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Cloning and expression of cardiac Kir2.1 and Kir2.2 channels in thermally acclimated rainbow trout

by

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Short title: Inward rectifiers of the fish heart

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Potassium currents are plastic entities which modify electrical activity of the heart in various physiological conditions including chronic thermal stress. We examined molecular basis of the inward rectifier K current (I_K1) in rainbow trout acclimated to cold (4°C, c.a.) and warm (18°C, w.a.) temperature. Kir2.1 and Kir2.2 transcripts were expressed in atrium and ventricle of the trout heart, Kir2.1 being the major component in both cardiac chambers. The relative expression of Kir2.2 was, however, higher (P<0.05) in atrium than ventricle. The density of ventricular I_K1 was about 25% larger (P<0.05) in w.a. than c.a. trout. Furthermore, the I_K1 of the w.a. trout was ten times more sensitive to Ba^{2+} (IC_{50}, 0.18 ± 0.42 µM) than the I_K1 of the c.a. trout (1.17 ± 0.44 µM) (P<0.05) and opening kinetics of single Kir2 channels was slower in w.a. than c.a. trout (P<0.05). When expressed in COS-1 cells, the homomeric Kir2.2 channels demonstrated higher Ba^{2+} sensitivity (2.88 ± 0.42 µM) than Kir2.1 channels (24.99 ± 7.40 µM) (P<0.05). Based on different Ba^{2+} sensitivities of omKir2.1 and omKir2.2 channels, it is concluded that warm-acclimation increases either number or activity of the omKir2.2 channels in trout ventricular myocytes. The functional changes in I_K1 are independent of omKir2 transcript levels which remained unaltered by thermal acclimation. Collectively, these findings suggest that thermal acclimation modifies functional properties and subunit composition of the trout Kir2 channels, which may be needed for regulation of cardiac excitability at variable temperatures.

Keywords: inward rectifier potassium channels, atrial myocytes, ventricular myocytes, thermal plasticity
INTRODUCTION

Strong inward rectifier potassium (Kir) channels conduct inward currents at membrane potentials negative to the $K^+$ reversal potential, but permit only limited $K^+$ efflux at more positive voltages (18, 37) based on the voltage-dependent block of the channels by intracellular Mg$^{2+}$ and polyamines (8, 9, 17). The small outward current is physiologically important, since it sets resting membrane potential (RMP), controls excitability and participates in diverse body functions in various organs. In the heart, the inward rectifier current ($I_{K1}$) clamps the RMP close to $K^+$ equilibrium potential and contributes to the late phase 3 repolarization of the action potential (AP) and thereby participates in the regulation of AP duration (18).

On the basis of sequence homology, inward rectifier K channels have been classified into seven subfamilies, Kir1-Kir7 (5, 18). Inward rectifiers of the mammalian heart are homo- or heterotetrameric assemblies of Kir2.1-3 subunits (16, 26, 31, 36, 40) with substantial variation between species (4, 42). Further complexity is generated by chamber-related differences in the expression of Kir2 subunits (11, 14, 36). It is, however, incompletely understood to what extent species-specific and regional differences in the properties of cardiac $I_{K1}$ are related to the relative expression of Kir2 subunits. For example the atrioventricular differences in density and rectification of $I_{K1}$ have been explained either by differences in free polyamine concentrations (39) or by variable expression of functionally different Kir2 subunits (4). Therefore, situations where the cardiac $I_{K1}$ is substantially modified within species by external or internal factors might shed new light on the relative importance of Kir subunits in the regulation of the cardiac $I_{K1}$.

Body temperature of ectothermic animals can vary quite considerably e.g. due to seasonal changes in ambient temperature which sets special demands on ion channel function to maintain adequate excitability of cardiac myocytes in various temperatures. Indeed,
prolonged exposure to low temperature induces compensatory shortening of AP which is associated with strong up-regulation of the delayed rectifier K current ($I_{Kr}$) (12, 34). Curiously, at the same time the density of the strong inward rectifier K$^+$ current, the $I_{K1}$, is reduced. The physiological importance of this cold-induced decrease is not completely understood, but it might increase cardiac excitability by reducing the demand for the depolarising Na$^+$ current. The objective of this study was to examine the molecular basis of the rainbow trout $I_{K1}$ current and to find out whether differences in molecular composition could explain temperature-induced changes and regional differences in $I_{K1}$ density of the trout heart. To this end, we cloned Kir2.1 and Kir2.2 genes, determined their expression and measured cardiac $I_{K1}$ at whole-cell and single-channel level from thermally acclimated trout.
MATERIALS AND METHODS

Fish

Rainbow trout (*Oncorhynchus mykiss*) were donated by a local fish farm (Kontiolahti, Finland). In the laboratory, the fish were kept in 500 l stainless steel tanks with aerated groundwater continuously delivered to the tanks at about 0.5 l/min, and were acclimated to 4°C (cold acclimation, c.a.) or 18°C (warm acclimation, w.a.) for at least three weeks before use in experiments. Fish were fed commercial trout fodder (Biomar, Brande, Denmark) to satiation three times a week. All experiments were conducted with consent of the local committee for animal experimentation.

Molecular methods

*Extraction of RNA and DNA.* Fish were stunned by a sharp blow to the head and the spine was cut. Atrium, ventricle, brain and a piece of gill, kidney, liver and skeletal muscle were quickly removed and immediately frozen in liquid nitrogen. Total RNA was extracted with Trizol Reagent (Invitrogen, Carlsbad, USA) according to manufacturer’s instructions. DNA was extracted from liver by the method of Sambrook et al. (1989) (30). RNA and DNA were qualified by gel electrophoresis and quantified by UV spectrophotometry.

