Cloning and expression of cardiac K\textsubscript{ir}2.1 and K\textsubscript{ir}2.2 channels in thermally acclimated rainbow trout

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Hassinen M, Paajanen V, Haverinen J, Eronen H, Vornanen M. Cloning and expression of cardiac K\textsubscript{ir}2.1 and K\textsubscript{ir}2.2 channels in thermally acclimated rainbow trout. Am J Physiol Regul Integr Comp Physiol 292: R2328–R2339, 2007. First published February 8, 2007; doi:10.1152/ajpregu.00354.2006.—Potassium currents are plastic entities that modify electrical activity of the heart in various physiological conditions including chronic thermal stress. We examined the molecular basis of the inward rectifier K\textsuperscript{+} current (I\textsubscript{K1}) in rainbow trout acclimated to cold (4°C, CA) and warm (18°C, WA) temperatures. Inward rectifier K\textsuperscript{+} channel (K\textsubscript{ir}2.1 and K\textsubscript{ir}2.2) transcripts were expressed in atrium and ventricle of the trout heart. K\textsubscript{ir}2.1 being the major component in both cardiac chambers. The relative expression of K\textsubscript{ir}2.2 was, however, higher (P < 0.05) in atrium than ventricle. The density of ventricular I\textsubscript{K1} was ~25% larger (P < 0.05) in WA than CA trout. Furthermore, the I\textsubscript{K1} of the WA trout was 10 times more sensitive to Ba\textsuperscript{2+} (IC\textsubscript{50} 0.18 ± 0.42 μM) than the I\textsubscript{K1} of the CA trout (1.17 ± 0.44 μM) (P < 0.05), and opening kinetics of single K\textsubscript{ir}2.2 channels was slower in WA than CA trout (P < 0.05). When expressed in COS-1 cells, the homomeric K\textsubscript{ir}2.2 channels demonstrated higher Ba\textsuperscript{2+} sensitivity (2.88 ± 0.42 μM) than K\textsubscript{ir}2.1 channels (24.99 ± 7.40 μM) (P < 0.05). In light of the different Ba\textsuperscript{2+} sensitivities of rainbow trout (om)K\textsubscript{ir}2.1 and omK\textsubscript{ir}2.2 channels, it is concluded that warm acclimation increases either number or activity of the omK\textsubscript{ir}2.2 channels in trout ventricular myocytes. The functional changes in I\textsubscript{K1} are independent of omK\textsubscript{ir}2 transcript levels, which remained unaltered by thermal acclimation. Collectively, these findings suggest that thermal acclimation modifies functional properties and subunit composition of the trout K\textsubscript{ir}2.2 channels, which may be needed for regulation of cardiac excitability at variable temperatures.

inward rectifier potassium channels; atrial myocytes; ventricular myocytes; thermal plasticity

STRONG INWARD RECTIFIER POTASSIUM (K\textsubscript{ir}) channels conduct inward currents at membrane potentials negative to the K\textsuperscript{+} reversal potential but permit only limited K\textsuperscript{+} efflux at more positive voltages (18, 37) based on the voltage-dependent block of the channels by intracellular Mg\textsuperscript{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\textsubscript{K1}) clamps the RMP close to K\textsuperscript{+} 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\textsuperscript{+} channels have been classified into seven subfamilies, K\textsubscript{ir}1–K\textsubscript{ir}7 (5, 18). Inward rectifiers of the mammalian heart are homomeric or heterotetrameric assemblies of K\textsubscript{ir}2.1–3 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 K\textsubscript{ir}2 subunits (11, 14, 36). It is, however, incompletely understood to what extent species-specific and regional differences in the properties of cardiac I\textsubscript{K1} are related to the relative expression of K\textsubscript{ir}2 subunits. For example, the atrioventricular differences in density and rectification of I\textsubscript{K1} have been explained either by differences in free polyamine concentrations (39) or by variable expression of functionally different K\textsubscript{ir}2 subunits (4). Therefore, situations in which the cardiac I\textsubscript{K1} is substantially modified within species by external or internal factors might shed new light on the relative importance of K\textsubscript{ir} subunits in the regulation of the cardiac I\textsubscript{K1}.

