Experimental mapping of the canine KCNJ2 and KCNJ12 gene structures and functional analysis of the canine KIR2.2 ion channel

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INTRODUCTION

In the mammalian heart, the resting membrane potential of cardiomyocytes is set and stabilized by the inward rectifier potassium current (I_K1; Dhamoon and Jalife, 2005). In addition, I_K1 contributes to outward potassium current during the last phase of action potential repolarization. The main molecular determinants of cardiac I_K1 are the KIR2.1 and KIR2.2 proteins expressed from the KCNJ2 and KCNJ12 genes respectively (De Boer et al., 2010a). Defective inward rectifier current may lead, amongst other features, to lethal cardiac arrhythmias in mice and man such as ventricular arrhythmias and atrial fibrillation (Anumonwo and Lopatin, 2010). ECG recording from neonatal mice homozygous for a KCNJ2 null mutation showed lengthening of RR, PR, and QT intervals and QRS broadening. Furthermore, isolated neonatal cardiomyocytes displayed action potential lengthening and ectopic activity (Zaritsky et al., 2001). In contrast, however, null mutation of KCNJ12 generated no cardiac abnormalities (Zaritsky et al., 2001). KCNJ2 loss of function mutation associated with Andersen–Tawil syndrome 1 regularly, but not in each case, displayed long repolarization times (LQT) and biventricular tachycardias. On the other hand, KCNJ2 gain of function mutations have been associated with short QT (Priori et al., 2005), and atrial fibrillation (Xia et al., 2005). Atrial fibrillation was also observed in a mouse model overexpressing KCNJ2 (Li et al., 2004). Finally, KCNJ2 mutations have been associated with Catecholaminergic Polymorphic Ventricular Tachycardia (Vega et al., 2009). Affected KIR2.1 and KIR2.2 functioning may be the result of amino-acid substitutions (Tristani-Firouzi and Etheridge, 2010), direct channel block (Rodriguez-Menchaca et al., 2008; De Boer et al., 2010b), or changes in expression regulation (Yang et al., 2007).

The dog (Canis lupus familiaris, Cf) with chronic complete atrial–ventricular block (cAVB) is a well established model for drug-induced arrhythmia (Thomsen et al., 2006a). The model acquires its sensitivity and specificity from bradycardia associated volume overload and subsequent cardiac remodeling (Thomsen et al., 2007). The latter process translates into modified contractile, structural, and electrical function (Oros et al., 2008). As a result of electrical remodeling, the so-called repolarization reserve (Roden, 1998; Michael et al., 2009) is diminished as evidenced by increased action potential duration and moreover by increased beat-to-beat variation of repolarization (Thomsen et al., 2006b).

For some species, the molecular basis underlying cardiac electrophysiology, e.g., ion channel, gap junction, and transporter genes and proteins, is well described. However, a functional integration of the individual components to explain a number of electrophysiological phenomena lags behind, despite many decades of research (Coronel, 2010). When considering the dog, the amount of electrophysiological studies by far outnumber those on the molecular make-up of the underlying ion currents. Nevertheless, the canine genes coding for the electrophysiological building blocks are becoming deciphered rapidly; genome project information can be found at http://www.broadinstitute.org/mammals/dog. The publication of the dog genome has provided an important additional tool that

For many model organisms traditionally in use for cardiac electrophysiological studies, characterization of ion channel genes is lacking. We focused here on two genes encoding the inward rectifier current, KCNJ2 and KCNJ12, in the dog heart. A combination of RTPCR, 5'-RACE, and 3'-RACE demonstrated the status of KCNJ2 as a two exon gene. The complete open reading frame (ORF) was located on the second exon. One transcription initiation site was mapped. Four differential transcription termination sites were found downstream of two consensus polyadenylation signals. The canine KCNJ2 gene was found to consist of three exons, with its ORF located on the third exon. One transcription initiation and one termination site were found. No alternative splicing was observed in right ventricle or brain cortex. The gene structure of canine KCNJ2 and KCNJ12 was conserved amongst other vertebrates, while current GenBank gene annotation was determined as incomplete. In silico translation of KCNJ12 revealed a non-conserved glycine rich stretch located near the carboxy-terminus of the KIR2.2 protein. However, no differences were observed when comparing dog with human KIR2.2 protein upon ectopic expression in COS-7 or HEK293 cells with respect to subcellular localization or electrophysiological properties.

