne lasers. Optical sections were taken through the entire volume of the cell with the XY frame set to 512 × 512 pixels and the Z-axis was changed in 0.5 to 1 micron increments. Sections were scanned sequentially to avoid bleeding artifacts. Images shown are individual confocal sections.

Electrophysiological recordings. Current measurements were obtained using the patch-clamp technique in excised patch configuration. Solutions were applied with a gravity flow system (speed 1–3 ml/min) to a 150 μl bath chamber. Electrode shanks were coated with dental wax and tips fire polished to a tip diameter of aprox. 1 μm. Electrode resistances were of 8–10 MΩ. Data acquisition and analysis: Experiments were controlled with an Axopatch 200A amplifier and pClamp 9.0/Digidata 1440A acquisition system (Molecular Devices). All experiments were acquired online for later analyses with Clampfit (Molecular Devices). Voltage activation curves for single-channel experiments were fitted with a two state Boltzmann equation of following form: $Po = Po_{max} + (Po_{min} - Po_{max})/1 + \exp[(V - V_{1/2})/dV]$, where Po_{max} and Po_{min} are the maximum and minimum Po asymptotes, respectively, V is the holding potential, $V_{1/2}$ is the voltage for half maximal activation, and dV represents the slope factor. Single channel data analysis was performed using Clampfit as previously described in Scornik et al.[24] Briefly, Po was determined by either all-point amplitude histogram or event detection with 50% amplitude criteria. All recordings were performed at 20–22°C.

Table 2. Splice variants identified in canine ICG and brain

Splice site	Splice variant	ICG	Brain
5'	MANG	–	–
	MSS	+	+
	MDALI	+ (?)	+ (?)
SS1	ZERO	+	–
	+4	+	+
SS2	ZERO	+	+
	+3	+	+
	STREX	+	–
	e22	–	–
SS3	ZERO	+	+
	+8	–	–
SS4	ZERO	+	+
	Slo27	+	+
3'	VYR	+	+
	DEC	+	+
	ERL	+	+

The table displays the results of sequencing the RT-PCR products for canine ICG and brain, as described in methods. The presence (+) or the absence (–) of each splice variant is indicated. Question marks are used to state that we cannot ensure the presence of transcripts starting with the MDALI start site (the three start sites are in the same reading frame, and cDNA sequencing does not allow discrimination). The STREX variant identified in ICG corresponds to the short isoform published (+58aa).

Solutions. Recordings were done in symmetrical solutions of the following composition (in mM): 140 KCl, 10 HEPES, 2 $MgCl_2$. Calcium concentration was titrated to 0.3, 3 or 10 μM with glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid or N-hydroxyethyl-ethylenediaminetriacetic acid and measured with a calcium electrode (Kwik-Tip, World Precision Instruments).

Drugs. Collagenase type I, trypsin and trypsin inhibitor (Worthtington). D-MEM, NGF 7S, FBS and glutamax (Invitrogen Corp.). All other drugs were obtained from Sigma.

Antibodies. Rabbit polyclonal anti-PGP9.5 was from EMD Chemical, mouse monoclonal anti β2, β3 and β4 were from

