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48	Abstract	<p>Electrophysiological properties and molecular background of the zebrafish (<i>Danio rerio</i>) cardiac inward rectifier current (<math>I_{K1}</math>) were examined. Ventricular myocytes of zebrafish have a robust (<math>-6.7 \pm 1.2</math> pA pF<sup>-1</sup> at <math>-120</math> mV) strongly rectifying and Ba<sup>2+</sup>-sensitive (<math>IC_{50} = 3.8</math> <math>\mu</math>M) <math>I_{K1}</math>. Transcripts of six Kir2 channels (drKir2.1a, drKir2.1b, drKir2.2a, drKir2.2b, drKir2.3, and drKir2.4) were expressed in the zebrafish heart. drKir2.4 and drKir2.2a were the dominant isoforms in both the ventricle (<math>92.9 \pm 1.5</math> and <math>6.3 \pm 1.5</math> %) and the atrium (<math>28.9 \pm 2.9</math> and <math>64.7 \pm 3.0</math> %). The remaining four channels comprised together less than 1 and 7 % of the total transcripts in ventricle and atrium, respectively. The four main gene products (drKir2.1a, drKir2.2a, drKir2.2b, drKir2.4) were cloned, sequenced, and expressed in HEK cells for electrophysiological characterization. drKir2.1a was the most weakly rectifying (passed more outward current) and drKir2.2b the most strongly rectifying (passed less outward current) channel, while drKir2.2a and drKir2.4 were intermediate between the two. In regard to sensitivity to Ba<sup>2+</sup> block, drKir2.4 was the most sensitive (<math>IC_{50} = 1.8</math> <math>\mu</math>M) and drKir2.1a the least sensitive channel (<math>IC_{50} = 132</math> <math>\mu</math>M). These findings indicate that the Kir2 isoform composition of the zebrafish heart markedly differs from that of the mammalian hearts. Furthermore orthologous Kir2 channels (Kir2.1 and Kir2.4) of zebrafish and mammals show striking differences in Ba<sup>2+</sup>-sensitivity. Those structural and functional differences needs to be taken into account when zebrafish is used as a model for human cardiac electrophysiology, cardiac diseases, and in screening cardioactive substances.</p>	

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49	Keywords	Zebrafish - Heart - Inward rectifier potassium current - Kir2 channel
	separated by ' - '	

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50	Foot note information	
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## Inward rectifier potassium current ( $I_{K1}$ ) and Kir2 composition of the zebrafish (*Danio rerio*) heart

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**Abstract** Electrophysiological properties and molecular background of the zebrafish (*Danio rerio*) cardiac inward rectifier current ( $I_{K1}$ ) were examined. Ventricular myocytes of zebrafish have a robust ( $-6.7 \pm 1.2$  pA pF<sup>-1</sup> at  $-120$  mV) strongly rectifying and Ba<sup>2+</sup>-sensitive ( $IC_{50} = 3.8$   $\mu$ M)  $I_{K1}$ . Transcripts of six Kir2 channels (drKir2.1a, drKir2.1b, drKir2.2a, drKir2.2b, drKir2.3, and drKir2.4) were expressed in the zebrafish heart. drKir2.4 and drKir2.2a were the dominant isoforms in both the ventricle ( $92.9 \pm 1.5$  and  $6.3 \pm 1.5$  %) and the atrium ( $28.9 \pm 2.9$  and  $64.7 \pm 3.0$  %). The remaining four channels comprised together less than 1 and 7 % of the total transcripts in ventricle and atrium, respectively. The four main gene products (drKir2.1a, drKir2.2a, drKir2.2b, drKir2.4) were cloned, sequenced, and expressed in HEK cells for electrophysiological characterization. drKir2.1a was the most weakly rectifying (passed more outward current) and drKir2.2b the most strongly rectifying (passed less outward current) channel, while drKir2.2a and drKir2.4 were intermediate between the two. In regard to sensitivity to Ba<sup>2+</sup> block, drKir2.4 was the most sensitive ( $IC_{50} = 1.8$   $\mu$ M) and drKir2.1a the least sensitive channel ( $IC_{50} = 132$   $\mu$ M). These findings indicate that the Kir2 isoform composition of the zebrafish heart markedly differs from that of the mammalian hearts. Furthermore orthologous Kir2 channels (Kir2.1 and Kir2.4) of zebrafish and mammals show striking differences in Ba<sup>2+</sup>-sensitivity. Those structural and functional differences needs

to be taken into account when zebrafish is used as a model for human cardiac electrophysiology, cardiac diseases, and in screening cardioactive substances.

**Keywords** Zebrafish · Heart · Inward rectifier potassium current · Kir2 channel

### Introduction

Zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), stickleback (*Gasterosteus aculeatus*), and other fish species have become popular model species for developmental biology, genetics, physiology, toxicology, evolutionary biology, and human diseases [32]. In particular, the zebrafish is a widely used animal model due to several technical advantages such as transparency in early life stages, well annotated genome, relative easiness of genetic manipulation, short generation time, and inexpensive and easy maintenance under laboratory conditions [6]. Understandably, zebrafish have become a popular vertebrate model also for cardiac development and regeneration, congenital and acquired human cardiac diseases, and drug screening [6, 27, 41]. Indeed, the zebrafish heart seems to be, in several respects, a better model than the murine heart for human cardiac electrophysiology. Heart rate (HR) in zebrafish is similar to that of humans (110–130 beats/min at 27 °C), and ventricular action potential (AP) of the zebrafish heart has a clear plateau phase with an AP duration of 270 ms (at 22–24 °C), which is similar to AP duration of the human ventricles (250–260 ms, at 37 °C) [4, 5, 37]. In strict sense, those functional similarities are, however, only valid when the comparisons are made at temperatures that differ more than 10°. If measured at the common experimental temperature (e.g., 20 °C), AP duration of the human heart would be much longer and HR much lower than in the zebrafish. Fishes are ectotherms, and their body temperature is often markedly

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68 lower and more variable than in mammals, and therefore, elec- 115  
69 trical excitability of the fish heart shows adaptation to function at 116  
70 colder temperatures [45]. Decisive for the use of zebrafish as a 117  
71 cardiac model for humans is whether ion current phenotypes are 118  
72 produced by orthologous genes in human, whether the gene 119  
73 products have similar biophysical properties and sensitivities to 120  
74 medicinal drugs, and whether the ion channel genes are under the 121  
75 same regulatory pathways. 122

