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A novel inwardly rectifying K^+ channel, Kir2.5, is upregulated under chronic cold stress in fish cardiac myocytes

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SUMMARY

A new member of the inward-rectifier K^+ channel subfamily Kir2 was isolated and characterised from the crucian carp (*Carassius carassius*) heart. When expressed in COS-1 cells this 422 amino acid protein produced an inward-rectifying channel with distinct single-channel conductance, mean open time and open probability. Phylogenetic sequence comparisons indicate that it is not homologous to any known vertebrate Kir channel, yet belongs to the Kir2 subfamily. This novel crucian carp channel increases the number of vertebrate Kir2 channels to five, and has therefore been designated as ccKir2.5 (cc for *Carassius carassius*). In addition to the ccKir2.5 channel, the ccKir2.2 and ccKir2.1 channels were expressed in the crucian carp heart, ccKir2.1 being present only in trace amounts (<0.8% of all Kir2 transcripts). Whole-cell patch clamp in COS-1 cells demonstrated that ccKir2.5 is a stronger rectifier than ccKir2.2 or ccKir2.1, and therefore passes weakly outward current. Single-channel conductance, mean open time and open probability of ccKir2.5 were, respectively, 1.6, 4.96 and 4.17 times as large as that of ccKir2.2. ccKir2.5 was abundantly expressed in atrium and ventricle of the heart and in skeletal muscle, but was a minor component of Kir2 in brain, liver, gill and kidney. Noticeably, ccKir2.5 was strongly responsive to chronic cold exposure. In fish reared at 4°C for 4 weeks, ccKir2.5 mRNA formed 59.1±2.1% and 65.6±3.2% of all ccKir2 transcripts in atrium and ventricle, respectively, while in fish maintained at 18°C the corresponding transcript levels were only 16.2±1.7% and 23.3±1.7%. The increased expression of ccKir2.5 at 4°C occurred at the expense of ccKir2.2, which was the main Kir2 isoform in 18°C acclimated fish. A cold-induced increase in the slope conductance of the ventricular I_{K1} from 707±49 to 1001±59 pS pF^{-1} (P<0.05) was thus associated with an isoform shift from ccKir2.2 towards ccKir2.5, suggesting that ccKir2.5 is a cold-adapted and ccKir2.2 a warm-adapted isoform of the inward-rectifying K^+ channel.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/211/13/2162/DC1

Key words: temperature acclimation, fish heart, repolarisation.

INTRODUCTION

Inward-rectifier potassium (Kir2) channels conduct large inward currents at membrane potentials negative to the K^+ reversal potential but, due to voltage-dependent block by Mg^{2+} and polyamines, permit only limited outward current at depolarised membrane potentials, thus generating a typical voltage dependence of the inward-rectifier current (I_{K1}) (Matsuda et al., 1987; Vandenberg, 1987; Ficker et al., 1994; Lopatin et al., 1994; Fakler et al., 1995; Kurata et al., 2006). The small outward current is, however, physiologically important in stabilising resting membrane potential close to the K^+ equilibrium potential and in regulating the duration of the cardiac action potential by phase-3 repolarisation (Shimoni et al., 1992; Lopatin and Nichols, 2001).

The Kir2 subfamily has four known members (Kir2.1–2.4), three of which (Kir2.1–2.3) are expressed in the heart. Kir2.1 is the dominating subunit in the mammalian heart, although Kir2.2 and Kir2.3 channels are variably expressed depending on the animal species and cardiac chamber (Liu et al., 2001; Preisig-Müller et al., 2002; Zobel et al., 2003; Dhamoon et al., 2004; Hume and Uehara, 1985; Wang et al., 1998; Melnyk et al., 2002). Each homotetrameric Kir2 channel has distinct kinetics, conductance and sensitivity to polyamines, enabling the regulation of I_{K1} density and inward rectification by variable expression and coassembly of the Kir2 subunits (Périer et al., 1994; Takahashi et al., 1994; Ishihara and Yan, 2007). Human diseases due to Kir2 mutations and knockout animal models of the Kir2 channels have demonstrated a vital role of I_{K1} in normal cardiac function (Plaster et al., 2001; Zaritsky et al., 2001). Furthermore, expression and function of the cardiac I_{K1} is altered in hypoxia, ischaemia and disease states (Piao et al., 2007; Liu et al., 2007; Ten Eick et al., 1992) indicating that Kir2 channels are plastic entities and are probably involved in cardiac remodelling in pathophysiological conditions.

