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Identification and functional characterization of zebrafish K_{2P}10.1 (TREK2) two-pore-domain K^+ channels

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ABSTRACT

Two-pore-domain potassium (K_{2P}) channels mediate K^+ background currents that stabilize the resting membrane potential and contribute to repolarization of action potentials in excitable cells. The functional significance of K_{2P} currents in cardiac electrophysiology remains poorly understood. *Danio rerio* (zebrafish) may be utilized to elucidate the role of cardiac K_{2P} channels in vivo. The aim of this work was to identify and functionally characterize a zebrafish otholog of the human K_{2P}10.1 channel. K_{2P}10.1 orthologs in the *D. rerio* genome were identified by database analysis, and the full zK_{2P}10.1 coding sequence was amplified from zebrafish cDNA. Human and zebrafish K_{2P}10.1 proteins share 61% identity. High degrees of conservation were observed in protein domains relevant for structural integrity and regulation. K_{2P}10.1 channels were heterologously expressed in *Xenopus* oocytes, and currents were recorded using two-electrode voltage clamp electrophysiology. Human and zebrafish channels mediated K^+ selective background currents leading to membrane hyperpolarization. Arachidonic acid, an activator of hK_{2P}10.1, induced robust activation of zK_{2P}10.1. Activity of both channels was reduced by protein kinase C. Similar to its human counterpart, zK_{2P}10.1 was inhibited by the antiarrhythmic drug amiodarone. In summary, zebrafish harbor K_{2P}10.1 two-pore-domain K^+ channels that exhibit structural and functional properties largely similar to human K_{2P}10.1. We conclude that the zebrafish represents a valid model to study K_{2P}10.1 function *in vivo*.

1. Introduction

Two-pore-domain potassium (K_{2P}) channels mediate K^+ background (or "leak") currents that stabilize the negative resting membrane potential (RMP) and contribute to repolarization of action potentials [1]. Tightly regulated leak currents control cellular excitability in the heart and other tissues [1–3]. Owing to their ubiquitous expression, K_{2P} channels are involved in multiple physiological functions including cardioprotection, neuronal plasticity, muscle contraction, and hormone secretion [4]. Repolarization of cardiomyocytes is mediated by different potassium channels [5]. The distinct cardiac plateau current (I_{KP}) is an instantaneously activating, non-inactivating K^+ current that modulates amplitude and duration of the cardiac action potential [6]. Based on common distribution and pharmacological and biophysical properties K_{2P} channels have been suggested as molecular counterparts of I_{KP} [7]. Expression of K_{2P}10.1 (TREK2; TWIK-related K^+ channel 2) has been detected in human, rat, and chicken heart, with predominant expression in atrial cells [8–12]. In chicken, K_{2P}10.1 and the related K_{2P}2.1 (TREK1) channels carry a thermosensitive and arachidonic acid (AA)-activated background current that is responsible for hyperpolarization and stabilization of RMP in embryonic atrial cardiomyocytes [12]. In addition, K_{2P}10.1 has been suggested to contribute to a neuronal background conductance [13] and is implicated in control of neuronal excitability and spatial learning [14]. K_{2P}10.1 activity is enhanced by a wide range of physical and chemical stimuli, including increase in temperature to 37 °C [15], mechanical stretch [13], intra- and extracellular acidification [8,16], bioactive lipids such as polyunsaturated fatty acids (PUFAs) [13] or lysophospholipids [8,17], and volatile anesthetics [8,10]. Stimulation of G protein-coupled membrane receptors by different neurotransmitters and hormones and activation of protein kinase pathways leads to inhibition of K_{2P}10.1 channels [8,10,18,19].

Zebrafish (*Danio rerio*) is an established model for human disease that is widely used in developmental biology [20]. Forward (phenotype-focused) [21] and reverse (candidate gene-centered) [22] genetic approaches render zebrafish a genetically tractable animal model. In addition, this technology offers experimental advantages relative to mammalian systems in drug discovery and safety pharmacology, as zebrafish embryos allow for rapid and high-throughput analysis using whole organism phenotypic assays [23]. Furthermore, zebrafish