76 The use of animal models gets its credence from the com- 123  
77 mon descent of animal species, i.e., common genetic and mo- 124  
78 lecular basis of physiological traits over the evolution. 125  
79 However, another equally important aspect of evolution is 126  
80 the diversity in form and function of animal species, which 127  
81 is based on the diversity of genomes as adaptation to different 128  
82 environments. Specifically, in teleost fishes, this diversity is 129  
83 considered to be largely based on the whole genome duplica- 130  
84 tion, which occurred about 320–350 million years ago [22]. 131  
85 The duplication event generated genetic material by diversifi- 132  
86 cation of duplicated genes for slightly different functions or to 133  
87 solve completely novel physiological problems (neo- and 134  
88 subfunctionalization) [43]. Therefore, it is not granted that 135  
89 ion currents of the heart are produced by orthologous genes 136  
90 in fishes and mammals, and the possibility remains that mam- 137  
91 malian and teleost ion channels have different biophysical 138  
92 properties and drug sensitivities, and they are regulated differ- 139  
93 ently under physiological stresses. To this end, the present 140  
94 study tests the hypothesis that the inward rectifier current 141  
95 ( $I_{K1}$ ) of the heart is produced by orthologous genes in 142  
96 zebrafish and mammals, and therefore, the zebrafish  $I_{K1}$  is 143  
97 functionally similar to its mammalian counterpart. Contrary 144  
98 to the hypothesis, it appears that in the zebrafish heart, the 145  
99 molecular background of  $I_{K1}$  is remarkably different from that 146  
100 of the mammalian heart including significant differences in 147  
101  $Ba^{2+}$ -sensitivity between orthologous Kir2 channels of 148  
102 zebrafish and mammals. 149

## 103 Methods

### 104 Animals

105 Wild type zebrafish (*Danio rerio*; strain AB/Nott) were reared 150  
106 at +28 °C in the zebrafish facilities at the University of 151  
107 Manchester, UK. Fish were killed by crushing the brains with 152  
108 forceps before the hearts were excised and rapidly frozen in 153  
109 liquid nitrogen for molecular studies or used for isolation of 154  
110 ventricular myocytes. All procedures adhere to the UK Home 155  
111 Office Animals Scientific Procedures Act of 1986. 156

### 112 Isolation of ventricular myocytes

113 Cardiac myocytes were enzymatically isolated at room tem- 157  
114 perature by retrograde perfusion of the heart. The heart was

115 gently excised and rinsed in the isolation saline solution (mM, 116  
100 NaCl; 10 KCl; 1.2  $KH_2PO_4$ ; 4  $MgSO_4$ ; 50 taurine; 20 117  
glucose; and 10 HEPES at pH 6.9 with NaOH). The heart was 118  
cannulated to a blunted 35G syringe needle, which was ad- 119  
vanced through the bulboventricular valve into the ventricular 120  
lumen. Enzyme perfusion with collagenase (Sigma Type 1A, 121  
0.2  $mg\ mL^{-1}$ ), trypsin (Sigma Type VI, 0.12  $mg\ mL^{-1}$ ), and 122  
fatty acid-free bovine serum albumin (Sigma, 1  $mg\ mL^{-1}$ ) was 123  
continued for 30 min. The digested ventricle was placed in 124  
0.5 mL of isolation solution, minced with scissors and tritu- 125  
rated using a Pasteur pipette. Cells were stored at +5 °C and 126  
used within 8 h from isolation. 127

### Molecular methods

**Cloning of zebrafish Kir2 genes** Excised hearts ( $n=5$ , five 128  
atria or ventricles pooled into one sample) were frozen in 129  
liquid nitrogen and stored at -70 °C for later use. Cardiac 130  
RNA was extracted from frozen tissue using TriReagent 131  
(Thermo Scientific), and genomic DNA (gDNA) was extract- 132  
ed from the myotomal muscle according to the method of 133  
Sambrook et al. [31]. Nucleic acids were quantified and qual- 134  
ified by NanoDrop ND-1000 spectrophotometer (NanoDrop 135  
Technologies, Wilmington, DE, USA) and agarose gel elec- 136  
trophoresis, respectively. Total RNA was treated with RQ1 137  
DNase (Promega) according to manufacturer's instructions 138  
and reverse transcribed to complementary DNA (cDNA) 139  
using RevertAid Premium Reverse Transcriptase (Thermo 140  
Scientific) and random hexamers (Thermo Scientific). 141  
Protein coding sequences of intronless drKir2.1a, drKir2.2a, 142  
and drKir2.2b were PCR-amplified from zebrafish gDNA and 143  
that of drKir2.4 from the cardiac cDNA using Phusion High 144  
Fidelity DNA Polymerase (Thermo Scientific) (primers 145  
shown in Table 1). The cycling parameters were as follows: 146  
initial denaturation at 98 °C for 1 min followed by 35 cycles at 147  
98 °C for 10 s, 63 °C for 30 s, and 72 °C for 45 s and final 148  
extension at 72 °C for 5 min. Overhang adenines were added 149  
to the 3'-ends of PCR products using Dynazyme II DNA po- 150  
lymerase (Thermo Scientific) and products were ligated to the 151  
pGEM-T Easy vector (Promega). Inserts were digested from 152  
pGEM-T Easy vector with EcoRI and SpeI and directionally 153  
cloned to pcDNA3.1/Zeo(+) digested with EcoRI and XbaI. 154  
The resulting plasmids were bidirectionally sequenced. The 155  
resulting sequences were assembled and aligned with the cor- 156  
responding target sequences using Geneious 7.0.4 [23]. 157

**Transcript expression** For quantitative RT-PCR (qPCR), 158  
five atrial and ventricular samples were prepared by pooling 159  
tissues from five fishes. RNA was extracted as described 160  
above and treated with DNase to avoid genomic DNA con- 161  
tamination. First-strand cDNA synthesis and qPCR reactions 162  
were conducted using DyNAmo™ HS SYBR® Green 2-step 163  
qRT-PCR Kit (Thermo Scientific). From every sample, a 164

**Q3** t1.1 **Table 1** Zebrafish Kir2 genes, their chromosomal location, and primer pairs used for cloning

t1.2	Protein name	Gene name	Gene number (ensemble)	Chromosomal location	Primers (5'–3')
t1.3	drKir2.1a	<i>KCNJ2a</i>	ENSDARG00000019418	12: 37 399 009-37 400 292	F: ATGGGAAGTGTGCGGG R: TCATATTCAGATCCCGCC
t1.4	drKir2.1b	<i>KCNJ2b</i>	ENSDARG00000038373	3: 12 404 618-12 405 940	
t1.5	drKir2.2a	<i>KCNJ12</i>	ENSDARG00000059822	24: 40 977 793-40 979 232	F: ATGAGCGTGGGTTCGGATT R: TCATATCTCCGACTCCCTGC
t1.6	drKir2.2b	<i>KCNJ12</i>	ENSDARG00000062618	3: 16 069 471-16 077 999	F: GCTGCGTTGGTACTCTCTCC R: AAACCCTGGGGCTAAAACCTG
t1.7	drKir2.3	<i>KCNJ4</i>	ENSDARG00000068110	3: 531 660-533 069	
t1.8	drKir2.4	<i>KCNJ14</i>	ENSDARG00000075914	16: 18 888 481-18 901 196	F: GTCACTCTGTGGGGGTCTGT R: TCAGTGTGTGTGGGTGTTT

165 control cDNA-reaction (-RT-control) containing all other  
166 components except RT-enzyme was prepared. Each sample  
167 was amplified in triplicates using Chromo4 Continuous  
168 Fluorescence Detector (MJ Research, Waltham,  
169 Massachusetts, USA) and primers represented in Table 2 under  
170 the following cycling parameters: 95 °C for 15 min  
171 followed by 40 cycles at 94 °C for 10 s, 61 °C for 20 s and  
172 72 °C for 30 s, then 72 °C for 10 min. After PCR, the speci-  
173 ficity of amplification was monitored by melting curve  
174 analysis.