Unlike mammalian hearts, the hearts of ectothermic vertebrates are naturally exposed to large temperature changes, which impose special requirements for cardiac ion channel function to maintain proper excitability and to prevent cardiac arrhythmias. For example, crucian carp (*Carassius carassius* L.) can tolerate temperatures between 0 and 38°C (Horoszewicz, 1973) and in their natural environment face seasonal temperature changes of over 20°C (Vornanen and Paajanen, 2004). Owing to its excellent thermal tolerance, crucian carp heart is an interesting subject for testing the plasticity of vertebrate cardiac phenotype and its molecular basis, in particular the responses of cardiac ion channels to temperature change. The objective of this study was to examine the contribution of different Kir2 subunits to putative temperature-induced changes in the cardiac I_{K1}. Three Kir2 channel genes were found in crucian carp heart, two of them being homologues of the mammalian Kir2.1 and Kir2.2 channel genes. The third one was a new, previously

unknown member of the *Kir2* subfamily and was designated as *ccKir2.5* (cc for *Carassius carassius*). The expression of *ccKir2.5* was strongly increased in the cold-acclimated fish (4°C), suggesting that the novel ccKir2.5 is intimately involved in cardiac adjustment to low temperature conditions by increasing the density of I_{K1}.

MATERIALS AND METHODS

Fish

Crucian carp (*Carassius carassius* L.; 20–50 g in body mass, *N*=46) were caught from a small lake and in the lab were reared in temperature-controlled 500 l stainless steel tanks at either 4°C (cold acclimation) or 18°C (warm acclimation) for a minimum of 4 weeks. Fish were stunned by a sharp blow to the head and killed by cutting the spine, and the organs needed in experiments were prepared. All experiments were carried out with the consent of the national committee for animal experimentation.

Molecular methods

Extraction of RNA and DNA

Atrium, ventricle and brains and a piece of gill, muscle, liver and kidney were homogenised under liquid nitrogen and RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA was extracted from liver by the method of Sambrook et al. (Sambrook et al., 1989). The quality and quantity of RNA and DNA were monitored by agarose gel electrophoresis and UV spectrophotometry, respectively.

Molecular cloning of cardiac *Kir* genes

The open reading frames (ORFs) for crucian carp *Kir2.1*, *Kir2.2* and *Kir2.5* and a 437 bp fragment for crucian carp *DnaJA2* gene were cloned by reverse transcriptase-PCR (RT-PCR). First strand cDNA synthesis was carried with RNAseH$^+$ (Finnzymes, Espoo, Finland) using random hexamers or oligo(dT) primers. Degenerative primers designed to the conserved regions of mammalian *Kir2.1*, *Kir2.2* and *Kir2.3* genes were used to get partial cDNA clones of crucian carp *Kir2* genes (Table 1). In spite of several trials, no *Kir2.3* products were found in the crucian carp heart. The first fragment of an unidentified *Kir* gene was obtained with degenerative primers to mammalian *Kir2.1*. New primers for further cloning were designed for each clone on the basis of sequences obtained. PCR was performed using a PTC-200 DNA Engine Cycler (MJ Research, Waltham, MA, USA) and conditions described previously (Hassinen et al., 2007). Oligo(dT) primers and a 3′-RACE kit (Invitrogen) were used to clone the 3′ end of the *ccKir2.2* and *ccKir2.5* gene, respectively (Table 1). Genome Walker kit (Clontech, Palo Alto, CA, USA) was used for cloning the 5′ ends. Four genomic libraries from crucian carp DNA were constructed and used as templates in PCR as previously described (Hassinen et al., 2007). Finally, the sequences were confirmed by cloning the whole coding region of the *ccKir2.1*, *ccKir2.2* and *ccKir2.5* genes.