*Cloning of Kir2.1 and Kir2.2 open reading frames (ORFs) from rainbow trout heart.* First-strand cDNA was prepared from the total cardiac RNA, and was treated with RQ1 RNase-Free DNase (Promega, Madison, Wisconsin, USA). Reverse transcription was performed by M-MuLV Reverse Transcriptase RNase H- (Finnzymes, Espoo, Finland) using an oligo(dT)$_{18}$ primer. Partial cDNAs corresponding to omKir2.1 and omKir2.2 were obtained by PCR using degenerative primers, designed to the homologous regions of corresponding mammalian genes (Table 1). PCR was performed in a 25 µl reaction mixture containing 50 mM Tris·HCl,
1.5 mM MgCl₂, 15 mM (NH₄)₂SO₄, 0.1% Triton X-100, 200 µM of each dNTP, 1 U DyNAzyme EXT (Finnzymes), 2 µl cDNA and 5 pmol each primer. Amplification was performed in PCR conditions: initial denaturation at 94°C for 2 min followed by 1-4 cycles with low annealing temperature at 94°C for 30 s, 40°C for 30 s and 72°C for 90 s further followed by 31 cycles with higher annealing temperature at 55°C and final extension at 72°C for 5 min. PCR products were checked on a 0.8% agarose gel and if no products were obtained, 0.5 µl of the PCR product was reamplified using PCR conditions: initial denaturation at 96°C for 2 min followed by 35 cycles at 95°C for 30s, 53°C for 30 s and 72°C for 90 s, and final extension at 72°C for 5 min. New forward primers were designed on the basis of the sequences obtained and used to clone the rest of the ORFs (Table 1). Oligo(dT) primer was used to clone the 3'-ends of the genes.

Genome walker kit (Clontech, Palo Alto, CA, USA) was used for the cloning of 5'-ends of the genes. To this end, high molecular mass genomic DNA was extracted from rainbow trout liver (30), and four genomic libraries were constructed by digesting genomic DNA with DraI, EcoRV, PvuII and StuI followed by ligation to the adaptor. Gene-specific primers and adaptor primers were used to amplify the 5'-ends of the genes. The first PCR was performed using GSP1 (gene-specific primer) and AP1 (adaptor primer) (Table 1) and a two-step-PCR protocol recommended by the manufacturer: 6 cycles at 94°C for 25 s and 72°C for 3 min followed by 31 cycles at 94°C for 25 s and 67°C for 3 min and final extension at 72°C for 7 min. Of this PCR product 0.5 µl was reamplified using GSP2 and AP2 (Table 1). The second PCR was identical to the first one, except that the annealing temperature of the first 6 cycles was 68°C and 63°C for the rest. The sequences were confirmed by cloning the whole coding region of the Kir2.1 and Kir2.2 genes from genomic DNA. PCR was performed as described in the use of degenerative primers, except that 50 ng of DNA was used as a template.
After amplification, all PCR products were analyzed by gel electrophoresis, extracted from gel by Qiaex II gel extraction kit (QIAGEN, Valencia, California, USA), cloned into the pGEM-T Easy Vector (Promega) and sequenced by ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, California, USA).

**Southern blotting.** Ten µg of genomic DNA was digested to completion with EcoRI, HindIII or NcoI. DNA fragments were size separated on a 1% agarose gel and blotted onto positively charged nylon membrane (Boehringer Mannheim, Mannheim, Germany). Probes were produced by PCR, amplifying the nucleotides 1026-1284 and 1119-1341 from omKir2.1 and omKir2.2 plasmids, respectively. Probes were labelled with [a-32P]dCTP using Ready-To-Go DNA Labelling Beads and purified by ProbeQuant G-50 Micro Columns (all obtained from Amersham Biosciences, England). Nylon membranes were prehybridized 2 hours at 42 °C in 20 ml of prehybridization solution containing 50% formamide, 3x SSC (0.45 M NaCl, 0.045 M Na citrate), 2.5x Denhardt’s (0.05% BSA, 0.05% polyvinylpyrrolidine, 0.05% Ficoll), 0.25% SDS and 0.1 mg/ml herring sperm DNA, followed by hybridization overnight with the probe at the same conditions. Thereafter they were washed twice for 15 min at room temperature and twice at 37°C with 1x SSC, 0.5% SDS solution followed by a final wash at 65°C for at least 15 min with 0.1x SSC, 1% SDS solution. Signals were detected by autoradiography.

**Quantitative RT-PCR.** For organ specific gene expression, total RNA was extracted from heart, brain, gill, kidney, liver and skeletal muscle of two w.a. trout and for the temperature-acclimation dependent gene expression from atrium and ventricle of three w.a. and three c.a. trout. Each RNA-sample (2 µg) was treated with RQ1 RNase-Free DNase (Promega), and the first strand cDNA synthesis was performed with random hexamers and MMuLV RNase H+
(Finnzymes) at the following conditions: 25°C for 10 min, 37°C for 30 min and 85°C for 5 min. A control run containing all other reaction components but RT enzyme was accomplished for every sample. Quantitative PCR was performed with DyNAmo HS SYBR Green qPCR Kit (Finnzymes) and Chromo4 Continuous Fluorescence Detector (MJ Research, Waltham, Massachusetts, USA) using primer pairs listed in Table 2 (primers for DnaJA2 were a kind gift from Dr. Aleksei Krasnov). Polymerase was activated at 94°C for 15 min and amplification was performed for 40 cycles at 94°C for 10 s, 57°C for 20 s and 72°C for 30 s. After PCR, the specificity of the reaction was monitored by melting curve analysis. Two controls, one containing all the reaction components except the template and the other one containing the product of cDNA synthesis performed without RT enzyme, was included in every experiment. DnaJA2 was selected as a reference gene. DnaJ is a small chaperonin, which belongs to the heat shock proteins, but unlike many commonly used reference genes, such as ribosomal proteins and β-actin, its expression remains quite constant in temperature acclimation (35). The suitability of DnaJA2 as an internal standard was further confirmed by a qPCR experiment using equal amount of RNA extracted from the tissues of c.a. and w.a. trout as a template.