Keywords: inward rectifier current, Canis familiaris, KCNJ2, KCNJ12, KIR2.1, KIR2.2, genes
sequencing method (BigDye®, Applied Biosystems, Nieuwerkerk a.d. IJssel, The Netherlands) on a 3730 Genetic Analyzer (Applied Biosystems). Sequences of primers used for RACE are depicted in Table 1. All PCR products were sequenced by the Dye-terminator method (Applied Biosystems). Sequences of primers used in this study, sequences are given in 5′–3′ orientation.

| KCNJ2 PRIMERS | KCNJ2#1se AGCTGGGTCTTGGGGATCTGG | KCNJ2#2se TTGCAGACGCGACTGAGGCC | KCNJ2#3as CATCCACCAGCCGGCAATGT | KCNJ2#4as ACTTGACGCTGACGCGGAGC | KCNJ2#5se TGGAGACTGACGGCGTAAT | KCNJ2#6se AGCAGTGAGCTTCCCGCGCC |
|----------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| KCNJ12 PRIMERS | KCNJ12#1se GCTGGGCTGACAGACGCTTG | KCNJ12#2se GTCGATGAGGTCTGAGGACG | KCNJ12#3as GACGTGAGCAGCGTAATGG | KCNJ12#4as GTTCCGGAGCGGAGGAGCC | KCNJ12#5se GGTGTTCGACGAGGCGCTTG | KCNJ12#6as GATCCACCAGAAGGTCGAGCC |
| SUPPLIED GENERACER™ PRIMERS USED | GeneRacer™ 5′ RNA primer GACUGAGGACGGGACACUGACAG | GeneRacer™ Oligo dT primer GACUGAGGAGACGAGACGAC | GeneRacer™ Oligo dT primer GACUGAGGAGACGAGACGAC | GeneRacer™ 5′ primer GACUGAGGAGACGAGACGAC | GeneRacer™ 5′ nested primer GACUGAGGAGACGAGACGAC |
|----------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| CLONING AND EXPRESSION ANALYSIS OF CfKCNJ12 | Complete CfKCNJ12 coding sequence was amplified as above using primers KCNJ12#10se and KCNJ12#12as as first and KCNJ12#11se and KCNJ12#13as as nested primers, and cloned in pGEM-T-Easy followed by subcloning in pcDNA3.1 using EcoRI. COS-7 cells were transfected with pcDNA–CfKCNJ12 or pcDNA–HsKCNJ12 (human KCNJ12) construct using Lipofectamine 2000 (Invitrogen) according to the manufacturers recommendations. Twenty-four hours post-transfection, cells were harvested in lysis buffer [20 mM HEPES, pH 7.6, 125 mM NaCl, 10% (v/v) glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM Dithiothreitol, 1% (v/v) Triton X-100]. Subsequently, 20 μg protein lysate was separated by 10% SDS-PAGE and blotted onto nitrocellulose membrane. Blots were blocked with 5% (v/v) fresh chicken egg yolk in TBST [20 mM Tris–Cl, pH 8.0, 150 mM NaCl, 0.05% (v/v) Tween-20] for 1 h at room temperature. KIR2.2 protein was detected using KIR2.1/2 (cat. no. sc-18708, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Donkey-anti-Goat horseradish peroxidase antibody (cat. no. sc-18708, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Standard ECL procedure was used for final detection (Santa Cruz Biotechnology). Localization studies were performed following transient transfection in HEK293 cells as described previously (Jansen et al., 2008).

| Patch clamp electrophysiology | HEK293T cells cultured on glass coverslips were co-transfected with 0.5 μg pcDNA–KIR2.2 and 0.5 μg pEGFP1 expression |
A. canine KCNJ2 gene structure

B. transcription initiation site

C. splice sites

D. poly A signal and transcript termination

FIGURE 1 | Canine KCNJ2 genomic structure. (A) Representation of the KCNJ2 gene indicating lengths of first and second exons, intron and the open reading frame (ORF). Relative position of KCNJ2 specific primers used for intron/exon mapping, 5' and 3' RACE are indicated by numbered box arrows. (B) Transcription initiation site (bold and underlined) with upstream (lower case) and downstream exon 1 (upper case) sequences. (C) Sequence surrounding exon 1–intron and intron–exon 2 splicing sites of dog, mouse, and chicken KCNJ2 genes. Hatched bar indicates intron region. Exon 1 and exon 2 sequences are indicated in upper case lettering, intron sequence in lower case. Consensus splicing sequences are indicated above the sites (underlined). (D) Four alternative 3' transcript termini (bold). Exon 2 sequences are indicated in upper case, downstream genomic sequences in lower case. Consensus termination signals are indicated above the sequences (underlined).

constructs as described above. Patch clamp measurements were done using an AxoPatch 200B amplifier controlled by pClamp 9 software (Molecular devices, Sunnyvale, CA, USA). Voltage clamp measurements of whole cell \( I_{K1} \) were performed by applying 1 s test pulses ranging between \(-120\) and \(+40\) mV, in 10 mV increments, from a holding potential of \(-40\) mV, and with series resistance compensation of at least 70%. Steady state current at the end of the pulse was normalized to cell capacitance and plotted versus test potential (corrected for liquid junction potential). Patch pipettes were made with a Sutter P-2000 puller (Sutter Instrument, Novato, CA, USA) and had resistances of 2–3 MΩ. Extracellular solution for whole cell \( I_{K1} \) measurements contained (in mmol/L): NaCl 140, KCl 5.4, CaCl\(_2\) 1, MgCl\(_2\) 1, glucose 6, NaHCO\(_3\) 17.5, HEPES 15, pH 7.4/NaOH. Pipette solution contained potassium gluconate 125, KCl 10, HEPES 5, EGTA 5, MgCl\(_2\) 2, CaCl\(_2\) 0.6, Na\(_2\)ATP 4, pH 7.20/KOH.