All PCR products were analysed by agarose gel electrophoresis, extracted from the gel by Qiaex II gel extraction kit (Qiagen, Valencia, CA, USA) and cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). DNA sequencing was conducted using the ABI PRISM BigDye Terminator cycle sequencing kit v2.0 (Applied Biosystems, Foster City, CA, USA) and the reactions were analysed using ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Phylogenetic analysis

The whole coding sequences of ccKir2 proteins, zebrafish Kir proteins and all known mouse Kir proteins were aligned by ClustalW and a phylogenetic tree was constructed in ClustalX (http://www.clustal.org/download/current/) using the neighbour-joining method. Sequence positions containing gaps in any of the *Kir* genes were ignored from the analysis. KirBac3.1 was used as an outgroup member and the analysis was performed with 1000 bootstrap replicates. Graphical presentation of the tree was produced in Treeview (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html/).

Quantitative PCR

Atrium, ventricle and brains and a piece of gill, muscle, liver and kidney were pooled from several fish for RNA sample preparation (*N*=3 for both acclimation groups). First strand cDNA synthesis was performed from DNAse-treated RNA (Hassinen et al., 2007) and DNA contamination was tested by a control cDNA synthesis containing all other reaction components except the RT enzyme. Quantitative RT-PCR was performed using Chromo4 Continuous Fluorescence Detector (MJ Research) under previously described conditions (Hassinen et al., 2007). Primers were designed to the

Table 1. Primers used to amplify the open reading frames for crucian carp *Kir2.1*, *Kir2.2* and *Kir2.5* and a fragment of crucian carp *DnaJA2*

Target gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplified region
ccKir2.1	ACTATAGGGCACGCGTGGT*	GCCACGGTGGCCAACTTCATACCGTGCTT	–244–74
ccKir2.1	ATGGGCAGTGTGAGAACCAACCGCTAC	GTCATGGCRGTSGCYTCSACCATGCC	1–917
ccKir2.1	CATCATCGGCGCCGTCATGGCCAAGAT	TCATATYTCYGAYTCWCGCCKYA	516–1284
ccKir2.1	ATGGGAAGTGTGCGGGC	TCATATTTCAGATTCTCGTCTTAGGG	1–1284
ccKir2.2	ATGAGTGTGGGGCGTCTCAACCGTTACA	GCACCAGCATGTAGCGCCAACGGAT	1–259
ccKir2.2	GGTGCCGCAACCGCTTYGTCAAGAA	TCCAGGATSACCACRATCTCAAAGTC	125–905
ccKir2.2	CGTGGGCTGCATCATTGACTGCTTCAT	TCAGATGGCAGAYTCCCTGCGRTA	507–1314
ccKir2.2	GACATGATTTCGAGCGACTGCAGAAC	TTTTTTTTTTTTTTTTT	1241–1450 (+136)
ccKir2.2	ATGAGTGTGGGGCGTCTCAACCGTTACA	TCATATCTCCGACTCCCTGCGGTAT	1–1314
ccKir2.5	ATGGGCAGTGTGAGAACCAACCGCTAC	GTCATGGCRGTSGCYTCSACCATGCC	1–929
ccKir2.5	TAAGCCCCGAATCACAGAAG	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT†	702–1723 (+454)
ccKir2.5	ATGAGTGTGGGCAAGCCCCA	TCATATTTCAGATTCTTTGTGATATG	1–1269
ccDnaJA2	CCAAATGCTGGHGACAARTTCAA	ACWGACTGCATCTGTTGDACCAT	121–557
Kir2.3	ATGCACGGRCACARCCGMAACGG	AAGCCGTTYACRTGCATGAT	
Kir2.3	CGCCGCAACCGCTTYGTCAAGAAGAA	ATGACMGCRTGGTGGCTGAACAGCA	
Kir2.3	ATGACCACHCAGGCMCGCAGCTCCTA	AGCATCCGGATRATGCCYGYCTCCT	
Kir2.3	CACTACAAGGTGGACTACTCACG	TCAGATGGCAGAYTCCCTGCGRTA	