Abbreviations: AA, arachidonic acid; aa, amino acids; CDS, coding sequence; E_{rev}, reversal potential; h, human; *I*, current; I_{KP}, cardiac plateau current; ISH, in situ hybridization; K_{2P} channel, two-pore-domain potassium channel; M1 and M2, first and second translation initiation sites; ORF, open reading frame; P, pore domain; PA, palmitic acid; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PUFA, polyunsaturated acid; r, rat; RMP, resting membrane potential; TASK, TWIK-related acid sensitive K^+ channel; TM, transmembrane domain; TRAAK, TWIK-related arachidonic acid-stimulated K^+ channel; TREK, TWIK-related K^+ channel; TWIK, tandem of P domains in a weak inward rectifying K^+ channel; UTR, untranslated region; *V*, voltage; z, zebrafish

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embryos are transparent and well suited for in vivo imaging studies. Recent studies established zebrafish as genetic and functional model for cardiac electrophysiology [24]. Mutations or pharmacological modulation of the repolarizing zebrafish ether-a-go-go-related gene (zERG/I_{Kr}) potassium channel result in long or short QT syndromes [23,25,26]. Electrophysiological similarities between zebrafish and humans were extended to SCN5A sodium channel subunits, aquaporins, connexin 43, transient receptor potential (TRP) channels, L-type calcium channels, and Kir1.1 inward rectifier channels [27–31]. To date, zebrafish has not been established to elucidate cardiac function of K_{2P}10.1 and other K_{2P} channels. Here we report cloning and functional characterization of zebrafish K_{2P}10.1 two-pore-domain K^+ channels. Human and zebrafish K_{2P}10.1 display similar electrophysiological properties and are sensitive to antiarrhythmic drugs. We conclude that the zebrafish represents an appropriate model to study in vivo function of K_{2P}10.1.

2. Material and methods

2.1. Molecular biology

Potential orthologs of hK_{2P} channels in the *D. rerio* genome were identified by reciprocal BLAST analysis (National Center for Biotechnology Information, Bethesda, USA). Human K_{2P} query sequences were included in TBLASTN search against a zebrafish nucleotide collection (nr/nt). Highest scoring hits were confirmed as potential orthologs if the reciprocal BLASTX screen recognized the initial hK_{2P} protein in non-redundant protein sequence database (nr). TBLASTN search using human K_{2P}10.1 isoform 2 (GenBank accession number NP_612190.1) as query sequence yielded two hits with high alignment scores (E-value $\leq 4e^{-162}$). Both hits represent nucleotide sequences of z*KCNK*10 genes. The first mRNA target (XM_686592.3) is encoded by the z*KCNK*10a gene on zebrafish chromosome 20, as assigned by Ensembl genome browser (Ensembl, HAVANA group, Welcome Trust Sanger Institute, Hinxton, UK). The second mRNA hit (XM_688902.2) is linked to the z*KCNK*10b gene on chromosome 17. Reciprocal BLASTX for z*KCNK*10a mRNA (XM_686592.3) and z*KCNK*10b mRNA (XM_688902.2) yielded hK_{2P}10.1 isoforms 1–3 as matching targets (E-values $\leq 8e^{-174}$), supporting z*KCNK*10a and z*KCNK*10b as putative orthologs to human *KCNK*10.

Our primary aim was to establish functional similarity between human and zebrafish K_{2P} channel orthologs, serving as proof-of-concept. Zebrafish K_{2P}10.1b produces an isoform with similar size compared to the protein length of three human K_{2P}10.1 isoforms. This study confirmed expression and function of zebrafish z*KCNK*10b channels, and extension to *KCNK*10a was not required. The entire open reading frame (ORF) of z*KCNK*10b was amplified in this work. Cloning primer sequences located in the proposed untranslated regions (UTR) were derived from the genomic sequence using automated and manually annotated transcript structures of z*KCNK*10b (Ensembl). The first hypothetical translation initiation site (M1) (TCGGCCATGC) annotated by Ensembl and vertebrate genome annotation (Vega; Welcome Trust Sanger Institute) databases has only a suboptimal Kozak context [32]. Thus, forward primers were designed to include a start codon with a strong Kozak sequence corresponding to the human clone that was identified 59 bases upstream. Reverse primers were situated within the proposed 3′-UTR between the translational stop sequence and a consensus sequence for polyadenlyation [33] 609 bases downstream. The entire coding sequence (CDS) of z*KCNK*10b corresponding to sequence XM_688902.2 was amplified by nested PCR from adult zebrafish cDNA using two sets of forward and reverse primers, respectively (F1, 5′-GCACATTTCAGAGCACTTTGTGAGACC-3′; R1, 5′-GCATTTAAAGCTCTCTCAGCCTCTTTATGAAAC-3′; F2, 5′-CCACCCAGTTCTGATCATCTCCGTCTC-3′; R2, 5′-GAATTTGGCCCTTCAGGTGTGATCG-3′). Resulting DNA was cloned into pCR2.1-TOPO (Invitrogen, Karlsruhe, Germany). The final z*KCNK*10b insert was amplified from pCR2.1-TOPO (F3, 5′-CACGACCTGGTCGACCAGACCAACATGGTCCCGCAG-3′; R3, 5′-GTGGTAACCAGATCTTTACTACGGTTGGTGTTCCTTCAGCTCCAG-3′) and subcloned into pRAT, a dual-purpose expression vector containing a CMV promoter for mammalian expression and a T7 promoter for cRNA synthesis. pCR2.1-TOPO and pRAT-z*KCNK*10 clones were sequenced.