Body temperature of ectothermic animals can vary quite considerably, e.g., because of seasonal changes in ambient temperature that set 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 that is associated with strong upregulation of the delayed-rectifier K\textsuperscript{+} current (I\textsubscript{Ks}) (12, 34). Curiously, at the same time the density of the strong inward rectifier K\textsuperscript{+} current, the I\textsubscript{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 depolarizing Na\textsuperscript{+} current. The objective of this study was to examine the molecular basis of the rainbow trout I\textsubscript{K1} and to find out whether differences in molecular composition could explain temperature-induced changes and regional differences in I\textsubscript{K1} density of the trout heart. To this end, we cloned K\textsubscript{ir}2.1 and K\textsubscript{ir}2.2 genes, determined their expression, and measured cardiac I\textsubscript{K1} at the whole cell and single-channel levels from thermally acclimated trout.

MATERIALS AND METHODS

Fish

Rainbow trout (Oncorhynchus mykiss) were donated by a local fish farm (Kontiolahiti, Finland). In the laboratory, the fish were kept in 500-liter stainless steel tanks with aerated groundwater continuously delivered to the tanks at ~0.5 l/min and were acclimated to 4°C (cold acclimation, CA) or 18°C (warm acclimation, WA) for at least 3 wk 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 and the Ministry of Agriculture and Forest Affairs (Finland).

**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 pieces 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, CA) according to the manufacturer’s instructions. DNA was extracted from liver by the method of Sambrook et al. (30). RNA and DNA were qualified by gel electrophoresis and quantified by UV spectrophotometry.

Cloning of K\textsubscript{r}.2.1 and K\textsubscript{r}.2.2 open reading frames from rainbow trout heart. First-strand cDNA was prepared from total cardiac RNA and was treated with RQ1 RNase-free DNase (Promega, Madison, WI). Reverse transcription was performed with M-MuLV Reverse Transcriptase RNase H– (Finnzymes, Espoo, Finland) and an oligo(dT)	extsubscript{18} primer. Partial cDNAs corresponding to rainbow trout omK\textsubscript{r}.2.1 and omK\textsubscript{r}.2.2 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 MgCl\textsubscript{2}, 15 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 0.1% Triton X-100, each dNTP at 200 μM, 1 U of DyNAzyme EXT (Finnzymes), 2 μl of cDNA, and 5 pmol of each primer. Amplification was performed under PCR conditions with 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 under PCR conditions with initial denaturation at 96°C for 2 min followed by 35 cycles at 95°C for 30 s, 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 open reading frames (ORFs) (Table 1). Oligo(dT) primer was used to clone the 3′-ends of the genes.

The Genome Walker Kit (Clontech, Palo Alto, CA) 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 with 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 with GSP2 and AP2 (Table 1). The second PCR was performed to the identical to the first, except that the annealing temperature was 68°C for the first six cycles and 63°C for the rest. The sequences were confirmed by cloning the whole coding region of the K\textsubscript{r}.2.1 and K\textsubscript{r}.2.2 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 with the Qiex II gel extraction kit (Qiagen, Valencia, CA), cloned into the pGEM-T Easy Vector (Promega), and sequenced by an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

**Southern blotting.** Ten micrograms of genomic DNA was digested to completion with EcoRI, HindIII, or NcoI. Genomic 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 nucleotides 1026–1284 and 1119–1341 from omK\textsubscript{r}.2.1 and omK\textsubscript{r}.2.2 plasmids, respectively. Probes were labeled with [α-\textsuperscript{32}P]dCTP, using Ready-To-Go DNA Labeling Beads, and purified by ProbeQuant G-50 Micro Columns (all obtained from Amersham Biosciences). Nylon membranes were prehybridized 2 h at 42°C in 20 ml of prehybridization solution containing 50% formamide, 3× SSC (0.45 M NaCl, 0.045 M Na citrate), 2.5× Denhardt’s solution (0.05% BSA, 0.05% polyvinylpyrrolidone, 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 1× SSC-0.5% SDS solution, followed by a final wash at 65°C for at least 15 min with 0.1× SSC-1% SDS solution. Signals were detected by autoradiography.