**Modelling of trout Kirs.** Homology models of omKir2.1 and omKir2.2 channels were constructed using DeepView 3.7 program (GlaxoSmithGline, Switzerland) and a SWISS-MODEL server. KirBac3.1 (PDB code 1XL4) and C-terminus of mammalian Kir3.1 (PDB code 1N9P) (21) were used as templates for the transmembrane segments (G50-N185 or G55-N195) and the C-terminus (N185-A358 or N195-A376) of omKir2.x, respectively. The fourfold rotational symmetry of the channels was adjusted manually by using KirBac3.1 and KcsA (PDB code 1BL8) (6) as templates.
Electrophysiological methods

Isolation of cardiac myocytes. Atrial and ventricular myocytes were isolated using previously published methods (33). Briefly, fish were stunned with a quick blow to the head, the spine was cut and the heart was excised. A metallic cannulae was brought through the bulbus arteriosus into the ventricle and the heart was retrogradely perfused with a nominally Ca\textsuperscript{2+}-free, low Na\textsuperscript{+}-solution for 10 min and then with proteolytic enzyme solution for 15 min. Solutions were continuously gassed with 100% O\textsubscript{2} and the enzyme solution was recycled using a peristaltic pump. The Ca\textsuperscript{2+}-free saline contained (mmol l\textsuperscript{-1}): 100 NaCl, 10 KCl, 1.2 KH\textsubscript{2}PO\textsubscript{4}, 4 MgSO\textsubscript{4}, 50 taurine, 20 glucose and 10 HEPES adjusted to pH 6.9 with KOH at 20\textdegree C. For enzymatic digestion, 0.75 mg/ml collagenase (Type IA, Sigma), 0.5 mg/ml trypsin (Type IX, Sigma) and 0.5 mg/ml fatty-acid-free bovine serum albumin (A6003, Sigma) were added to this solution. After enzymatic digestion, atrium and ventricle were chopped in small pieces in the Ca\textsuperscript{2+}-free solution and myocytes were released by agitating tissue pieces through the opening of a Pasteur pipette. Isolated myocytes were stored at 6\textdegree C and used within 8 h from the isolation.

Whole-cell patch clamp. The whole-cell voltage clamp recording of the I\textsubscript{K1} was performed using an Axopatch 1-D (Axon Instruments, CA, USA) or an EPC-9 (HEKA Instruments, Germany) amplifier (22). Myocytes were superfused in a small recording chamber with a pre-cooled external saline solution at the rate of 1.5-2.0 ml min\textsuperscript{-1}. Temperature was adjusted to 4\textdegree C and 18\textdegree C for c.a. and w.a. trout, respectively, using circulating water baths or a Peltier device (TC-10, Dagan Corporation, USA) and was continuously monitored by a thermistor positioned closed to the myocyte. The external saline solution contained (mmol l\textsuperscript{-1}): 150 NaCl, 5.4 KCl, 1.8 CaCl\textsubscript{2}, 1.2 MgCl\textsubscript{2}, 10 glucose and 10 HEPES (pH adjusted to 7.7 with NaOH). Patch pipettes were pulled from borosilicate glass (Garner, Claremont, Calif., USA) and filled with K\textsuperscript{+}-based electrode solution (mmol l\textsuperscript{-1}: 140 KCl, 1 MgCl\textsubscript{2}, 5 EGTA, 4 MgATP and 10 HEPES adjusted to pH 7.2 with KOH) giving a mean (± SEM) pipette resistance of 2.81 ± 0.07 M\textOmega}s. After getting access to the
cell, pipette capacitance (7.55 ± 0.14 pF, n = 48) and series resistance (9.96 ± 0.48 MΩ, n=48) were compensated. The inward rectifier current was determined as a Ba^{2+} (0.1 mM) sensitive current in the presence of tetrodotoxin (0.5 µM, Tocris Cookson, UK), nifedipine (10 µM, Sigma), glibenclamide (10 µM, Sigma) and E-4031 (1 µM, Alomone labs) to block Na^{+}, Ca^{2+}, ATP-sensitive K^{+} and delayed rectifier K^{+} (I_{Kr}) current, respectively. The I_{K1} was elicited every 5 seconds by repolarising ramps or square wave pulses from the holding potential of -80 mV.

Barium inhibition of the I_{K1} was determined in the presence of cumulatively added concentrations of BaCl_2 (10^{-9}-10^{-4} M). The cell was exposed to each Ba^{2+} concentration for 4 minutes. Dose-response curves were fitted with a Hill equation:

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

where \( I_{\text{min}} \) is the minimum I_{K1} at the highest Ba^{2+} concentration, \( I_{\text{max}} \) the I_{K1} before Ba^{2+} addition, \( IC_{50} \) the drug concentration which causes half-maximal inhibition of the I_{K1}, [Ba] the Ba^{2+} concentration, and \( H \) the Hill slope of the line.

Voltage dependence of inward rectification was determined as a portion of the Ba^{2+}-sensitive current that was inhibited at depolarising voltages relative to the unblocked current. The unblocked (non-rectifying) inward current was obtained from the current-voltage relationship between -120 mV and the reversal potential (V_{rev}) of the I_{K1} and extrapolated to the voltage area of inward rectification. Scattering data points around the V_{rev} were omitted and the current was fitted the sum of two Boltzmann functions:

\[ I_{K1} = (A_1 (1 + \exp(V - V_{h1})/S_1)) + (A_2 (1 + \exp(V - V_{h2})/S_2)) \]

where A_1, V_{h1} and S_1 are amplitudes, voltages and slopes of half-block for steep and shallow components of the polyamine block, respectively.
Single-channel patch clamp. Single-channel properties of the omKir2 channels were recorded in the cell-attached configuration using an EPC-9 amplifier and Pulse software, and analyzed using TAC, TACFIT (Bruxton) and SigmaPlot 6.0 (SPSS) programs as described previously (23). Pipettes were pulled from a thick-walled borosilicate glass (Garner, Claremont, Calif., USA) using a two-state puller (PP-83, Narishige, Tokyo, Japan), coated with Sylgard (WPI, UK) and fire-polished on a microforge (MF-83, Narishige, Tokyo, Japan). The mean resistance of the pipettes was 9.2 ± 0.6 MΩ when filled with the K-based solution (in mM): 134 KCl, 1.8 CaCl₂, 2 MgCl₂, 10 glucose and 10 HEPES adjusted to pH 7.6 with KOH ([K⁺] = 141 mM). Physiological saline solution was used as the bath solution. The activity of unwanted channels was abolished by including specific ion channel blockers in the pipette and Tyrode solutions (see Whole-cell patch clamp). All single-channel recordings were sampled at 4 kHz and low-pass filtered at 2 kHz. Single-channel conductance was determined by applying 5 s square pulses from -200 to -20 mV in 20 mV increments every 10 s from the holding potential of -80 mV. Distributions of open and closed times were obtained from 20 s recordings at -100 mV. Open time and closed time analyses were performed on patches, which had only a single open current level. Open and closed times were detected with time-course fitting, and probability density functions (pdf) were analyzed from idealized data with Log-likelihood method on log (event times) (TACFIT).