To evaluate the blocking effect of polyamine on KIR2.2 channel currents from both human and dog KCNJ12 genes, we analyzed chord conductance values from \( I–V \) relationship by inside-out patch clamp experiments as previously described (Ishihara and...
FIGURE 2 | Canine KCNJ12 genomic structure. (A) Representation of the KCNJ12 gene indicating lengths of first, second, and third exons, first and second intron, and the open reading frame (ORF). Relative position of KCNJ12 specific primers used for intron/exon mapping, 5′ and 3′ RACE are indicated by numbered box arrows 1–9. Relative positions of KCNJ12 specific primers for cloning the ORF are indicated by numbered box arrows 10–13. (B) Transcription initiation site (bold and underlined) with upstream (lower case) and downstream exon 1 (upper case) sequences. (C) Sequence surrounding exon 1–intron 1, intron 1–exon 2, exon 2–intron 2, and intron 2–exon 3 splicing sites of dog KCNJ12. Open bars indicate intron regions, hatched bar indicates exon 2 region. Exon sequences are indicated in upper case lettering, intron sequences in lower case. Consensus splicing sequences are indicated above the sites (underlined). (D) 3′ transcript terminus (bold). Exon 3 sequence is indicated in upper case, downstream genomic sequence in lower case. Consensus termination signals are indicated above the sequences (underlined).

Ehara, 2004). The pipette (extracellular) solution contained (mM): 145 KCl, 1 CaCl₂, and 5 HEPES (pH 7.4 with KOH). The bath (intracellular) solution contained (mM) 125 KCl, 4 EDTA (2K), 7.2 K₂HPO₄, and 2.8 KH₂PO₄ (pH 7.2 with KOH). Spermine was used in the concentration of 0.1, 1, and 10 μM. Currents were recorded from inside-out membrane patches at room temperature (22°C; De Boer et al., 2010b). The holding potential was set to 0 mV, and test pulses were applied between −60 and +90 mV in 5 mV steps with −40 mV hyperpolarizing pre-pulse. Current amplitude was measured at 2 s after the onset of the test pulse. For this evaluation, we used the equation \[ G = I / (V - V_{rev}) \]. In our study, the values of \( V_{rev} \) measured by a ramp protocol (40 mV/s) were always near 0 mV (between ±2 mV). Conductance values for each test voltage were normalized by the maximum value. Furthermore, we evaluated the time-dependent decay of KIR2.2 channel current just after the onset of test pulse. Single exponential fitting of the current was performed by Microcal Origin (ver.8, Microcal Software, Northampton, MA, USA).
RESULTS AND DISCUSSION

MAPPING OF THE KCNJ2 GENE

To experimentally map the canine KCNJ2 gene (Figure 1A), we first retrieved the putative KCNJ2 genomic sequence from chromosome 9 using the canine genome project data (accession number NC_006591). Homology comparison (Vector NTI suite 8, operating on Clustal W algorithm) with the mapped murine KCNJ2 (Redell and Tempel, 1998) indicated putative regions for a first and second exon. Based on these, PCR primers were designed for RT-PCR, 5′- and 3′-RACE (Table 1). RNA was isolated from canine ventricular cardiomyocytes. RT-PCR using primer KCNJ2#1se and KCNJ2#3as designed at the putative exon 1 and exon 2 sequences respectively revealed one single product of approximately 590 bp. Exon/intron boundaries were determined by sequencing of the products and aligned against the genomic DNA sequences. Boundaries were found to conform to consensus exon/intron and intron/exon sites, and high levels of homology were found at the splice sites when compared with mouse and chicken (Figure 1C). No additional exons were found in this intron region. Differences with respect to the genome reference sequence were observed.

The transcription initiation site (TIS) was mapped by 5′-RACE using primer KCNJ2#3as and the nested primer KCNJ2#4as originated from exon 2. Only a single TIS was obtained in this way (Figure 1B). RT-PCR using primer KCNJ2#5se, located directly upstream of the TIS and KCNJ2#4as yielded no product. In the mouse KCNJ2 gene, four TISs were determined in a stretch of approximately 590 bp. Exon/intron boundaries were determined by inter-exon RT-PCR. Exon/intron boundaries were determined by sequencing PCR products and compare these to genomic DNA.