175 To select a stably expressed reference gene for the qPCR  
176 experiments, five generally used reference genes: beta-actin  
177 (ACTB), DnaJ homologue subfamily A member 2 (DnaJA2),  
178 eukaryotic translation elongation factor 1 alpha (EEF1A1),  
179 glyceraldehyde phosphate dehydrogenase (GAPDH), and  
180 ubiquitin C (UBC) were tested. Each sample was amplified  
181 with specific primers for these genes (Table 2), and the results  
182 were analyzed with NormFinder [2], Genorm [42], the com-  
183 parative delta-Ct method [35], and BestKeeper [29] software.  
184 Depending on the evaluation method used, DnaJA2, ACTB,  
185 or GAPDH was ranked as the most stable control gene,  
186 EEF1A1 and UBC showing the most variable expression (da-  
187 ta not shown). All four approaches ranked DnaJA2 as the best

or second best reference gene, whereas ACTB and GAPDH 188  
were ranked as the best or third best reference genes. Thus, 189  
DnaJA2 appeared to be the most stably expressed gene in 190  
zebrafish cardiac tissues. To minimize the effect of potential 191  
differences in reference gene expression on results, the geo- 192  
metric mean of DnaJA2 and ACTB were used for normaliza- 193  
tion of the drKir2 transcript expression. 194

**Heterologous expression of cardiac drKir2 genes** Human 195  
embryonic kidney (HEK293; ECACC) cells were grown in 196  
DMEM (EuroClone) supplemented with 10 % fetal bovine 197  
serum (FBS; Euroclone) and 100 U/ml penicillin and strepto- 198  
mycin (EuroClone). HEK cells were transiently cotransfected 199  
with pEGFP-N1 (Clontech), and either drKir2.1a, drKir2.2a, 200  
drKir2.2b, or drKir2.4 were cloned to the pcDNA3.1/Zeo(+) 201  
using TurboFect transfection reagent (Thermo Scientific). 202  
Whole cell patch-clamp experiments were conducted 24– 203  
56 h after transfection. 204

**Electrophysiological experiments** 205

For whole-cell patch-clamp recording of  $I_{K1}$  ventricular 206  
myocytes ( $33.3 \pm 2.4$  pF) and HEK cells ( $9.4 \pm 0.9$  pF) 207

t2.1 **Table 2** Primer pairs used for  
t2.2 quantitative PCR

		Forward primer (5'–3')	Reverse primer (5'–3')	Product (bp)
t2.3	drKir2.1a	GTGGCCCTTCAAAACAAGA	GCCTGGCTGTGTTTCAGAGT	104
t2.4	drKir2.1b	CGGAGGATGATGATGATGAC	AAGCTGTGCTTTTGACATCG	102
t2.5	drKir2.2a	CCAGAACGGATAAAGCCAGA	CCTTTGTTCTGTGCATCGAG	102
t2.6	drKir2.2b	CGGTGCCAACTTCTGCTAT	GTCTCTAGCTCAGTCCCCCT	100
t2.7	drKir2.3	AGAAAATGCTCCAGGACTCG	ATGGTGGAGTGGAGGATGTC	103
t2.8	drKir2.4	CTGCAGATCTCCTCCTCTGT	AGGAGTCTTGTCGAGGTGGT	103
t2.9	drACTB	CTTCCAGCAGATGTGGATCA	GCCATTAAAGGTGGCAACA	102
t2.10	drDnaJA2	CTATGGGGAACAGGGTCTGC	GTCCACCCATGAAACCAAAC	104
t2.11	drEEF1A1	CAGTCAAGGACATCCGTCGT	AGGGTGGTTCAGGATGATGAC	104
t2.12	drGAPDH	TTGACGCTGGTGTGTTATT	CCATCAGGTCACATACACGGT	102
t2.13	drUBC	GAGTCCACCTTGCATCTGGT	GTGTCGCTTGGCTCTACCTC	104

208 expressing the cloned drKir2 channels were superfused with  
 209 external saline solution (mM, 150 NaCl; 5.4 KCl; 1.8 CaCl<sub>2</sub>;  
 210 1.2 MgCl<sub>2</sub>; 10 glucose; and 10 HEPES in mM at pH 7.6 with  
 211 NaOH) at room temperature (21–23 °C). Tetrodotoxin  
 212 (1 μM), nifedipine (10 μM), and E-4031 (2 μM) were added  
 213 into this solution to prevent contamination of the recordings  
 214 by Na<sup>+</sup>, Ca<sup>2+</sup>, and delayed rectifier K<sup>+</sup> currents (*I<sub>Kr</sub>*), respec-  
 215 tively. Patch pipettes were filled with intracellular saline solu-  
 216 tion of the following composition (mM, 140 KCl; 4 MgATP; 1  
 217 MgCl<sub>2</sub>; 5 EGTA; and 10 HEPES at pH 7.2 with KOH). *I<sub>K1</sub>*  
 218 was elicited from the holding potential of -80 mV by  
 219 repolarizing voltage ramps (+60–120 mV for 1 s) for every  
 220 10 s. Ba<sup>2+</sup>-sensitivity of the ventricular *I<sub>K1</sub>* and current gener-  
 221 ated by each of the four most abundantly expressed drKir2  
 222 channels was determined by exposing the cells to cumulatively  
 223 increasing BaCl<sub>2</sub> concentrations (10<sup>-9</sup>–10<sup>-3</sup> M). Cells were  
 224 exposed to each Ba<sup>2+</sup> concentration until the current inhibition  
 225 leveled out (about 2.5 min). The normalized *I<sub>K1</sub>* was plotted as  
 226 a function of Ba<sup>2+</sup> concentration and fitted to the sigmoidal  
 227 equation

$$I = I_{\min} + \frac{I_{\max} \times [\text{Ba}^{2+}]^H}{(IC_{50}^H + [\text{Ba}^{2+}]^H)}$$

228 where *I<sub>min</sub>* is the residual *I<sub>K1</sub>* at the highest Ba<sup>2+</sup> concentration,  
 229 *I<sub>max</sub>* the maximum *I<sub>K1</sub>* before Ba<sup>2+</sup> addition, *IC<sub>50</sub>* the Ba<sup>2+</sup>  
 230 concentration which causes half-maximal inhibition of the  
 231 *I<sub>K1</sub>*, [Ba<sup>2+</sup>] the molar concentration of Ba<sup>2+</sup>, and *H* the Hill  
 232 slope factor of the line.

233  
 234 Inward rectification of the Ba<sup>2+</sup>-sensitive *I<sub>K1</sub>* was deter-  
 235 mined for the ventricular current and each of the four cloned  
 236 drKir2 channels. The non-rectifying current, obtained from  
 237 the current-voltage relationship at the negative side of the  
 238 reversal potential of the *I<sub>K1</sub>*, was extrapolated to the voltage  
 239 area of inward rectification (positive to the reversal potential).  
 240 The measured (rectifying) *I<sub>K1</sub>* was divided with the linear  
 241 (non-rectifying) current to obtain inward rectification. The  
 242 normalized current (relative chord conductance, *I/I<sub>max</sub>*) was  
 243 plotted as a function of membrane voltage, and the  
 244 Boltzmann equation (below) was fit to the data [11, 34].

$$\frac{I}{I_{\max}} = (1 + \exp((V - V_{0.5})/k))^{-1}$$

245 In the equation, *V* is membrane potential, and *V<sub>0.5</sub>* and *k* are  
 246 the midpoint voltage and the slope of the curve, respectively.