Functional expression of omKir2 genes in COS-1 cells. ORF sequences for putative ion channel forming genes omKir2.1 and omKir2.2 were subcloned into the pcDNA3.1/Zeo (+) vector (Invitrogen) for expression in a COS-1 cell line. Correctness of the sequences was confirmed by DNA sequencing. COS-1 cells (ECACC) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, BioWhittaker, Cambrex Verviers, Belgium) containing 10 % fetal bovine serum (FBS, Euroclone, Milano, Italy) and 3000 units ml⁻¹ of both penicillin and
streptomycin (EuroClone). Cells were transiently cotransfected with pEGFP-N1 (Clontech) and either omKir2.1- or omKir2.2-pcDNA3.1/Zeo (+) using Effectene Transfection Reagent (QIAGEN). Function of the cloned channels was tested with the whole-cell patch-clamp method at room temperature (20°C) 48-64 hours after transfection. The same external and internal solutions were used as in measurement of endogenous currents (see Whole-cell patch clamp, page 9).

Statistics. Differences between mean values from w.a. and c.a trout and between atrial and ventricular $I_{K1}$ were assessed by Student’s $t$-test. If the data were not normally distributed, differences were tested with the non-parametric Mann-Whitney-test. A $P$-value of 0.05 was regarded as a limit of statistical significance.
RESULTS

Rainbow trout Kir2.1 and Kir2.2 genes. Coding regions of Kir2.1 (KCNJ2) and Kir2.2 (KCNJ12) genes were cloned from rainbow trout cDNA and genomic DNA. Genomic clones indicated that the coding regions of these genes were intronless. Two different alleles both for Kir2.1 and Kir2.2 were found and are named as omKir2.1a, omKir2.1b, omKir2.2a and omKir2.2b. The nucleotide sequences for omKir2.1a and omKir2.1b ORF include 1278 and 1284 bp, respectively, omKir2.1a lacking the nucleotides 93-98 of the omKir2.1b (Fig. 1A). The nucleotide sequences for omKir2.2a and omKir2.2b ORF include 1341 bp coding 446 amino acid residues (Fig. 1B). Nucleotide sequences of the two alleles are 96.6% and 97.4% identical for omKir2.1 and omKir2.2, respectively, and show even higher amino acid identity. Southern hybridization of the genomic DNA with omKir2.1 or omKir2.2 probes revealed only one band for each gene, suggesting that the two alleles are located in equivalent loci of the genome (data not shown).

Amino acid alignments of omKir2.1 and omKir2.2 with mammalian Kir proteins reveal about 82% and 66% sequence similarity, respectively (Fig. 2). The lowest homology with mammalian Kirs is seen in the C-terminus and in the M1-P loop (“the turret”), both in omKir2.1 and omKir2.2. Interestingly, the M1 transmembrane domain of omKir2.2 also differs greatly from the corresponding mammalian sequence. A structural model of the omKir2.2 (Fig. 3) shows that the differences are located in the lipid-bilayer facing side of the membrane spanning α-helix, where almost one third (6) of the amino acids are leucines (Fig. 1). In the M2 transmembrane domain, the glycine G169 of the mammalian Kir2.2 - the so-called glycine hinge - is replaced by serine (S173) in the omKir2.2. The most prominent structural difference between omKir2.1 and omKir2.2 is the extracellular mouth formed by the M1-P loop, which in omKir2.1 is oriented straight upwards and does not form any
secondary structures, whereas in omKir2.2 it forms a short β-sheet and is turned towards the membrane being possibly in contact with the lipid bilayer.

Functionally important sequences that are common to all Kir subfamilies, including the pore domain with $K^+$-selectivity signature sequence (GYG), were identified in the rainbow trout Kir2s using multiple sequence alignments (Figs. 1 and 2). Amino acids, critical for polyamine, Mg$^{2+}$ and PIP$_2$ binding were the same as in mammalian Kir2.1 and Kir2.2 channels. In contrast, differences existed in extracellular Ba$^{2+}$ binding sites between fish and mammalian Kirs: in omKir2.1 glutamate E125 was replaced with asparagine and in omKir2.2 threonine T141 was replaced with serine.

*Expression of omKir2 mRNAs.* The expression of omKir2.1 and omKir2.2 transcripts was determined from different organs by quantitative RT-PCR and normalized to the abundance of DnaJA2 mRNA. DnaJA2 expression was quite constant at both acclimation temperatures (data not shown) and therefore a suitable reference gene, as confirmed by qPCR using the same amount of RNA from the tissues of c.a. and w.a. trout as a template. Both omKir2.1 and omKir2.2 were expressed in all examined organs including heart, brain, gill, kidney, liver, and skeletal muscle (Fig. 4A). Of the two genes, omKir2.1 was more abundant in all other organs except the brain, where omKir2.2 was the dominant transcript. The expression of omKir2.2 in the brain was ten times higher than in the atrium and much lower in ventricle, gill, skeletal muscle and liver. The expression of omKir2.1 was highest in the kidney, being 7.1 times higher than in the atrium. In general, the total amount of omKir2 mRNAs in cells was very low in all tissues, e.g. in the atrium only about 1% of the DnaJA2 expression level, which is not a very abundant protein either.