Next, sense (KCNJ2#1se) and antisense (KCNJ2#6as) primers were derived from chromosome 5 (accession number NC_006587). By sequencing PCR products and compare these to genomic DNA, we first retrieved the putative exon 3 and the nested primer KCNJ2#8as located in exon 3, an additional exon of 406 bps was discovered (Figure 1B). Upon sequencing, the latter was found as being identical to the ventricular product. No evidence was found for alternative splicing, either by 5′-RACE neither by inter-exon RT-PCR. Exon/intron boundaries were determined by sequencing PCR products and compare these to genomic DNA (Figure 2C). As for KCNJ2, boundaries were found to conform to consensus exon/intron and intron/exon sites. 5′-RACE using primer KCNJ12#7as and primer KCNJ12#8as identified one TIS (Figure 2B). No putative TATA box was found upstream of the initiation site. 3′-RACE using KCNJ12#3se and two different nested primers (KCNJ12#4se and KCNJ12#5se), all located in exon 3, identified only one termination site (Figure 2D). No genuine termination signal was identified in this gene. Finally, no differences with respect to the genome reference sequence were observed.

To our knowledge, no KCNJ2 gene structure from other species has been published. Indirectly however, Ryan et al. (2010) indicate that the genomic structure of KCNJ2 is very similar with that of KCNJ16. They indicate that KCNJ12 is a three exon gene...
with strong homology to KCNJ16 except for exon 1, which is longer in KCNJ12 than in KCNJ16. KCNJ12 intron lengths were not reported. When comparing our dog KCNJ12 sequence with that of human, strong similarity is seen for the length of exon 2
and 3 (Table 2). Furthermore, dog KCNJ12 is a three exon gene too, with its entire ORF located on Exon 3. Unfortunately, due to the lack of more specific sequence information of the human form, no reliable comparisons can be made further. Finally, the gene structure as presented here is different from the one that is annotated in NCBI Entrez Gene (GeneID 403760, accessed at July 19, 2011), that annotates the coding region on five separate exons. Furthermore, no 5′ UTR is presented, while the 3′ UTR is ∼22 bp in length.

**ELECTROPHYSIOLOGICAL ANALYSIS OF KIR2.2**

Translating genomic KCNJ12 sequence revealed a KIR2.2 protein containing a peculiar stretch of glycine residues near the carboxy-terminus. Apparently this was not a sequencing artifact as RT-PCR using primers located in exon 1 and 3 confirmed the finding in mRNA derived from left ventricle and cortex (Figure 3A). Although the glycine rich region was not identified in other species (Figure 3B), it is found in a less-conserved region of the protein. Glycine rich amino-acid stretches are found in many other proteins, however no function has been addressed. To investigate a potential functional consequence, the complete channel was cloned. Expression in COS-7 cells revealed a protein with an apparent Mw of ∼50 kDa (Figure 4A). Upon transfection in HEK293 cells, strong expression of canine KIR2.2 was seen at the plasma membrane, comparable with that of human KIR2.2 (Figure 4B; Kaibara et al., 2002). When expressed in HEK293T cells, patch clamp analysis demonstrated the presence of a typical barium sensitive inward rectifying current (Figure 4C) which was indistinguishable from human KIR2.2 carried IK1 (Figure 4D).

KIR2.x channels show dose-dependent changes of blocking profile by spermine (SPM) and spermidine (Ishihara and Yan, 2007). To evaluate the electrophysiological properties of canine KIR2.2 into more detail, voltage-dependent blockade of KIR2.2 channel current by different concentrations of SPM was analyzed by determining the V–G relation (Figures 5A,B). The representative current traces measured by inside-out patch clamp show that outward IK1 currents mediated by human and dog KCNJ12 are blocked in the presence of 0.1–10 μM of SPM in a dose-dependent manner. The V–G curve shifted to more negative voltages upon increasing the SPM concentration. No significant difference in V–G relations at either SPM concentration was observed (Figure 5B). In the presence of physiological levels of SPM (1 and 10 μM; Yan et al., 2005), human and dog KIR2.2 channels displayed similar time-dependent decay at +80 mV | 1 μM: 10 μM.

**FIGURE 5** Spermine-induced block of human (Hs) and dog (Cf) KIR2.2 channels. (A) Representative traces of KIR2.2 channel currents in the absence and presence of 0.1, 1, and 10 μM of spermine. Inset: pulse protocol, currents were elicited by 10 mV step test pulses from −60 to +90 mV. (B) Comparison of V–G relationships of human and dog KIR2.2. The scale of G/Gmax is logarithmic. Test pulses were applied in 5 mV steps from −60 to +90 mV, and G was calculated for each voltage. (C) Comparison of time constant of KIR2.2 channel currents at +80 mV in the presence of 1 and 10 μM SPM.
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