## 250 Statistics

251 Results are given as means±SEM. Differences in drKir2 tran-  
 252 script expressions between atrium and ventricle were tested  
 253 using Student's *t* test. If the data was not normally distributed,

Mann-Whitney *U* test was used. A *P* value of 0.05 was  
 regarded as a limit of statistical significance.

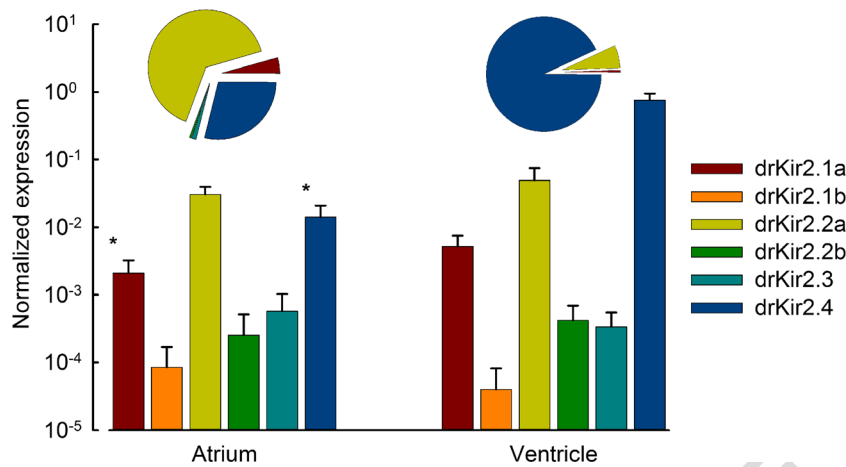
## 256 Results

### 257 Expression pattern of zebrafish Kir2 genes in cardiac 258 tissues

259 Sequences for zebrafish Kir2 (drKir2) genes were searched  
 260 from the Ensembl Genome Browser (<http://www.ensembl.org/index.html>). Altogether, six drKir2 genes were found.  
 261 Two gene paralogues (a and b) existed for drKir2.1 and  
 262 drKir2.2, whereas no duplicates were found for drKir2.3 and  
 263 drKir2.4. In contrast, no orthologues to Kir2.6 were found  
 264 from the zebrafish genomes. All zebrafish Kir2 genes, with  
 265 the exception of ENSDARG00000062618, were already  
 266 annotated and named. ENSDARG00000062618 showed  
 267 higher homology with zebrafish (72.1 %) and human (71.  
 268 5 %) Kir2.2 than other Kir2 genes (51.1–65.9 %) and was  
 269 therefore named as drKir2.2b. This is consistent with the  
 270 annotation of Leong et al. (2014) who regarded it as a Kir2.  
 271 2 paralogue. drKir2.2b is also highly homologous (71.5 %) to  
 272 human Kir2.6, which in turn is nearly identical to human  
 273 Kir2.2 and possibly a duplicate to it [30]. Even if human Kir2.6 and  
 274 zebrafish Kir2.2b were duplicates of Kir2.2, they are not  
 275 orthologues to each other because they are outcomes from  
 276 separate duplication events. Human Kir2.6 is aroused from  
 277 duplication of a limited chromosomal region, whereas  
 278 drKir2.2b is assumed to be an outcome of the whole genome  
 279 duplication (2R) in the teleost fish lineage. drKir2.2b shares  
 280 high homology (87.9 %) with crucian carp Kir2.5  
 281 (EU182584) which is presumably also a duplicate of Kir2.2  
 282 [17] and therefore renamed as ccKir2.2b.

283  
 284 Next, we examined the transcript levels of the six drKir2  
 285 genes in the zebrafish heart. The main Kir2 subunits of  
 286 zebrafish heart were drKir2.4 and drKir2.2a, which jointly  
 287 represented about 99 and 94 % of ventricular and atrial  
 288 drKir2 transcripts, respectively (Fig. 1). In the ventricle,  
 289 drKir2.4 was clearly the main drKir2 isoform, comprising  
 290 92.9±3.4 % of the total drKir2 transcripts. In the atrium,  
 291 drKir2.2a was the dominant drKir2 channel subunit with a  
 292 transcript expression level of 64.7±3.1 %. drKir2.1 was a  
 293 minor component in ventricle, where drKir2.1a and  
 294 drKir2.1b comprised only 0.61±0.08 % of the drKir2 tran-  
 295 scripts. In the atrium, the relative proportion of drKir2.1 was  
 296 slightly higher (4.39±0.98 %) than in the ventricle (*P*<0.05).  
 297 drKir2b and drKir2.3 were expressed in atrium and ventricle  
 298 only in trace amounts.

299 Sequence comparisons showed several consensus sites for  
 300 inward rectification and Ba<sup>2+</sup> block in all drKir2 channels  
 301 (Fig. 2a, b). Eight amino acids critical for inward rectification  
 302 have been previously identified for mammalian Kir2 channels



**Fig. 1** Normalized transcript expression of drKir2 genes in atrium and ventricle of the zebrafish heart. The bar chart shows mean ( $\pm$  SEM,  $n=5$ ) transcript levels for each drKir2 gene normalized to the geometric mean of DnaJA2 and ACTB reference genes. Note the logarithmic scale of the

y-axis. The pie charts depict relative portions (%) of different drKir2 transcripts in the total drKir2 pool. An asterisk indicates statistically significant difference between atrial and ventricular gene expression ( $p < 0.05$ )

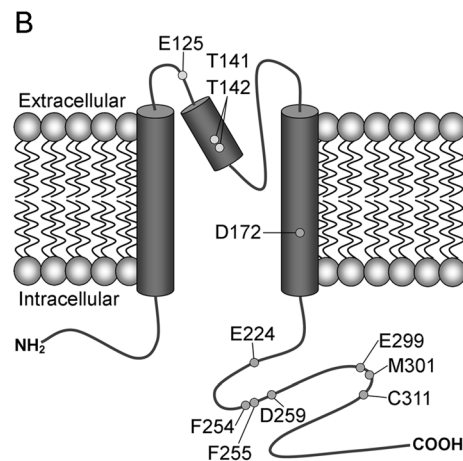
303 [28]. Six of them (D172, E224, F255, D259, E299, and  
304 M301) were identical in all drKir2 channels (Fig. 2c).  
305 drKir2.2b differed from drKir2.2a and mammalian Kir2.2 in

that F254 was replaced by Y254. Similar to mammalian 306  
Kir2.2 channels, drKir2.2a and drKir2.2b do not have C311 307  
which is considered to affect inward rectification. Three 308