**Quantitative RT-PCR.** Total RNA was extracted from heart, brain, gill, kidney, liver, and skeletal muscle of two WA trout for organ-specific gene expression and from atrium and ventricle of three WA and three CA trout for temperature acclimation-dependent gene expression. Each RNA sample (2 μg) was treated with RQ1 RNase-free DNase (Promega), and first-strand cDNA synthesis was performed with random hexamers and MuLV 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 performed for every sample. Quantitative PCR (qPCR) was performed with the DyNAamo HS SYBR Green qPCR Kit (Finnzymes) and the Chromo4 Continuous Fluorescence Detector (MJ Research, Waltham, MA), using primer pairs listed in Table 2 (primers for DnaJA2 were a kind gift from Dr. Aleksei Krasnov, University of Kuopio, Finland). 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 containing the product of cDNA synthesis performed without RT enzyme, were included in every experiment. DnaJA2 was selected as a reference gene. DnaJ is a small chaperonin, which belongs to the

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Amplified Region</th>
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<tr>
<td>K\textsubscript{r}.2.1</td>
<td>ATGGGCGGAGTGAGAGAGAAGACAGCGCTAC</td>
<td>GCCATGGCGRGTGTCGGTCTACCATGCGCC</td>
<td>1–911</td>
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<td>K\textsubscript{r}.2.1</td>
<td>GGATTTGAGATGATGCTGATCATAGGAGAA</td>
<td>TCATTACCTTGAATGCAACGCCYYA</td>
<td>858–1278</td>
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<td>K\textsubscript{r}.2.1</td>
<td>GCGGCGAGATGAACCCAGAACAGAATA</td>
<td>TTTT TTTTTTT TTTTTT</td>
<td>1178–UTRx</td>
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<tr>
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Nucleotide numbering starts with the initiator methionine. ORF, open reading frame; UTR, untranslated region. *AP1, †AP2 primer of the Genome Walker Kit.
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 DnaJ2A as an internal standard was further confirmed by a qPCR experiment using equal amounts of RNA extracted from the tissues of CA and WA trout as a template.

**Modeling of trout K_\text{as.}** Homology models of omK_2.1 and omK_2.2 channels were constructed with the DeepView 3.7 program (GlauxoSmithKline) and a SWISS-MODEL server. K_\text{as} model (PDB code 1XL4) and COOH terminus of mammalian K_\text{as} 3.1 (PDB code 1N9P) (21) were used as templates for the transmembrane segments (G50-N185 or G55-N195) and the COOH terminus (N185-A358 or N195-A376) of omK_2.2, respectively. The fourfold rotational symmetry of the channels was adopted manually by using K_\text{as} Bac3.1 and KcsA (PDB code 1BL8) (6) as templates.

**Electrophysiological Methods**

**Isolation of cardiac myocytes.** Atrial and ventricular myocytes were isolated by 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 cannula was brought through the bulbus arteriosus into the ventricle, and the heart was retrogradely perfused with a nominally Ca^{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 with a peristaltic pump. The Ca^{2+}-free saline contained (mM) 100 NaCl, 10 KCl, 1.2 KH\textsubscript{2}PO\textsubscript{4}, 4 MgSO\textsubscript{4}, 50 taurine, 20 glucose, and 10 HEPES (pH adjusted to 7.2 with KOH). 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^{2+}-free solution, and myocytes were released by agitation tissue pieces through the opening of a Pasteur pipette. Isolated myocytes were stored at 6°C and used within 8 hr of the isolation.