Since thermal acclimation changes $I_{K1}$ of the trout heart and as there are striking differences in density and rectification of the $I_{K1}$ between atrium and ventricle, Kir2
expression was studied in both cardiac chambers of w.a. and c.a. trout (Fig. 4B). Although omKir2.1 was the dominant isoform in both chambers, the atrium expressed relatively more omKir2.2 than the ventricle (P=0.021), the transcripts of omKir2.1 being 2.7, 1.7, 7.3 and 6.1 times more abundant than omKir2.2 mRNA in c.a. atrium, w.a. atrium, c.a. ventricle and w.a. ventricle, respectively. Quite surprisingly, no significant differences in omKir2 expression existed between c.a. and w.a. trout hearts.

**Density and inward rectification of the $I_{K1}$.** In accordance with previous findings (34) the peak outward and inward $I_{K1}$ density of trout ventricular myocytes was 22-25% larger (P<0.05) in w.a. than c.a. fish (Fig. 5A). The charge transfer of the outward current between $V_{rev}$ and 0 mV did not, however, differ between c.a. and w.a. trout (P=0.12). There was a striking difference in current size between ventricular and atrial myocytes (Fig. 5A). The peak inward density and outward charge transfer of the atrial $I_{K1}$ were only about 3% and 10%, respectively, of the values of the ventricular $I_{K1}$.

The current-voltage relationships indicate that the ventricular $I_{K1}$ rectifies completely at positive voltages, while inward rectification of the atrial $I_{K1}$ is incomplete (Fig. 5A). Two Boltzmann functions were needed to fit the steady-state inward rectification of both atrial and ventricular $I_{K1}$, which alike the mammalian $I_{K1}$ consists of a steep and a shallow component (Fig. 5B). The weaker inward rectification of atrial $I_{K1}$ in comparison to ventricular $I_{K1}$ is mainly due to the differences in the shallow component, which is larger and shallower in atrial than ventricular myocytes. Thermal acclimation did not affect inward rectification either in atrial or ventricular myocytes. Taken together, these findings indicate striking differences in $I_{K1}$ between atrial and ventricular myocytes, and suggest that warm-acclimation slightly increases the number of active Kir channels in ventricular myocytes.
Different Kir2 channels vary in regard to their response to external K\(^+\) and Ba\(^{2+}\). To examine putative atrioventricular differences in Kir composition, K\(^+\) dependence and Ba\(^{2+}\) sensitivity of the rainbow trout cardiac I\(_{K1}\) were determined by whole-cell patch clamp. Doubling of [K\(^+\)]\(_o\) from 5.4 to 10.8 mM resulted in a Nernstian shift of the reversal potential (E\(_{rev}\)) from about -80 to -60 mV (Table 3), and 1.64-1.74 fold and 1.70-2.43 fold increase of the inward I\(_{K1}\) in ventricular and atrial myocytes, respectively (Fig. 6). The effect of high [K\(^+\)]\(_o\) on the outward I\(_{K1}\) was, however, weak and opposite in the two cardiac chambers. In ventricular myocytes the outward I\(_{K1}\) was marginally (1.05-1.15 fold) increased, while in atrial myocytes it was slightly (0.79-0.93 fold) depressed. As an outcome of this difference, the current-voltage relationships of the outward I\(_{K1}\) crossed each other at the two [K\(^+\)]\(_o\) in ventricular but not in atrial myocytes. The ventricular crossover points differed between c.a. (-39.75 ± 1.77 mV) and w.a. trout (-45.88 ± 1.66 mV) (P<0.05), but when the crossover points were expressed relative to the reversal potential of the I\(_{K1}\) in 5.4 mM [K\(^+\)]\(_o\), i.e. corrected for the temperature-dependent shift of the reversal potential, the difference disappeared (33.3 ± 1.5 and 32.5 ± 1.4 mV for c.a. and w.a trout, respectively; P = 0.70). Taken together, these experiments indicate marked chamber-specific differences in K\(^+\) sensitivity in the trout cardiac I\(_{K1}\).

The trout atrial I\(_{K1}\) was so small, that the effects of [Ba\(^{2+}\)]\(_o\) could be reliably measured only in ventricular myocytes. The Ba\(^{2+}\) concentration required for half-maximum block of the I\(_{K1}\) was almost an order of magnitude lower in w.a. (IC\(_{50}\), 0.18 ± 0.13 µM) than in c.a. (1.17 ± 0.15 µM) (P<0.05) trout ventricular myocytes (Fig. 7A). Moreover, the Hill slopes differed, being -0.69 ± 0.08 and -0.97 ± 0.08 (P<0.05) in w.a. and c.a. trout, respectively. Differences in Ba\(^{2+}\) sensitivity of the native I\(_{K1}\) suggest temperature-related variation in omKir2 channel composition. To test this possibility omKir2.1 and omKir2.2 channels were expressed in COS-1 cells and their Ba\(^{2+}\) sensitivity was measured. Both genes formed K\(^+\) selective ion
channels with clear inward rectifying properties (Fig. 7B). Current amplitude at -120 mV was 2.5 ± 0.5 (mean ± S.E.M) and 2.2 ± 0.3 nA and reversal potential -70.2 ± 3.6 and -73.7 ± 2.0 mV for omKir2.1 (n=7) and omKir2.2 (n=10), respectively (P>0.05). No inward rectifying K⁺ current was detected in un-transfected COS-1 cells (data not shown). Sensitivity of omKir2.1 to Ba²⁺ was almost an order of magnitude lower (IC₅₀, 24.99 ± 7.40 µM) than in omKir2.2 (IC₅₀, 2.88 ± 0.42 µM) (P<0.05) (Fig. 7b), but Hill slopes did not differ between omKir2.1 and omKir2.2 (1.15 ± 0.22 vs. 0.997 ± 0.064; P>0.05). 