Numbering starts with the initiator methionine. The numbering for *ccDnaJA2* is according to the zebrafish *DnaJA2* gene (NM_213493).
*AP2 primer of the Genome Walker kit, †AP primer of 3′-RACE kit (see Materials and methods).

non-conserved N- or C-termini of the *ccKir2.1*, *ccKir2.2* and *ccKir2.5* and to the cloned region (nucleotides 121–557; GenBank accession number EU191947) of the crucian carp *DnaJA2* (Table 2). *DnaJA2* was used as a reference gene because it is more stable in thermal acclimation than the conventional reference genes (Vornanen et al., 2005). Transcript abundance of the *ccKir2* genes was normalised to the *DnaJA2* expression level.

Electrophysiological methods

Heterologous expression of the ccKir2 proteins

ORF sequences for the putative ion channel-forming genes *ccKir2.1*, *ccKir2.2* and *ccKir2.5* were subcloned into the pcDNA3.1/Zeo (+) vector (Invitrogen) for expression in a COS-1 cell line (Hassinen et al., 2007). Electrophysiological experiments were made 48–72 h after transfection.

Whole-cell patch clamp

Ventricular myocytes were enzymatically isolated (Vornanen, 1997) and used within 8 h of isolation. Whole-cell voltage clamp experiments were done using an EPC9 patch clamp amplifier (HEKA Instruments Inc., Lambrecht/Pfalz, Germany), a PC-16 solution exchanger (Bioscience Tools, San Diego, CA, USA) and Pulse acquisition software (HEKA Instruments). External saline solution contained (mmol l^{-1}): 150 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose and 10 Hepes (pH 7.6). Patch pipettes were pulled from borosilicate glass (Garner, Claremont, CA, USA) and filled with K^+-based electrode solution (mmol l^{-1}: 140 KCl, 1 $MgCl_2$, 5 EGTA, 4 MgATP and 10 Hepes at pH 7.2). For experiments with ventricular myocytes, tetrodotoxin (0.5 μmol l^{-1}, Tocris Cookson, Bristol, UK) (Haverinen et al., 2007), nifedipine (10 μmol l^{-1}, Sigma, Helsinki, Finland), glibenclamide (10 μmol l^{-1}, Sigma) and E-4031 (1 μmol l^{-1}, Alomone labs, Jerusalem, Israel) were added to the extracellular solution to block Na^+, Ca^{2+}, ATP-sensitive K^+ and delayed-rectifier K^+ (I_{Kr}) current, respectively. The mean resistance of the pipettes was 2.52±0.07 MΩ.

I_{K1} was elicited every 10 s by repolarising ramps or square-wave pulses from the holding potential of –80 mV. Barium inhibition of I_{K1} was determined in the presence of cumulatively added concentrations of $BaCl_2$ (10^{-9} to 3×10^{-4} mol l^{-1}). Concentration–response curves were fitted with a Hill equation:

$$I = I_{min} + I_{max}\,[Ba^{2+}]^H / (K_d{}^H + [Ba^{2+}]^H) ,$$

where I_{min} is the minimum I_{K1} at the highest Ba^{2+} concentration, I_{max} is the I_{K1} before Ba^{2+} addition, K_d is the drug concentration that causes half-maximal inhibition of I_{K1} and H is the Hill coefficient.