**Whole cell patch clamp.** Whole cell voltage-clamp recording of the I_{K1} was performed with an Axopatch 1-D (Axon Instruments) or an EPC-9 (HEKA Instruments) amplifier (22). Myocytes were superfused in a small recording chamber with a precooled external saline solution at the rate of 1.5–2.0 ml/min. Temperature was adjusted to 4°C and 18°C for CA and WA trout, respectively, with circulating solution at the rate of 1.5–2.0 ml/min. Temperature was adjusted to pH 7.2 with KOH giving a mean (\textpm S.E.) pipette resistance of 2.81 \pm 0.07 \textOmega. After gaining access to the cell, pipette capacitance (7.55 \pm 0.14 pF, n = 48) and series resistance (9.96 \pm 0.48 M\textOmega, 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 \textmu M, Tocris Cookson), nifedipine (10 \textmu M, Sigma), glibenclamide (10 \textmu M, Sigma), and E-4031 (1 \textmu M, Alomone Labs) to block Na\textsuperscript{+}, Ca\textsuperscript{2+}, and ATP-sensitive K\textsuperscript{+} current and I_{K1}, respectively. The I_{K1} was elicited every 5 s by repolarizing ramps or square wave pulses from a holding potential of \textpm 80 mV. Ba^{2+} inhibition of the I_{K1} was determined in the presence of cumulatively added concentrations of BaCl\textsubscript{2} (10^{-9}–10^{-4} M). The cell was exposed to each Ba^{2+} concentration for 4 min. Dose-response curves were fitted with the Hill equation I = I_{\text{min}} + \frac{I_{\text{max}} \cdot [\text{Ba}^{2+}]}{IC_{50} + [\text{Ba}^{2+}]}, 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 that 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 depolarizing voltages relative to the unblocked current.** The unblocked (non-rectifying) inward current was obtained from the current-voltage relationship relative to -120 mV and the reversal potential (E_{rev}) of the I_{K1} and extrapolated to the voltage area of inward rectification. Scattering data points around the E_{rev} were omitted, and the current was fitted as the sum of two Boltzmann functions: I_{K1} = A1 \times \frac{\text{exp}(V - V_{0.5}1)/S_1 + A2 \times \text{exp}(V - V_{0.5}2)/S_2}{A1 + \text{exp}(V - V_{0.5}1)/S_1 + A2 + \text{exp}(V - V_{0.5}2)/S_2}, where A1, V_{0.5}, and S 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 omK_2 channels were recorded in the cell-attached configuration with an EPC-9 amplifier and Pulse software and analyzed with TAC, TACFIT (Bruxton), and SigmaPlot 6.0 (SPSS) programs as described previously (23). Pipettes were pulled from thick-walled borosilicate glass (Garner, Claremont, CA) with a two-state puller (PP-83, Narishige, Tokyo, Japan), coated with Sylgard (WPI), and fire polished on a microforge (MF-83, Narishige). The mean resistance of the pipettes was 9.2 \pm 0.6 M\textOmega when they were filled with K\textsuperscript{+}-based solution (mM: 134 KCl, 1.8 CaCl\textsubscript{2}, 2 MgCl\textsubscript{2}, 10 glucose, and 10 HEPES adjusted to pH 7.6 with KOH ([K\textsuperscript{+}] = 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 \textpm 80 mV. Distributions of open and closed times were obtained from 20-s recordings at \textpm 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 omK_{2.1} genes in COS-1 cells.** ORF sequences for putative ion channel-forming genes omK_{2.1} and omK_{2.2} 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, Bio-Whittaker, Cambrex Verviers, Belgium) containing 10% fetal bovine serum (FBS; EuroClone, Milan, Italy) and 3000 U/ml of both penicillin and streptomycin (EuroClone). Cells were transiently cotransfected with PEGFP-N1 (Clontech) and either omK_{2.1} or omK_{2.2}-pcDNA3.1/Zeo(+), using Effectene Transfection Reagent (Qiagen).

### Table 2. Primers used for quantitative PCR

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<tr>
<th>Gene</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
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<tr>
<td>K_{2.1}</td>
<td>GCCGAGATGGTAAACCCGACAGACAATA</td>
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<td>TTGTTATGGGAGAAGGGGAAGAGG</td>
<td>TGGGGCGCGGTTTTGTTTAGT</td>
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omK_{2.1} and omK_{2.2} primers are specific for the 3’ end of the coding region of the genes, which is identical in the 2 alleles.
Function of the cloned channels was tested with the whole cell patch-clamp method at room temperature (20°C) 48–64 h after transfection. The same external and internal solutions were used as in measurement of endogenous currents (see Whole cell patch clamp).

Statistics

Differences between mean values from WA and CA 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 nonparametric Mann-Whitney test. A \( P \) value of 0.05 was regarded as a limit of statistical significance.