**Fig. 2** Amino acid residues important in  $Ba^{2+}$  sensitivity and rectification in vertebrate Kir2 channels. **a** Amino acid alignment of genes represented in Table 3. Amino acids important for  $Ba^{2+}$  sensitivity and rectification are identified with light and dark gray, respectively. Amino acids are numbered according to human Kir2.1 (*h* human, *cp* guinea-pig (*Cavia porcellus*), *rn* rat (*Rattus norvegicus*), *cc* crucian carp (*Carassius carassius*)). **b** Schematic presentation of transmembrane topology of Kir2 channel. Cylinders indicate the transmembrane  $\alpha$ -helices and the  $\alpha$ -helix of the P-loop. Amino acids involved in  $Ba^{2+}$  sensitivity and rectification are indicated. **c** Conserved amino acid residues involved in inward rectification of the vertebrate Kir2 channels [28] and their presence in zebrafish Kir2 channels

**A**

	E125	T141 T142	D172	E224	F254 F255	D259	E299 M301	C311
hKir2.1	VSEVNSFTA	AFLSIETQ	TTIGYG...	CIIDA...LVEAH...	GFD	SGIDR...	LEG	MVEATAMTTQCR
cpKir2.1	VSEVNSFTA	AFLSIETQ	TTIGYG...	CIIDA...LVEAH...	GFD	SGIDR...	LEG	MVEATAMTTQCR
rnKir2.1	VSEVNSFTA	AFLSIETQ	TTIGYG...	CIIDA...LVEAH...	GFD	SGIDR...	LEG	MVEATAMTTQCR
ccKir2.1	VSNVSTFTA	AFLSIETQ	TTIGYG...	CIIDA...LVEAH...	GFD	SGIDR...	LEG	MVEATAMTTQCR
drKir2.1a	VSNVSSFTA	AFLSIETQ	TTIGYG...	CIIDA...LVEAH...	GFD	SGIDR...	LEG	MVEATAMTTQCR
hKir2.2	VMQVHGFM	AFLFSIETQ	TTIGYG...	CIIDS...IVEAH...	GFD	KGLDR...	LEG	MVEATAMTTQAR
cpKir2.2	VMQVHGFM	AFLFSIETQ	TTIGYG...	CIIDS...IVEAH...	GFD	KGLDR...	LEG	MVEATAMTTQAR
rnKir2.2	VLQVHGFM	AFLFSIETQ	TTIGYG...	CIIDS...IVEAH...	GFD	KGLDR...	LEG	MVEATAMTTQAR
ccKir2.2a	VLQVNGFIA	AFLFSIETQ	TTIGYG...	SIIDC...IVEAH...	GFD	KGLDR...	LEG	MVEATAMTTQAR
drKir2.2a	VLQVNGFIA	AFLFSIETQ	TTIGYG...	SIIDC...IVEAH...	GFD	KGLDR...	LEG	MVEATAMTTQAR
ccKir2.2b	VMQVNSFIA	AFLFSVETQ	TTIGYG...	CIIDS...IVEAH...	GFD	QGLDR...	LEG	MVEATAMTTQVR
drKir2.2b	VMQVNSFVA	AFLFSVETQ	TTIGYG...	CIIDS...IVEAH...	GFD	QGLDR...	LEG	MVEATAMTTQVR
hKir2.3	IMHVNGLF	GAFLFSVETQ	TTIGYG...	CVIDS...IVEAH...	GFD	IGLDR...	LEG	MVEATAMTTQAR
cpKir2.3	IMHVNGLF	GAFLFSVETQ	TTIGYG...	CVIDS...IVEAH...	GFD	IGLDR...	LEG	MVEATAMTTQAR
cpKir2.4	FSHVASFL	AFLFALETQ	TSIGYG...	CVLDA...LVEAH...	GFD	GGTDR...	LEG	MVEATAMTTQCR
rnKir2.4	FSQVASF	LAAFLFALETQ	TSIGYG...	CVLDA...LVEAH...	GFD	GGTDR...	LEG	MVEATAMTTQCR
drKir2.4	FQQVNSFM	AFLFSLETQ	TSIGYG...	CIIDA...LVEAH...	GFD	TGTDR...	LEG	MVEATAMTTQCR



**C**

	drKir2.1a	drKir2.2a	drKir2.2b	drKir2.4
D172	+	+	+	+
E224	+	+	+	+
F254	+	+	Y254	+
D255	+	+	+	+
D259	+	+	+	+
E299	+	+	+	+
M301	+	+	+	+
C311	+	A311	V311	+



309 amino acid residues, E125, T141, and T142, are considered to  
 310 be crucial for Ba<sup>2+</sup> sensitivity [1, 8]. drKir2.1a and b differ  
 311 from the mammalian Kir2.1 in that glutamate E124 is replaced  
 312 by asparagine N124.

313 **Inward rectifier current (*I<sub>K1</sub>*) of zebrafish ventricular**  
 314 **myocytes**

315 Zebrafish cardiac myocytes showed a robust *I<sub>K1</sub>* with typical  
 316 electrophysiological characteristics of the vertebrate cardiac *I<sub>K1</sub>*  
 317 (Fig. 3). The zebrafish ventricular *I<sub>K1</sub>* had a reversal potential  
 318 (−81±1.1 mV) close to the theoretical reversal potential (*E<sub>rev</sub>*)  
 319 of K<sup>+</sup> ions (−84.7 mV) (Fig. 3a), a large inward current at neg-  
 320 ative side of the *E<sub>rev</sub>* (−6.7±1.2 pA pF<sup>−1</sup> at −120 mV) and a peak  
 321 outward current at the positive side of the *E<sub>rev</sub>* (0.68±0.1 pA pF<sup>−1</sup>  
 322 at −59 mV) (Fig. 3b). The maximum outward current was  
 323 10.1 % of the inward current at −120 mV. There was clear neg-  
 324 ative slope conductance positive to −59 mV, but the current did  
 325 not completely rectify at 0 mV. Half-maximal inward rectifica-  
 326 tion occurred at the voltage of −79.3±1.1 mV and with a slope of  
 327 6.9±0.6 (Fig. 3c). The current was completely inhibited by ext-  
 328ernal Ba<sup>2+</sup> with the IC<sub>50</sub> value of 3.8 μM (Fig. 3d).

329 ***I<sub>K1</sub>* of the cloned drKir2 channels**

330 The four most abundant Kir2 channels (drKir2.1a, drKir2.2a,  
 331 drKir2.2b, drKir2.4) of the zebrafish heart were expressed in  
 332 HEK cells for electrophysiological characterization (Fig. 4).  
 333 All drKir2 channels generated strongly inwardly rectifying  
 334 currents, which reversed direction at around −80 mV, the

Nernst equilibrium potential of K<sup>+</sup> ions (Fig. 4a). drKir2.1a  
 channels passed more outward current (25 % of the current  
 amplitude at −120 mV) than other drKir2 channels, i.e., it was  
 the weakest inward rectifier. drKir2.2b was clearly the stron-  
 gest inward rectifier as the maximum outward current was  
 only 7 % of the current density at −120 mV. drKir2.2a and  
 drKir2.4 were intermediate between those two channels (out-  
 ward current 16 and 12 % of the current at −120 mV, respec-  
 tively). In regard to the voltage-dependence of inward rectifica-  
 tion drKir2.1a, drKir2.2a and drKir2.4 were similar with  
 voltage for half-maximal inactivation at around −77 mV,  
 while inactivation of the drKir2.2b occurred at more negative  
 voltages (−82 mV) (Fig. 4b).