The voltage dependence of inward rectification was measured separately using a single high dose of Ba^{2+} (0.1–0.3 mmol l^{-1}) for a complete and reversible block of the current within 45 s of the onset of the whole-cell configuration. Ba^{2+}-sensitive current was compared with the unblocked (non-rectifying) current, which was obtained from the current–voltage relationship between –120 mV and the reversal potential (V_{rev}) of I_{K1} and extrapolated to the voltage area of inward rectification. Scattering data points around V_{rev} (±1.5 mV) were omitted and the current was fitted with a Boltzmann function:

$$I_{K1} = I_{max} / \{1 + \exp[zF / RT(V - V_{1/2})]\} ,$$

where I_{K1} is current at each membrane potential (V), I_{max} is the unblocked current, $V_{1/2}$ is the membrane potential where 50% of the channels are blocked and z is the effective valency of the block. R, T and F represent the gas constant, absolute temperature and Faraday's constant, respectively, with their usual values.

Single-channel patch clamp

Single-channel properties of the cloned ccKir2 channels were recorded at room temperature (21±1°C) in inside-out configuration with an EPC-9 amplifier and Pulse software and analysed with TAC, TACFit (Bruxton Corporation, Seattle, WA, USA) and SigmaPlot 6.0 (SPSS, Chicago, IL, WA) software (Paajanen and Vornanen, 2004). The pipette solution was composed of (mmol l^{-1}): 134 KCl, 1.8 $CaCl_2$, 2 $MgCl_2$, 10 glucose and 10 Hepes adjusted to pH 7.6 with KOH ($[K^+]$=141 mmol l^{-1}). The composition of the bath solution in the inside-out experiments was (mmol l^{-1}): 140 KCl, 2 EGTA, 1 EDTA and 5 Hepes adjusted to pH 7.6 with KOH. EGTA and EDTA were included to prevent endogenous Ca^{2+}-dependent currents of the COS-1 cells. Inside-out patches with any outward current were excluded from further analyses.

Native Kir2 channels of fish ventricular myocytes were measured in inside-out and cell-attached configurations. Inside-out experiments were made under the same experimental conditions as the experiments with the cloned channels. Cell-attached recordings were conducted at 11°C (temperature in the middle of the acclimation temperatures of 4 and 18°C) by using the same external saline in the bath as in the whole-cell experiments. Pipettes were pulled (PP-83 puller, Narishige, Tokyo, Japan) from thick-walled borosilicate glass (Garner), coated with Sylgard (WPI, Stevenage, UK), fire polished on a microforge (MF-83, Narishige) and filled with the same high K^+ solution that was used for the inside-out patches. The mean resistance of the pipettes was 25.1±1.8 MΩ. Because low external K^+ causes a voltage offset, the Nernst potential of K^+ ions (–80 mV) was added to the membrane voltage.

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 –120 to +80 or –200 to –20 mV in 20 mV increments every 10 s for inside-out and cell-attached patches, respectively. Distributions of open and closed times were obtained from 20 to 120 s recordings at –100 mV. Open and closed time analyses were performed on patches that had only a single open current level. Open and closed times were detected with time-course fitting, and probability density functions (pdf) were analysed (TACFit) from idealised data with the log-likelihood method on log(event times). Histograms of single-channel conductance were constructed from cell-attached and inside-out recordings of the endogenous inward-rectifier channels from cold- and warm-acclimated fish to see the natural variation of conductance levels.