RESULTS

Rainbow Trout \( K_{ir} \) 2.1 and \( K_{ir} \) 2.2 Genes

Coding regions of \( K_{ir} \) 2.1 (KCNJ2) and \( K_{ir} \) 2.2 (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 \( K_{ir} \) 2.1 and \( K_{ir} \) 2.2 were found and named om\( K_{ir} \)2.1a, om\( K_{ir} \)2.1b, om\( K_{ir} \)2.2a, and om\( K_{ir} \)2.2b. The nucleotide sequences for om\( K_{ir} \)2.1a and om\( K_{ir} \)2.1b ORF include 1,278 and 1,284 bp, respectively, om\( K_{ir} \)2.1a lacking nucleotides 93–98 of the om\( K_{ir} \)2.1b (Fig. 1A). The nucleotide sequences for om\( K_{ir} \)2.2a and om\( K_{ir} \)2.2b ORF include 1,341 bp coding 446 amino acid residues (Fig. 1B). Nucleotide sequences of the two alleles are 96.6% and 97.4% identical for om\( K_{ir} \)2.1 and om\( K_{ir} \)2.2, respectively, and show even higher amino acid identity. Southern blot hybridization of the genomic DNA with om\( K_{ir} \)2.1 or om\( K_{ir} \)2.2 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 om\( K_{ir} \)2.1 and om\( K_{ir} \)2.2 with mammalian \( K_{ir} \) proteins reveal about 82% and 66% sequence similarity, respectively (Fig. 2). The lowest homology with mammalian \( K_{ir} \)s is seen in the COOH terminus and in the M1-P loop (“the turret”), both in om\( K_{ir} \)2.1 and om\( K_{ir} \)2.2. Interestingly, the M1 transmembrane domain of om\( K_{ir} \)2.2 also differs greatly from the corresponding mammalian sequence. A structural model of om\( K_{ir} \)2.2 (Fig. 3) shows that the differences are located in the lipid bilayer-facing side of the membrane-spanning \( \alpha \)-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 \( K_{ir} \)2.2—the so-called glycine hinge—is replaced by serine (S173) in om\( K_{ir} \)2.2. The most prominent structural difference between om\( K_{ir} \)2.1 and om\( K_{ir} \)2.2 is the extracellular mouth formed by the M1-P loop, which in om\( K_{ir} \)2.1 is oriented straight upward and does not form any secondary structures, whereas in om\( K_{ir} \)2.2 it forms a short \( \beta \)-sheet and is turned toward the membrane, being possibly in contact with the lipid bilayer.

Functionally important sequences that are common to all \( K_{ir} \) subfamilies, including the pore domain with \( K^{+} \) selectivity signature sequence (GYG), were identified in the rainbow trout \( K_{ir} \)2.2s with multiple sequence alignments (Figs. 1 and 2). Amino acids, critical for polyamine, Mg\( ^{2+} \), and phosphatidylinositol 4,5-bisphosphate (PIP\( \_ \)) binding were the same as in mammalian \( K_{ir} \)2.1 and \( K_{ir} \)2.2 channels. In contrast, differences existed in extracellular Ba\( ^{2+} \) binding sites between fish and mammalian \( K_{ir} \): in om\( K_{ir} \)2.1 glutamate E125 was replaced with asparagine, and in om\( K_{ir} \)2.2 threonine T141 was replaced with serine.

Expression of om\( K_{ir} \)2 mRNAs

The expression of om\( K_{ir} \)2.1 and om\( K_{ir} \)2.2 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 CA and WA trout as a template. Both om\( K_{ir} \)2.1 and om\( K_{ir} \)2.2 were expressed in all examined organs including heart, brain, gill, kidney, liver, and skeletal muscle (Fig. 4A). Of the two genes, om\( K_{ir} \)2.1 was more abundant in all other organs except the brain, where om\( K_{ir} \)2.2 was the dominant transcript. The expression of om\( K_{ir} \)2.2 in the brain was 10 times higher than in the atrium and much lower in ventricle, gill, skeletal muscle, and liver. The expression of om\( K_{ir} \)2.1 was highest in the kidney, being 7.1 times higher than in the atrium. In general, the total amount of om\( K_{ir} \)2 mRNAs in cells was very low in all tissues, e.g., in the atrium only ~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 because there are striking differences in density and rectification of the \( I_{K1} \) between atrium and ventricle, \( K_{ir} \)2 expression was studied in both cardiac chambers of WA and CA trout (Fig. 4B). Although om\( K_{ir} \)2.1 was the dominant isoform in both chambers, the atrium expressed relatively more om\( K_{ir} \)2.2 than the ventricle (\( P = 0.021 \)), the transcripts of om\( K_{ir} \)2.1 being 2.7, 1.7, 7.3, and 6.1 times more abundant than om\( K_{ir} \)2.2 mRNA in CA atrium, WA atrium, CA ventricle, and WA ventricle, respectively. Quite surprisingly, no significant differences in om\( K_{ir} \)2 expression existed between CA and WA trout hearts.