Single-channel conductance of the native Kir channels. To separate acute temperature effects from putative acclimation effects, single channel currents were measured at the same experimental temperature (11°C) for both acclimation groups. Single-channel conductance was practically the same for c.a. (18.0 ± 1.3 pS, n=24) and w.a. trout (18.0 ± 0.7 pS, n=32) (P>0.05) (Fig. 8). In contrast, there were clear temperature-related differences in gating kinetics. Time-constant for opening was about four times slower in w.a. trout (23 ms) than in c.a. trout (6 ms) (P<0.05), and three functions were needed to describe closing kinetics in w.a. trout (0.58, 5.37 and 11.3 ms), while two was sufficient in c.a. trout (0.96 and 6.04 ms) (Fig. 8).
DISCUSSION
Rainbow trout are moderately eurythermal fish which maintain activity throughout their thermal tolerance range (0-25°C). This requires thermal plasticity in various body functions including the cardiovascular system, i.e. the trout genome must be able to produce different phenotypes by differential gene expression according to thermal conditions of the environment (35). Shortening of the trout cardiac AP under chronic cold exposure (4°C) enables higher heart rates and larger cardiac output, and is indicative for compensatory changes in cardiac ion channel function (1, 12). The inward rectifier, \( I_{K1} \), does not contribute to AP shortening, as it is decreased by cold-acclimation (34; present study). \( I_{K1} \) has stabilizing effect on membrane potential, and therefore the reduction of \( I_{K1} \) might increase excitability of trout ventricular myocytes in the cold. Here we show that cardiac \( I_{K1} \) of the rainbow trout is produced by omKir2.1 and omKir2.2 channels and that \( Ba^{2+} \) sensitivity and single channels kinetics of the ventricular \( I_{K1} \) are modified by thermal acclimation. Based on different \( Ba^{2+} \) sensitivities of homomeric omKir2.1 and omKir2.2 channels, it is concluded that warm-acclimation increases either number or activity of omKir2.2 channels in trout ventricular myocytes. These functional changes are independent of omKir2 transcript levels which remained unaltered by thermal acclimation.

Expression of omKir2.1 and omKir2.2. Kir2 inward rectifier K\(^+\) channels are involved in a number of cellular functions, and hence they are ubiquitously distributed in the animal body (29). In addition to their expression in heart, liver and skeletal muscle, transcripts of omKir2.1 and omKir2.2 were especially abundant in brain, gill and kidney. Trout brain was the only organ where omKir2.2 was more abundant than omKir2.1, suggesting a significant role for omKir2.2 in trout brain function (27, 40). In the kidney, \( I_{K1} \) is involved in the regulation of renin secretion and consequently in blood pressure regulation and electrolyte homeostasis
Consistent with earlier findings from the rat kidney (15), omKir2.1 is also the main Kir2 transcript in the trout kidney. The relatively high expression of omKir2.1 in trout gill suggests its participation in ion regulation. Previously, Suzuki et al. (32) cloned a homolog of the mammalian Kir7.1 from the gills of the sea water adapted eel. Thus, the present study extends the expression of fish gill K^+ channels to the Kir2 subfamily.

**Sequence structures of omKir2.1 and omKir2.2.** Amino acid alignments show that omKir2.1 and omKir2.2 are relatively highly homologous to corresponding mammalian proteins. Even so, some interesting sequence differences exist, particularly in the transmembrane domains. For example, several amino acids in the M1 domain of the omKir2.2 are different from those of the mammalian Kir2.2. Growing evidence suggests that ion channels are regulated by their lipid environment (19, 28). Considering the large difference in body temperature between mammals and ectotherms, and associated differences in chemical composition of lipid membrane (3), it is not unexpected the amino acids facing towards the lipid bilayer differ. Kir2 channels are almost exclusively located in the cholesterol rich lipid rafts and their activity is strongly modulated by membrane cholesterol (28). As temperature acclimation has clear impact on cholesterol content of the lipid rafts in rainbow trout (41), the importance of membrane cholesterol on temperature-dependent regulation of the trout cardiac I_K1 warrants further study.

In the mammalian Kir2.1, glutamates E224 and E299 of the inner vestibule are involved in polyamine binding and positioned so that the access of polyamines to aspartate D172, the blocking site in the transmembrane pore region, is enhanced (38). All three amino acids exist both in omKir2.1 (D168, E220 and E295) and omKir2.2 (D178, E230 and E305). Magnesium, the other important intracellular blocking agent of Kir2 channels, binds to the serine S165 in the mammalian Kir2.1 (10). This serine is also conserved in trout Kir2
channels: S161 in omKir2.1 and S171 in omKir2.2. Taken together these findings indicate that amino acids critical for inward rectification are highly conserved from fish to mammals. This structural similarity is reflected in inward rectification of the \( I_{K1} \) which is composed of steep and shallow components in both vertebrate classes (24)(this study).

In contrast, amino acid residues involved in \( \text{Ba}^{2+} \) binding are partially different in mammalian and fish Kirs. In the mammalian Kir2.1, glutamate E125 in the extracellular loop between M1 and the pore region interacts with \( \text{Ba}^{2+} \) and facilitates its access to the plugging site, threonine T141 in the pore (2). In the omKir2.1, E125 is replaced by asparagine. The Kir2.2 subunit does not have the glutamate E125, only the threonine T141. In the omKir2.2, the T141 is replaced by serine. Analysis of cloned omKir2 channels showed that omKir2.2 is about 10 times more sensitive to \( \text{Ba}^{2+} \) than omKir2.1. This agrees with previous studies that have established that homomeric Kir2.1 channels are 5 to 10 fold more resistant to blockade by \( \text{Ba}^{2+} \) than Kir2.2 channels (16, 26). Interestingly, \( I_{K1} \) of the w.a. trout heart was almost tenfold more sensitive to \( \text{Ba}^{2+} \) than \( I_{K1} \) of the c.a. trout. This strongly suggests that warm acclimation increases either number or activity of the omKir2.2 subunits in ventricular myocytes of the trout heart. It is possible that in c.a. trout practically all Kir channels are composed of the omKir2.1 subunits and thus have low \( \text{Ba}^{2+} \) affinity, whereas in w.a. trout some portion of the channels might be homomeric Kir2.2 channels and therefore more sensitive to \( \text{Ba}^{2+} \). The low Hill slope value of the w.a. trout suggests more than one type of \( \text{Ba}^{2+} \) binding site with different affinities to \( \text{Ba}^{2+} \) might exist in w.a. trout Kirs.