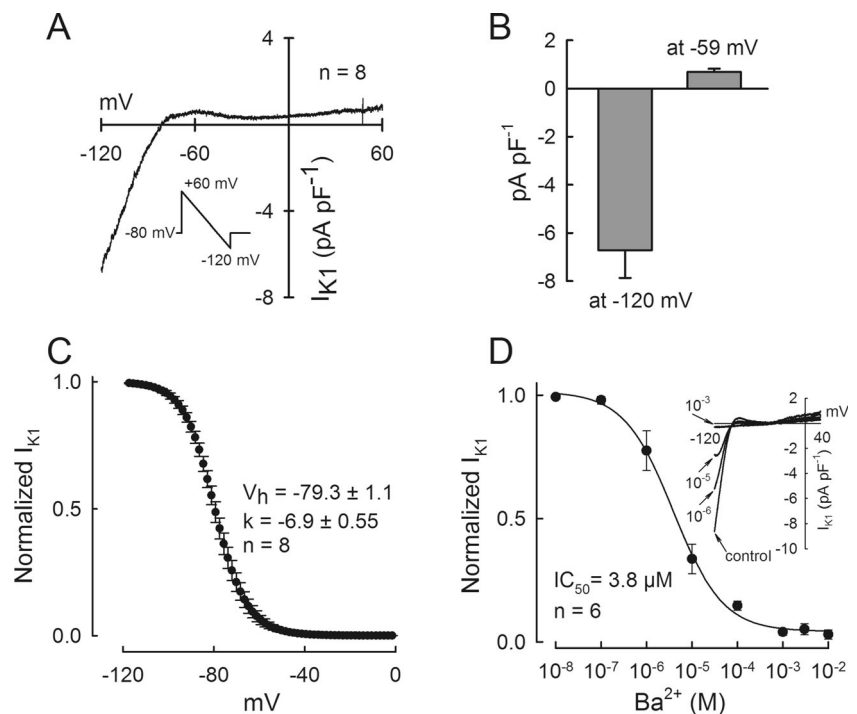
External Ba<sup>2+</sup> completely blocked all four drKir2 channels  
 (Fig. 4c). There were, however, prominent differences in Ba<sup>2+</sup>-  
 sensitivity between the drKir2 isoforms. drKir2.1a was the most  
 insensitive channel to Ba<sup>2+</sup> block with IC<sub>50</sub> of 132±14 μM,  
 while drKir2.4 was the most Ba<sup>2+</sup>-sensitive channel with the  
 IC<sub>50</sub>-value almost two orders magnitude higher (1.8±1.1 μM)  
 than that of the drKir2.1a. IC<sub>50</sub>-values for drKir2.2a and  
 drKir2.2b channels were 14±5.1 μM and 21±8.5 μM,  
 respectively.

**Discussion**

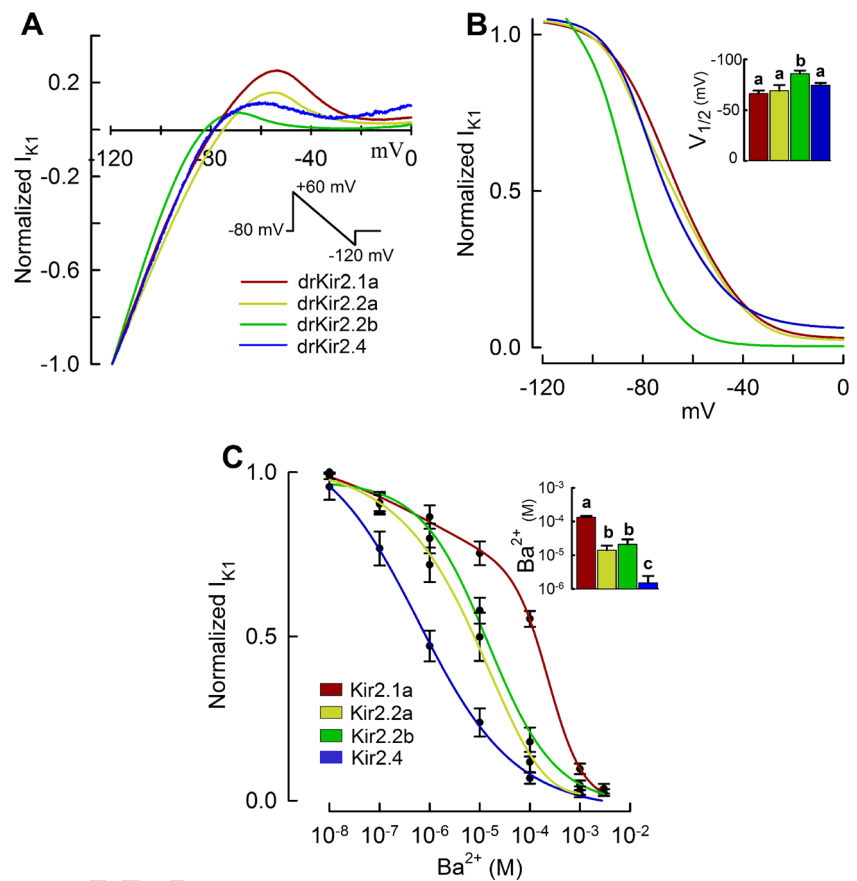
**Kir2 composition of the zebrafish heart**

The present results show that ventricular myocytes of the  
 zebrafish heart have a robust inward rectifier K<sup>+</sup> current, *I<sub>K1</sub>*,

**Fig. 3** The inward rectifier current (*I<sub>K1</sub>*) of the zebrafish ventricular myocytes. **a** A mean current voltage relationship of *I<sub>K1</sub>* from eight ventricular myocytes. **b** Maximum inward current density at −120 mV and the maximum outward current density at −59 mV. The results are means±SEM from eight myocytes. **c** Voltage-dependence of inward rectification of the Ba<sup>2+</sup>-sensitive *I<sub>K1</sub>* (means±SEM, n=8). **d** A concentration-response curve of *I<sub>K1</sub>* to external Ba<sup>2+</sup> at −120 mV (n=6)



**Fig. 4** Electrophysiological properties of the cloned drKir2 channels in HEK cells. **a** Mean current-voltage relationships of the  $I_{K1}$  generated by drKir2.1a, drKir2.2a, drKir2.2b and drKir2.4 channels ( $n=10-14$ ). The currents were normalized to the maximum inward current at  $-120$  mV. **b** Voltage-dependence of inward rectification of the current produced by the cloned drKir2 channels. The inset shows the voltage for half-maximal inactivation of the current ( $V_{1/2}$ ). The results are means $\pm$ SEM of 10-14 cells. **c** Ba<sup>2+</sup>-sensitivity of the  $I_{K1}$  generated by the cloned drKir2.4 channels. The inset shows concentration for half-maximal inhibition of the current ( $IC_{50}$ ) by Ba<sup>2+</sup>. The results are means $\pm$ SEM of 10-12 cells. Statistically significant differences ( $p<0.05$ ) between mean values are shown by dissimilar letters



361 with typical characteristics for vertebrate cardiac  $I_{K1}$  including  
 362 strong inward rectification and block by external Ba<sup>2+</sup>.  
 363 Interestingly, the zebrafish cardiac  $I_{K1}$  is largely produced by  
 364 drKir2.4 and drKir2.2a isoforms, thereby strongly deviating  
 365 from the Kir2 subunit composition of the mammalian hearts  
 366 [20]. Furthermore, some Kir2 orthologues of the zebrafish  
 367 genome (drKir2.1a, drKir2.4) clearly differ from their mam-  
 368 malian counterparts in regard to the Ba<sup>2+</sup>-sensitivity of the  
 369 generated  $I_{K1}$ .