Density and Inward Rectification of \( I_{K1} \)

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 WA than CA fish (Fig. 5A). The charge transfer of the outward current between \( E_{K1} \) and 0 mV did not, however, differ between CA and WA 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 like the mammalian \( I_{K1} \) consists of a steep and a shallow component (Fig. 5B). The weaker inward rectification of atrial \( I_{K1} \) compared with 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. 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 \( K_{ir} \) channels in ventricular myocytes.

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Different Kir2 channels vary in regard to their response to external K⁺ and Ba²⁺. To examine putative atrioventricular differences in Kir composition, K⁺ dependence, and Ba²⁺ sensitivity of the rainbow trout, cardiac Iₖ¹ were determined by whole cell patch clamp. Doubling of external K⁺ concentration ([K⁺]₀) from 5.4 to 10.8 mM resulted in a Nernstian shift of the (Eᵢₖ¹) from about -80 to -60 mV (Table 3), and 1.64- to 2-fold increase in peak Iₖ¹ amplitude (n = 9).
Fig. 2. Alignment of vertebrate inward rectifier K⁺ channel (Kᵢ)₂.1 (A) and Kᵢ₂.2 (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), and rainbow trout (om; DQ435674 and DQ435675 for omKir2.1a and omKir2.1b, respectively) and for Kir2.2 human (NP_066292), guinea pig (AAG17048), mouse (NP_034733), rat (P52188), and rainbow trout (DQ435676 and DQ435677 for omKir2.2a and omKir2.2b, respectively). Amino acids critical for Ba²⁺, polyamine, and PIP₂ binding are indicated with F, c₁, and *, respectively. C: nucleotide and amino acid sequence identities of the Kir2.1 (top) and Kir2.2 (bottom) subfamilies in different vertebrates. Nucleotide identities (%) are given at top right and amino acid identities are given at bottom left of each table.
The trout atrial $I_{K1}$ was so small that the effects of external $\text{Ba}^2+\$ concentration ([Ba$^2+\$]_o) could be reliably measured only in ventricular myocytes. The [Ba$^2+\$] required for half-maximum block of the $I_{K1}$ was almost an order of magnitude lower in WA (IC$_{50}$ 0.18 ± 0.13 μM) than in CA (1.17 ± 0.15 μM) ($P < 0.05$) trout ventricular myocytes (Fig. 7A). Moreover, the Hill slopes differed, being $-0.69 \pm 0.08$ and $-0.97 \pm 0.08$ ($P < 0.05$) in WA and CA trout, respectively. Differences in $\text{Ba}^2+$ sensitivity of the native $I_{K1}$ suggest temperature-related variation in omKir2.2 channel composition. To test this possibility omKir2.1 and omKir2.2 channels were expressed in COS-1 cells and their $\text{Ba}^2+$ sensitivity was measured. Both genes formed $\text{K}^+$-selective ion channels with clear inward rectifying properties (Fig. 7B). Current amplitude at $-120$ mV was $2.5 \pm 0.5$ (mean ± SE) and $2.2 \pm 0.3$ nA and reversal potential $-70.2 \pm 3.6$ and $-73.7 \pm 2.0$ mV for omKir2.1 ($n = 7$) and omKir2.2 ($n = 10$), respectively ($P > 0.05$). No inward rectifying $\text{K}^+$ current was detected in nontransfected COS-1 cells (data not shown). Sensitivity of omKir2.1 to $\text{Ba}^2+$ was almost an order of magnitude lower (IC$_{50}$ 24.99 ± 7.40 μM) than in omKir2.2 (IC$_{50}$ 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 Native $K_r$ 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 CA (18.0 ± 1.3 pS, $n = 24$) and WA (18.0 ± 0.7 pS, $n = 32$) ($P > 0.05$) trout (Fig. 8). In contrast, there were clear temperature-related differences in gating kinetics. The time constant for opening was about four times slower in WA trout (23 ms) than in CA trout (6 ms) ($P < 0.05$), and three functions were needed to describe closing kinetics in WA trout (0.58, 5.37, and 11.3 ms), while two were sufficient in CA trout (0.96 and 6.04 ms) (Fig. 8).