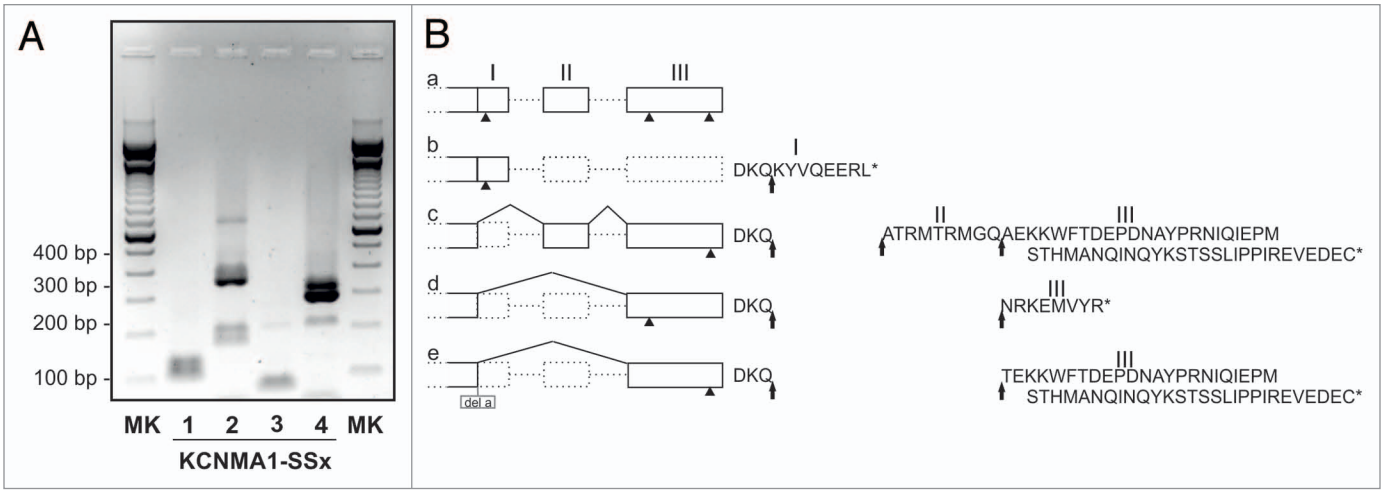

Figure 3. Multiple BK channel splice variants are expressed in canine ICG. (**A**) Picture depicts the results of RT-PCRs performed as described in Methods using specific primers for some BK channel splice sites (SS). A molecular marker (MK) and the PCR products were resolved in 2% agarose gels. Expected amplicons, in bp: SS1: 107 (without insert, –) and 119 (including insert, +); SS2: 172 (–), 346 (+, STREX-short isoform), 355 (+, STREX-long isoform), 259 (+, e22), and 181 (+, +3); SS3: 79 (–) and 103 (+); SS4: 208 (–) and 289 (+). n = 1–4. (**B**) Schematic representation of the genomic organization that leads to the alternative C-terminus identified in canine ICG. The uppermost scheme (a) illustrates the genomic organization of KCNMA1 C-terminus (not in actual scale). The schemes below represent the assemblies identified in ICG (b, c and d) and those previously reported for canine tissue (d and e). Boxes represent exons and are labeled with roman numbers, and dashed lines represent introns. Arrowheads mark the approximate localization of stop codons. Exons that are included in a given splice variant are connected by solid lines. Exons not included are dashed. In e, the box labeled "del a" indicates a deletion of an adenine at the indicated splice site. The resulting aminoacid sequences are shown on the right. The arrows indicate splice sites and asterisks mark stop codons. Note that only one of the assemblies previously described (d) was present in ICG. The other two assemblies identified (b and c) had not been formerly reported in canine tissue.

NeuroMab and secondary Alexa conjugated antibodies were from Invitrogen.

RT-PCR. Total RNA was extracted from ICG using the RNeasy Micro Kit (Qiagen) and from brain using the RNeasy Fibrous tissue Mini Kit (Qiagen), following the manufacturer's recommendations. DNase treatment of the RNA samples was performed using the DNA-free Kit from Ambion. Reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as per manufacturer's directions. Primers used are available upon request. The PCR products were analyzed by electrophoresis on 2% agarose gels.

Sequencing. DNA was extracted from bands in the agarose gels using the PCR clean-up Gel extraction kit (Macherey-Nagel GmbH and Co. KG). All DNAs were directly sequenced in both directions (Big Dye Terminator v3.1 cycle sequencing kit and 3130XL Genetic Analyzer, both from Applied Biosystems) and the obtained sequences were compared with their respective reference sequences (KCNMA1 from *Canis lupus familiaris* when available or alternatively from *Mus musculus;* GenBank gene IDs 403984 and 16531, respectively. *Canis lupus familiaris* KCNMB1 to B4 GenBank gene IDs: 403983, 488179, 488085 and 474447, respectively).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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