For *in vitro* transcription, pRAT-z*KCNK*10b (FJ888634.1) was linearized with *Not*I and transcribed using T7 RNA polymerase and the mMessage mMachine kit (Ambion, Austin, USA). Transcripts and control samples were separated by agarose gel electrophoresis and quantified by spectrophotometry. For heterologous expression 46 nl of cRNA were injected into stage V–VI defolliculated *Xenopus* oocytes and incubated for 2 to 3 days at 18 °C. This study has been carried out in accordance with the Council Directive 86/609/EEC on the protection of animals used for scientific purposes released by the European Commission.

2.2. In situ hybridization

A digoxigenin-labeled z*KCNK*10b antisense probe (607 bases) was synthesized using T7 polymerase from z*KCNK*10b in pCR2.1-TOPO vector linearized with SgrAI as template. Whole-mount in situ antisense RNA hybridization (ISH) was carried out as described [34,35].

2.3. Electrophysiology

Whole cell currents were measured by two-electrode voltage clamp as published previously [36]. Briefly, currents were recorded with an Oocyte Clamp amplifier (Warner Instruments, Hamden, USA) using pCLAMP (Axon Instruments, Foster City, USA) and Origin (OriginLab, Northampton, USA) software for data acquisition and analysis. Data were sampled at 2 kHz, filtered at 1 kHz. Current amplitudes were determined at the end of + 60 mV pulses. No leak current subtraction was performed. Two-electrode voltage clamp electrode pipettes were produced from glass capillary tubes GB100F-10 (Science Products, Hofheim, Germany) using a P-87 micropipette puller (Sutter Instruments, Novato, USA). Pipettes were filled with 3 M KCL and had a tip resistance of 1–5 MΩ. All experiments were conducted at room temperature (20–22 °C).

2.4. Solutions and drug administration

The standard physiological extracellular solution contained 96 mM NaCl, 4 mM KCl, 1.1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES and was adjusted to pH 7.4 with NaOH. Stock solutions of arachidonic acid (AA; 20 mM), palmitic acid (PA; 100 mM), quinidine (100 mM), and amiodarone (10 mM; all agents obtained from Sigma-Aldrich, Steinheim, Germany) were prepared in ethanol. Phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and Ro-32-0432 (Calbiochem, Darmstadt, Germany) were reconstituted in DMSO to stock solutions of 10 mM. $ZnCl_2$ (Sigma-Aldrich) was dissolved in standard extracellular solution (100 mM). Aliquots of stock solutions were stored at − 20 °C and diluted to desired concentrations in the standard extracellular solution on the day of experiment. Baseline currents were measured before drug administration, and drug effects on channel activity were assessed following incubation periods of 30 min (PMA), 3 h (AA, PA, quinidine, amiodarone), and 4 h (Ro-32-0432). Corresponding control periods of 30 min, 3 h, and 4 h in standard extracellular solution had no significant effects on hK_{2P}10.1 or zK_{2P}10.1 currents ($n = 5–28$; data not shown). Application of 100 μM Zn^{2+} (30 min) activated endogenous currents by 4.68 ± 1.15-fold ($n = 5$; $p = 0.026$). This affect, however, did not significantly affect the assessment of K_{2P}10.1 regulation, as endogenous currents after application of Zn^{2+} constituted a negligible fraction of outward currents in the presence of heterologously expressed hK_{2P}10.1 and zK_{2P}10.1 currents (3.1% and 12.9%, respectively). The remaining agents used in this work were tested on mock (0.1 M KCl) injected cells as well, excluding