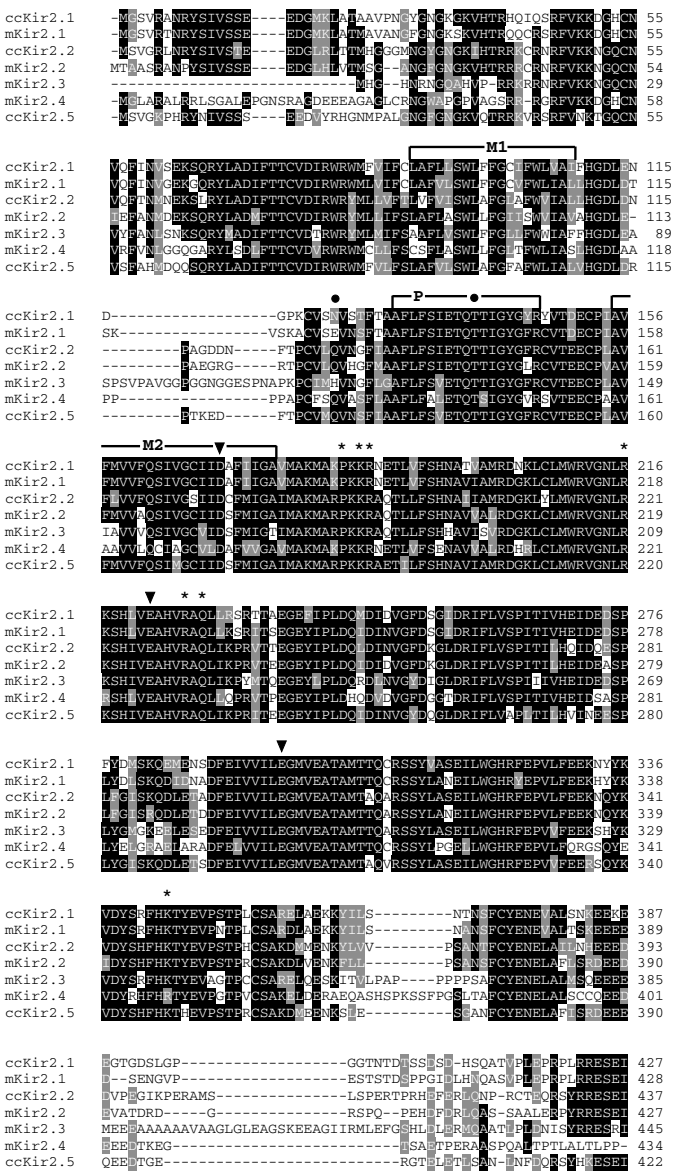
Table 2. Primers used in quantitative RT-PCR analysis

Target gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplicon size (bp)
ccKir2.1	GGAGAAAGAGGAAGGGAACG	GCGGTTCTAAGGGAATGGTA	104
ccKir2.2	ATGAGCTGGCAATCCTGAAC	TCATGTCGAGGGGTTCTCTC	103
ccKir2.5	AGCAATCTCAGCGCTACCTC	AGCTCAGGACAAATGCAAGG	99
ccDnaJA2	AGGACTTGTACGACCGTTATGG	CGCCAAAGATATGGGAAAAGAT	93

Statistics

Differences between mean current values from warm- and cold-acclimated carp ventricular I_{K1} were assessed by Student's t-test, whereas mean current values of the channels encoded by the cloned *ccKir2.1*, *ccKir2.2* and *ccKir2.5* genes were evaluated by using one-way analysis of

A



B

	ccKir2.1	omKir2.1a	hKir2.1	gpKir2.1	rKir2.1	mKir2.1
ccKir2.1		82.3	76.8	74.9	74.8	75.5
omKir2.1a	90.2		75.2	74.2	75.7	74.6
hKir2.1	83.7	82.6		91.4	88.7	88.4
gpKir2.1	83.5	82.6	99.8		87.4	87.0
rKir2.1	82.8	82.1	98.8	99.1		94.1
mKir2.1	83.0	81.9	98.4	98.6	99.1	

	ccKir2.2	omKir2.2a	hKir2.2	gpKir2.2	rKir2.2	mKir2.2
ccKir2.2		75.7	74.5	73.8	73.5	73.7
omKir2.2a	80.5		75.4	73.8	72.1	72.5
hKir2.2	77.2	66.8		90.1	88.2	88.6
gpKir2.2	77.4	66.1	97.9		87.9	88.6
rKir2.2	76.9	65.8	96.1	95.8		95.0
mKir2.2	76.9	65.6	95.8	96.1	99.3	