370 In mammalian hearts, three major Kir2 channels are  
 371 expressed, Kir2.1, Kir2.2, and Kir2.3. In mammalian ventricu-  
 372 lar myocytes, Kir2.1 channels are the predominating isoform  
 373 with a smaller contribution by Kir2.2 and Kir2.3 channels  
 374 [46]. For example in the right ventricle of the human heart,  
 375 Kir2.1, Kir2.2, and Kir2.3 transcripts form 47, 29, and 24 % of  
 376 the total Kir2 transcripts, respectively [13]. In mammalian  
 377 atria, Kir2.3 channels are abundantly expressed [26]. In the  
 378 right atrium of the human heart, Kir2.3 forms 56 % of all Kir2  
 379 transcripts, while the relative portion of Kir2.2 and Kir2.1 is  
 380 31 and 13 %, respectively [13]. Contrary to the mammalian  
 381 cardiac Kir2 composition, drKir2.1a, drKir2.1b, and drKir2.3  
 382 formed less than 6 and 1 % of the total drKir2 population in  
 383 atrium and ventricle, respectively. Two homologues to the  
 384 mammalian Kir2.2 channel, drKir2.2a and drKir2.2b, were  
 385 present in the zebrafish heart. drKir2.2a was the main isoform

of the zebrafish atrium (64.7 %) and expressed also in the  
 ventricle (6.3 %). Synteny data strongly suggests that  
 drKir2.2a and drKir2.2b are paralogues from a gene duplica-  
 tion event [24], but probably, the regulation of their gene ex-  
 pression have diverged from each other [17].

Surprisingly, an orthologue to the mammalian isoform of  
 Kir2.4, drKir2.4, was the main Kir2 isoform in the zebrafish  
 fish ventricle (93 %) and the second largest isoform in the  
 zebrafish atrium (28.9 %). In mammals, Kir2.4 is strongly  
 expressed in brain and retina [21, 39], but is not present or is  
 weakly expressed in mammalian hearts [12, 36]. Even in the  
 heart it may be confined to neuronal elements only [25]. Thus,  
 our analysis of Kir2 channel composition in zebrafish heart  
 reveals marked differences from mammals which may affect  
 the extrapolation of zebrafish heart electrophysiology to  
 human.

**Inward rectification**

Functionally, Kir2 channels are inward rectifiers, i.e., they  
 pass little or no outward K<sup>+</sup> current at the plateau voltage of  
 cardiac AP while allowing some K<sup>+</sup> efflux at more negative  
 voltages. By this means  $I_{K1}$  enables long plateau duration and  
 accelerates the final phase 3 repolarization of the cardiac AP.  
 However, Kir2 isoforms markedly differ in their inward

409 rectifier properties [11, 28]. Similar to mammalian Kir2.1  
 410 channels, the zebrafish drKir2.1a subunit allows significant  
 411 outward  $I_{K1}$  around  $-60$  mV, shows a steep negative slope  
 412 conductance between  $-60$  and  $0$  mV, and completely rectifies  
 413 at  $0$  mV. drKir2.2b channels are strong rectifiers, as are their  
 414 mammalian counterparts [11, 28], passing relatively little out-  
 415 ward current close to the  $E_{rev}$  of  $K^+$  ions and completely rec-  
 416 tifying at  $0$  mV. They will contribute to repolarization of the  
 417 cardiac AP at the very late phase, when membrane potential  
 418 approaches RMP. However, drKir2.1a and drKir2.2b are  
 419 weakly expressed in the zebrafish heart and therefore unlikely  
 420 to have any significant effect on atrial or ventricular  $I_{K1}$ . The  
 421 prevailing Kir2 isoforms of the zebrafish heart drKir2.2a and  
 422 drKir2.4 are intermediate between drKir2.1a and drKir2.2b  
 423 channels in their rectification properties. It is notable, howev-  
 424 er, that unlike other drKir2 channels, drKir2.4 subunits and the  
 425 native  $I_{K1}$  of zebrafish ventricular myocytes do not completely  
 426 rectify at  $0$  mV, i.e., the negative slope conductance is  
 427 shallower than that of the drKir2.1a. In this regard,  
 428 drKir2.4 isoform seems to be more similar to the mam-  
 429 malian Kir2.3 channels, which are mainly expressed in  
 430 mammalian atria [11].

431 Inward rectification of Kir2 channels is produced by  
 432 voltage-dependent block of the channel by intracellular poly-  
 433 amines and  $Mg^{2+}$  ions. Several critical amino acid residues  
 434 necessary for polyamine block of Kir2 channels have been  
 435 found and examined including D172, E224, F254, D255,  
 436 D259, E299, M301, and C311 (Fig. 2). All these critical res-  
 437 idues also exist in drKir2.1a and drKir2.4 channels. drKir2.2a  
 438 and drKir2.2b differ in regard to one of those residues: in  
 439 drKir2.2b, the nonpolar phenylalanine in position 254  
 440 (F254) is replaced by a polar amino-acid tyrosine (Y254).  
 441 Similar to the mammalian Kir2.2 and Kir2.3 channels, the  
 442 zebrafish drKir2.2a and drKir2.2b do not have cysteine in  
 443 the position 311. The polar cysteine is replaced by nonpolar  
 444 amino-acids alanine and valine in drKir2.2a and drKir2.2b,  
 445 respectively. Site-directed mutagenesis is needed to examine  
 446 what kind of effects those two residues (254, 311) might have  
 447 on inward rectification and other electrophysiological proper-  
 448 ties of the zebrafish channels.

#### 449 $Ba^{2+}$ sensitivity of drKir2 channels

450 There were two striking features in  $Ba^{2+}$ -sensitivity of  
 451 zebrafish drKir2 channels. Divergent from the mammalian  
 452 Kir2.4 channels, which are characterized by low sensitivity  
 453 to  $Ba^{2+}$  block [21, 38, 39], the zebrafish drKir2.4 was highly  
 454 sensitive to  $Ba^{2+}$ . The difference between mammalian and  
 455 zebrafish Kir2.4 is almost two orders of magnitude  
 456 (Table 3). Comparison of the amino acid residues E125,  
 457 T141, and T142, known to be important for  $Ba^{2+}$  sensitivity  
 458 [1, 7], shows that these amino acid residues are identical in  
 459 zebrafish and rat Kir2.4 (Fig. 2a). Evidently, other amino-acid

**Table 3** Comparison of  $Ba^{2+}$ -sensitivities ( $IC_{50}$ -values,  $\mu M$ ) between mammalian and fish Kir2 channels together with zebrafish ventricular  $I_{K1}$