**Chamber-specific differences of \( I_{K1} \).** Contradiction between \( I_{K1} \) density and Kir2 expression is especially striking regionally, i.e. between atrium and ventricle of the trout heart. In spite of about 30 times larger \( I_{K1} \) in ventricular than atrial myocytes, the summed expression levels of omKir2.1 and omKir2.2 transcripts were not higher in the ventricle. The higher relative
expression of omKir2.2 transcripts in the atrium cannot explain the large difference in $I_{K1}$ density. There are several possible explanations for this contradiction. First, mRNA levels do not necessarily correlate with protein levels, as has been shown for canine Kir2.1 and Kir2.3 (36). Thence, regional differences in the number of omKir2 proteins might exist. Even Kir2 protein expression does not always correlate well with the $I_{K1}$ density, suggesting that in addition to protein density of Kir channels, subcellular distribution of channels or other factors are involved in producing the whole-cell $I_{K1}$ (20). Second, polyamines regulate the rectification of Kir channels and affect the amplitude of both inward and outward $I_{K1}$. This raises the possibility that regional differences in $I_{K1}$ might be explained by variation in free polyamine pool. Recently, Yan et al. (39) have provided evidence that in the guinea-pig lower spermine and spermidine concentrations of the ventricular tissue might explain the twice larger $I_{K1}$ of the ventricular myocytes in comparison to the atrial $I_{K1}$. Atrioventricular differences in the density of $I_{K1}$ are, however, much more extreme in trout than guinea-pig, and therefore it is questionable whether differences in the amount of free polyamines alone could account for this large difference. Third, membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP$_2$) binds directly to Kir channels and activates them by stabilizing the open state (13). Therefore, one possible explanation for the atrioventricular difference in $I_{K1}$ density may lie in the PIP$_2$ regulation of Kir2-channels. All Kir2 channels are sensitive to PIP$_2$, but their affinity to PIP$_2$ differs. Kir2.1 has much higher affinity to PIP$_2$ than Kir2.2 and Kir2.3 (7). Accordingly, the low $I_{K1}$ current density in atrial myocytes could result either from a higher relative proportion of low-affinity Kir2.2 channels and/or low atrial PIP$_2$ level. Fourth, trout heart might have other Kir2 channels in addition to Kir2.1 and Kir2.2, which could account for the regional differences in $I_{K1}$. We were unable to detect Kir2.3 mRNA from the trout heart in the present study, but cannot completely exclude the possibility that Kir2.3 is involved in the formation of the trout $I_{K1}$.
**Functional studies.** As species-specific antibodies were not available for omKir2.1 and omKir2.2 to determine omKir2-protein expression, we tried to resolve regional and temperature-related differences of the I_{K1} with functional studies. Because homomeric Kir2 channels differ in respect to external K^+ dependence, single-channel conductance and Ba^{2+} sensitivity, electrophysiological studies could reveal functional differences which may underlie Kir subunit variation of the I_{K1} between c.a. and w.a. trout. The drastic difference in Ba^{2+} sensitivity of the I_{K1} between c.a. and w.a. trout, as discussed above (see page 20), is indicative of a higher omKir2.2 expression in w.a. ventricle. Temperature-related differences were also evident in single channel properties of the trout ventricular I_{K1}. The mammalian Kir channels differ in respect to their single channel conductance, Kir2.2 having the largest and Kir2.3 the smallest conductance, respectively, and Kir2.1 being intermediate between the two (16). The similarity of single-channel conductances of the native omKir2 channels in w.a. and c.a. trout does not provide any clues to the temperature-related changes in trout Kir channel composition. However, the kinetics of omKirs differed between w.a. and c.a. trout, the latter having faster gating. Whether this is due to higher omKir2.2 subunit expression, lipid-protein interaction, polyamine block or regulatory mechanisms, needs to be resolved by further experiments. Collectively, the electrophysiological studies suggest that functional heterogeneity of trout I_{K1} may originate from differential expression or assembly of omKir2.1 and omKir2.2 subunits in c.a. and w.a. trout.

A hallmark of the strong inward rectifier channels is the crossover of current-voltage curves upon increase in extracellular K^+. This crossover effect originates from the polyamine and Mg^{2+} dependent negative slope conductance of the I_{K1} and an increase in the peak outward current in high K^+ solution. Atrial and ventricular I_{K1} of the trout heart had distinctly different properties in that the current-voltage curves of the atrial I_{K1}, unlike those of the
ventricular $I_{K1}$, did not cross at different $K^+$ concentrations. The absence of the cross-over effect in atrial myocytes was caused by a decrease of the outward current in high $K^+$ solution. Previous studies on mammalian Kirs imply that the outward current magnitude increases in Kir2.1 but not in Kir2.3 channels (4). If this were the case Kir2.3 might be involved in the formation of trout atrial $I_{K1}$ and it was only due to the methodological limitations that Kir2.3 was not found in the trout heart. However, recordings of the $I_{K1}$ in cell-free outside-out patches indicate that in all Kir2 channels current-voltage relationships display the characteristic crossover effect (24). Accordingly, the absence of the cross-over effect cannot be ascribed to any specific Kir2 channel, but may be an outcome of an interaction of Kir2 channels with some intracellular regulatory factors. Taken together, the prominent differences in density and rectification of the trout $I_{K1}$ between atrial and ventricular myocytes cannot be ascribed to different omKir2 transcripts, but rather, may depend on differences in polyamines and other intracellular regulators that interact with omKir2 subunits.

**Conclusions.** Kir2 transcript expression suggests that rainbow trout cardiac $I_{K1}$ is composed of omKir2.1 and omKir2.2 subunits. The relative mRNA expression of the two subunits is not affected by temperature acclimation, while electrophysiological experiments indicate distinct functional properties of the $I_{K1}$ in c.a. and w.a. trout, especially in regard to $Ba^{2+}$ sensitivity and single channel kinetics. Considering the different $Ba^{2+}$ sensitivities of the homomeric omKir2 channels, functional variation of the native $I_{K1}$ suggests that either activity or number of the omKir2.2 channels is increased by warm-acclimation. Although the relative expression of the omKir2.2 was higher in atrium than ventricle, the atrioventricular differences in $I_{K1}$ density cannot be explained by expression levels of omKir2.1 and omKir2.2 transcripts and may involve differences in polyamine-dependent regulation of atrial and ventricular Kirs or differential co-assembly of the omKir2 subunits.
ACKNOWLEDGMENTS

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GRANTS

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FIGURE LEGENDS

Figure 1. Open reading frame nucleotide sequences and deduced amino acid sequences for the two alleles of omKir2.1 (A) and omKir2.2 (B) cloned from the rainbow trout heart. Dissimilar nucleotides are shown in lower case. Whole amino acid sequences are shown only for a-alleles (upper sequence) and only dissimilar amino acids for b-alleles (lower sequence). Numbering starts with the initiator methionine. The following functionally important domains are shown: the transmembrane regions M1 and M2 and the intervening pore domain (P) are indicated by a line; the conserved GYG motif for K⁺ selectivity filter is shown in bold; amino acids critical for Ba²⁺, polyamine and PIP₂ binding are indicated with ●, ▼ and *, respectively.