DISCUSSION

Rainbow trout are moderately eurythermal fish that 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 because it is decreased by cold acclimation (Ref. 34; present study). \( I_{K1} \) has a 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-channel kinetics of the ventricular \( I_{K1} \) are modified by thermal acclimation. On the basis of the 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

Ko2 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 in which 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 (25). Consistent with earlier findings from the rat kidney (15), omKir2.1 is also the main \( K_{o2} \) 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 Ko7.1 from the gills of the seawater-adapted eel. Thus the present study

Table 3. Effects of temperature and external potassium concentration on reversal potential of inward rectifier current and Nernstian reversal potential of potassium ions

<table>
<thead>
<tr>
<th>Temperature</th>
<th>( [K_0] )</th>
<th>( E_{K1} )</th>
<th>( E_{Nernst} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>WA trout ventricle at +18°C</td>
<td>5.4 mM</td>
<td>-78.2±1.4 (–81.6)</td>
<td>-62.3±1.4 (–64.2)</td>
</tr>
<tr>
<td>CA trout ventricle at +4°C</td>
<td>10.8 mM</td>
<td>-73.4±1.4 (–77.7)</td>
<td>-59.5±1.4 (–61.1)</td>
</tr>
<tr>
<td>WA trout atrium at +18°C</td>
<td>5.4 mM</td>
<td>-81.8±2.5 (–81.6)</td>
<td>-61.6±0.9 (–64.2)</td>
</tr>
<tr>
<td>CA trout atrium at +4°C</td>
<td>10.8 mM</td>
<td>-77.5±3.2 (–77.6)</td>
<td>-57.0±1.2 (–61.1)</td>
</tr>
</tbody>
</table>

Values (in mV) are means ± SE. \( [K_0] \), external potassium concentration; \( E_{K1} \), reversal potential of inward rectifier current; \( E_{Nernst} \), Nernstian reversal potential of potassium ions; WA, warm acclimated; CA, cold acclimated. *Statistically significant difference (\( P < 0.05 \)) from CA fish value.
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 their activity is strongly modulated by 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. 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 (Ref. 24; this study).

In contrast, amino acid residues involved in Ba²⁺ 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 Ba²⁺ and facilitates its access to the plugging site, threonine T141 in the pore (2). In omKir2.1, E125 is replaced by asparagine. The Kir2.2 subunit does not have the glutamate E125, only the threonine T141. In omKir2.2, T141 is replaced by serine.

Analysis of cloned omKir2 channels showed that omKir2.2 is 10 times more sensitive to Ba²⁺ 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 Ba²⁺ than Kir2.2 channels (16, 26). Interestingly, I_K1 of the WA trout heart was almost 10-fold more sensitive to Ba²⁺ than I_K1 of the CA 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 CA trout practically all Kir channels are composed of the omKir2.1 subunits and thus have low Ba²⁺ affinity, whereas in WA trout some portion of the channels might be homomeric Kir2.2 channels and therefore more sensitive to Ba²⁺. The low Hill slope value of the WA trout suggests that more than one type of Ba²⁺ binding site with different affinities to Ba²⁺ might exist in WA 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. Despite ~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). Thus regional differences in the number of omKir2 proteins might exist. Even Kir2 protein expression does not always correlate well with I_K1 density, suggesting that, in addition to protein density of Kir channels, subcellular distribution of channels or other factors is involved in producing the whole cell I_K1 (20). Second, polyamines regulate the rectifica-
tion 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) 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 compared with 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, the membrane phospholipid PIP2 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 PIP2 regulation of Kir2 channels. All Kir2 channels are sensitive to PIP2, but their affinity to PIP2 differs. Kir2.1 has much higher affinity to PIP2 than Kir2.2 and Kir2.3 (7). Accordingly, the low $I_{K1}$ density in atrial myocytes could result from a higher relative proportion of low-affinity Kir2.2 channels and/or low atrial PIP2 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
that may underlie Kir subunit variation of the physiological studies could reveal functional differences in temperature-related changes in trout Kir channel composition. The drastic difference in Ba2⁺ sensitivity of the trout ventricles 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

omKir2 transcript expression suggests that rainbow trout cardiac I\(_{K_1}\) 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\(_{K_1}\) in CA and WA trout, especially in regard to Ba²⁺ sensitivity and single-channel kinetics. Considering the different Ba²⁺ sensitivities of the homomeric omKir2 channels, functional variation of the native I\(_{K_1}\) 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\(_{K_1}\) 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 Kᵦᵦ₆ or differential coassembly of the omKir2 subunits.

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