	Human <sup>a</sup>	Guinea-pig <sup>b</sup>	Rat <sup>c</sup>	Crucian carp <sup>d</sup>	Zebrafish <sup>e</sup>	
Kir2.1	16.2	3.2	8	22.2	132 (drKir2.1a)	t3.3
Kir2.2	2.3	0.5	6	–	–	t3.4
Kir2.2a	–	–	–	3.5	14	t3.5
Kir2.2b	–	–	–	2.4	21	t3.6
Kir2.3	18.3	10.2	–	–	–	t3.7
Kir2.4		235	390		1.8	t3.8
$I_{K1}$					3.8	t3.9

$Ba^{2+}$ -sensitivity of  $I_{K1}$  was measured at  $-100$  or  $-120$  mV

<sup>a</sup> [33]

<sup>b</sup> [25]

<sup>c</sup> [39]

<sup>d</sup> [17]

<sup>e</sup> Present study

460 residues in addition to those three sites must be involved in  
 461 regulation of Kir2  $Ba^{2+}$  binding. Another marked deviation  
 462 appeared in  $Ba^{2+}$  sensitivity of the drKir2.1a, because of its  
 463 low affinity to  $Ba^{2+}$  in comparison to Kir2.1 channels of mam-  
 464 mals and other fish species [17]. Both crucian carp Kir2.1 and  
 465 drKir2.1a have asparagine instead of E125 of the mammalian  
 466 Kir2.1 channels. However, this residue is unlikely to be asso-  
 467 ciated with lower  $Ba^{2+}$  sensitivity of the drKir2.1a, because  
 468 the crucian carp (*Carassius carassius*) orthologue is five times  
 469 more sensitive to  $Ba^{2+}$  than the drKir2.1a (Table 3).

#### 470 Implications for a zebrafish model

471  $I_{K1}$  is involved in some ion channel diseases of the human  
 472 heart [10, 40]. A long QT7 (Andersen-Tawil) syndrome, a  
 473 short QT syndrome, catecholaminergic polymorphic ventricu-  
 474 lar tachycardia and familial atrial fibrillation of the human  
 475 heart, are all due to mutations of the main ventricular isoform,  
 476 Kir2.1 and thus associated with the ventricular  $I_{K1}$ , [3].  
 477 Because of short AP duration, high HR, and divergent reper-  
 478 toire of the repolarizing  $K^+$  currents, the murine heart may not  
 479 always be a useful arrhythmia model despite similarities in  
 480 Kir2 channel composition between human and murine hearts  
 481 [46]. Zebrafish is increasingly used as model for human car-  
 482 diac electrophysiology and drug screening due to its amena-  
 483 bility for genetic modification and similarities to human car-  
 484 diac excitation. Recently, an orthologue to human *KCNJ2*  
 485 gene (drKir2.1a) was cloned from the zebrafish and the mu-  
 486 tated gene with delta95–98 deletion (producing an Andersen-  
 487 Tawil syndrome in humans), was introduced into zebrafish  
 488 embryos [24]. Although several dysmorphologies and  
 489 malfunctions of skeleton and skeletal muscles, typical for the

490 syndrome, appeared in the fish embryos, the cardiac pheno- 538  
 491 type was almost untouched. The current study shows that 539  
 492 drKir2.1a forms less than 0.7 % of the total drKir2 transcripts, 540  
 493 and therefore, it is likely that the trafficking-defect mutant of 541  
 494 the drKir2.1a is either not produced in cardiac myocytes or it 542  
 495 does not co-assemble with the dominant cardiac isoforms 543  
 496 drKir2.4 and drKir2.2a channels. In order to manipulate the 544  
 497 zebrafish cardiac  $I_{K1}$ , the target for manipulation should be the 545  
 498 main cardiac isoforms drKir2.4 and/or drKir2.2a. Although 546  
 499 drKir2.4 and drKir2.2a channels are stronger rectifiers than 547  
 500 the Kir2.1 isoforms, loss and gain of drKir2.4 and/or 548  
 501 drKir2.2a function might produce cardiac phenotypes similar 549  
 502 to long QT and short QT syndromes of the human heart, 550  
 503 respectively. 551

504 **Why drKir2.4 is the dominant isoform in zebrafish** 552  
 505 **ventricle?** 553

506 Kir2.1–3 subunits are expressed in mammalian hearts with 554  
 507 some clear differences in Kir2 channel composition between 555  
 508 species [20]. Kir2 composition of the zebrafish heart markedly 556  
 509 deviate from the mammalian cardiac Kir2 composition in that 557  
 510 drKir2.4 is the main subunit. This raises a question about 558  
 511 possible physiological significance of the special Kir2 com- 559  
 512 position. Also, there exists clear interspecies difference in car- 560  
 513 diac Kir2 composition among fish species. For example, in the 561  
 514 heart of rainbow trout (*Oncorhynchus mykiss*), Kir2.1 chan- 562  
 515 nels are dominating while in crucian carp (*Carassius* 563  
 516 *carassius*), Kir2.2a and Kir2.2b are the main cardiac isoforms 564  
 517 [16, 17]. As noted above (*Inward rectification*), inward recti- 565  
 518 fication properties of the drKir2.4 are not strikingly different 566  
 519 from those of other drKir2 channels but rather an intermediate 567  
 520 between the extremes. drKir2.4 channels have a clear negative 568  
 521 slope conductance which provides repolarizing power during 569  
 522 phase 3 of the cardiac AP and passes less outward current at 570  
 523 the plateau level. Fish are ectotherms, and thermal tolerance 571  
 524 range of the zebrafish extends from +6 to +36 °C [9]. Since 572  
 525 Kir2 channel composition and  $I_{K1}$  density of fish hearts is 573  
 526 strongly affected by environmental temperature [14, 17, 19], 574  
 527 it remains to be shown what significance of drKir2.4 and 575  
 528 drKir2.2a channels might have in thermal acclimation of the 576  
 529 tropical zebrafish. Temperature changes are also associated 577  
 530 with variation of blood pH. In this regard, the high pH sensi- 578  
 531 tivity of Kir2.4 channels [21] might play some role in excit- 579  
 532 ability of the fish heart. 580

533 **Conclusions** 581

534 The  $I_{K1}$  current of the zebrafish heart is produced by markedly 582  
 535 different Kir2 channel composition in comparison to mamma- 583  
 536 lian hearts. This difference emphasizes the importance of clar- 584  
 537 ifying the molecular genetic background of zebrafish ion 585

channels, when using zebrafish as a model for human cardiac 538  
 electrophysiology and cardiac diseases. Furthermore, signifi- 539  
 cant differences are evident in  $Ba^{2+}$ -sensitivity between 540  
 orthologous mammalian and zebrafish Kir2 gene products 541  
 which suggests that the sensitivity of zebrafish cardiac ion 542  
 channels to ion channel blockers can markedly differ from 543  
 those of the human heart. This is consistent with the previous 544  
 studies which have shown marked differences in chromanol 545  
 239B sensitivity of the delayed rectifier  $K^+$  current ( $I_{Ks}$ ) and 546  
 tetrodotoxin sensitivity of  $Na^+$  current ( $I_{Na}$ ) between fish and 547  
 mammalian hearts [15, 18, 44]. 548

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**Conflict of interest** The authors declare that they have no conflict of 555  
 interest. 556

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