Figure 2. Alignment of vertebrate Kir2.1 (A) and Kir2.2 (B) amino acid sequences and their sequence homologies (C). Identical amino acids are shown with black shading and similar amino acids with gray shading. Species included and their GenBank accession numbers are for Kir2.1 human, h (NP_000882); guinea pig, gp (Q549A2); mouse, m (NP_032451); rat, r (NP_058992); chicken, c (P52186); rainbow trout, om (DQ435674 and DQ435675 for omKir2.1a and omKir2.1b, respectively) and for Kir2.2: human, h (NP_066292); guinea pig, gp (AAG17048); mouse, m (NP_034733); rat, r (P52188) and rainbow trout, om (DQ435676 and DQ435677 for omKir2.2a and omKir2.2b, respectively). Amino acids critical for Ba²⁺, polyamine and PIP₂ binding are indicated with ●, ▼ and *, respectively. (C) Nucleotide and amino acid sequence identities of the Kir2.1 and Kir2.2 subfamilies in different vertebrates. Nucleotide identities (%) are given in the top right and amino acid identities are given in the bottom left of each table.
Figure 3. Homology models of omKir2.1 and omKir2.2. Modeling has been done using KirBac3.1 and C-terminus of the mammalian Kir3.1 as templates for the transmembrane segments, and the C-termini of the mammalian Kir2.1 or Kir2.2 as templates for the C-terminus of omKir2.x. Different amino acids between trout and mammalian channel are shown in red.

Figure 4. Expression profiles (means ± S.E.M.) of omKir2.1 and omKir2.2 mRNAs in different tissues of the warm-acclimated rainbow trout (A), and in atrium and ventricle of warm- and cold-acclimated trout (B). The amounts of omKir2 mRNAs are normalized to the expression of the DnaJA2. An asterisk (*) indicates a statistically significant difference (P<0.05) between two groups.

Figure 5. Comparison of the whole-cell I\textsubscript{K1} of atrial and ventricular myocytes from c.a and w.a. rainbow trout heart at the same experimental temperature (11°C). (A) Current-voltage relationships indicate striking differences in current density and inward rectification between atrial and ventricular I\textsubscript{K1}. The inset at the left shows the atrial I\textsubscript{K1} density with an expanded y-axis scale. Bar graphs indicate the outward charge transfer by the I\textsubscript{K1} between the reversal potential and 0 mV. An asterisk indicates a statistically significant difference (P<0.05 between w.a. and c.a. trout. (B) Steady-state inward rectification of the I\textsubscript{K1} is composed of steep and shallow components which can be fitted as the sum of two Boltzmann functions. Relative sizes of the two components (A), slopes (S) and half-voltages (V\textsubscript{h}) of the inward rectification are indicated in the figure. The results are means from 9-12 myocytes.

Figure 6. Current-voltage relations of ventricular and atrial I\textsubscript{K1} in 5.4 mM and 10.8 mM extracellular K\textsuperscript{+} solution. Currents were elicited by voltage ramps (A). The cross-over effect
is evident in ventricular myocytes (B) but absent in atrial myocytes (C). The results are means of 10 myocytes for each group. Note the different y-axis scale for atrial and ventricular $I_{K1}$.

**Figure 7.** Dose-dependent block of the native $I_{K1}$ of rainbow trout ventricular myocytes and the cloned omKir2.1 and omKir2.2 $I_{K1}$ in COS-1 cells by $Ba^{2+}$. (A) Representative tracings indicating different sensitivities of cold- and warm-acclimated trout $I_{K1}$ to $Ba^{2+}$ (left) and mean results ($\pm$ S.E.M) from 8 myocytes (right). The numbers on the left show $Ba^{2+}$ concentration in $\mu$M. (B) Dose-dependent inhibition of cloned omKir2.1 and omKir2.2 channels in COS-1 cells (right). The results are means $\pm$ S.E.M. of 7 and 10 cells for omKir2.1 and omKir2.2, respectively. Tracings on the left show inward rectifying currents generated by the cloned channels.

**Figure 8.** Single-channel characteristics of the trout $I_{K1}$. (Top) Representative single channel recordings of the native omKir2 from w.a. trout at membrane potentials between -200 and -40 mV. Vertical lines on the left indicate a closed state of the channel. (Bottom) Mean current-voltage relation of single trout $I_{K1}$ channel (right), and distributions of open and closed times (left).
References


10. **Fujiwara Y and Kubo Y.** Ser165 in the second transmembrane region of the Kir2.1 channel determines its susceptibility to blockade by intracellular Mg²⁺. *J Gen Physiol* 120: 677-693, 2002.


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Table 1 PCR-primers used to clone the ORFs of omKir2.1 and omKir2.2. Nucleotide numbering starts with the initiator methionine.

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*AP1 and **AP2 primer of the GenomeWalker Kit
**Table 2.** Primers used for quantitative PCR. OmKir2.1 and omKir2.2 primers are specific for the 3’-end of the coding region of the genes, which is identical in the two alleles.

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**Table 3.** Effects of temperature and external potassium concentration ([K]_o) on the reversal potential of the inward rectifier current (E_{IK1}) and Nernstian reversal potential of potassium ions (E_K).

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An asterisk (*) indicates a statistically significant difference (P<0.05) from the value of the c.a. fish.