	Amino acid identity (%)	Amino acid similarity (%)	Nucleic acid identity (%)
mKir1.1	40.7	59.8	51.2
mKir2.1	67.7	81.0	66.3
mKir2.2	71.3	83.9	70.9
mKir2.3	58.8	69.4	60.8
mKir2.4	55.6	70.2	59.8
mKir3.1	37.4	55.1	50.0
mKir3.2	45.3	65.7	56.9
mKir3.3	44.7	64.3	55.2
mKir3.4	45.8	66.6	58.0
mKir4.1	36.6	55.4	52.9
mKir4.2	35.0	56.9	51.6
mKir5.1	42.4	57.4	55.0
mKir6.1	40.7	59.3	54.6
mKir6.2	41.8	59.3	53.8
zKir6.3	37.8	57.5	52.6
mKir7.1	30.5	46.0	48.3

Fig. 1. Amino acid alignment of ccKir2.5 channel with other members of the vertebrate Kir2 subfamily (A) and sequence similarities of vertebrate Kir channels (B). (A) Functionally important transmembrane domains (M1 and M2) and the intervening pore domain (P) are indicated with horizontal lines. Amino acids involved in Ba^{2+}, polyamine and phosphatidylinositol 4,5-bisphosphate binding are identified with filled circles, filled triangles and asterisks, respectively. (B) Nucleic acid identity (above the line) and amino acid identity (below the line) of vertebrate Kir2.1 (top) and Kir2.2 (middle). Species included are: crucian carp (cc; EU182582 for Kir2.1 and EU182583 for Kir2.2), rainbow trout (om; DQ435674 and DQ435676), human (h; NP_000882 and NP_066292), guinea-pig (gp; Q549A2 and AAG17048), rat (r; NP_058992 and NP_446433) and mouse (m; NP_032451 and NP_034733). Nucleotide and amino acid sequence identities and similarities of crucian carp Kir2.5 (EU182584) with all known mouse Kirs are shown at the bottom, including Kir1.1 (NM_001012387), Kir2.1 (NM_008425), Kir2.2 (NM_010603), Kir2.3 (NM_008427), Kir2.4 (NM_145963), Kir3.1 (NM_008426), Kir3.2 (NM_001025584), Kir3.3 (NM_008429), Kir3.4 (NM_010605), Kir4.1 (NM_001039484), Kir4.2 (NM_001039057), Kir5.1 (NM_010604), Kir6.1 (NM_008428), Kir6.2 (NM_010602) and Kir7.1 (XM_001473740). Zebrafish Kir6.3 (NM_001012387) is also included.

variance. Differences in *Kir2* transcript abundances were tested using Student's *t*-test. A P value of 0.05 was regarded as the limit of statistical significance.

RESULTS

Identification of crucian carp cardiac *Kir2* genes

Mammalian cardiac Kir2 channels are composed of Kir2.1, Kir2.2 and Kir2.3 subunits. Three partial cDNAs for *Kir2* genes were also obtained from crucian carp heart using degenerative primers in RT-PCR. Two of the completely sequenced ORFs showed high predicted amino acid sequence identity with mammalian (about 83% and 77%) and fish (*Oncorhynchus mykiss*) (90% and 80%) Kir2.1 and Kir2.2 channels, respectively, suggesting that they are homologues of the vertebrate *Kir2.1* and *Kir2.2* genes (Fig. 1). Accordingly, these crucian carp *Kir* genes were designated as *ccKir2.1* (EU182582) and *ccKir2.2* (EU182583). Surprisingly, the third clone of the crucian carp Kir channel genes had a relatively low predicted amino acid sequence identity (59%) with the mammalian Kir2.3 channel and with other members of the Kir2 subfamily (68%, 71% and 56% for Kir2.1, Kir2.2 and Kir2.4, respectively). Even lower identity values were obtained with another six Kir subfamilies (Fig. 1B). These findings suggest that *Kir2.3* is not expressed in the crucian carp heart, and the third gene is a member of the *Kir2* subfamily, even if it is not homologous to any known *Kir2* gene. We assumed it to be a novel gene of the vertebrate Kir2 subfamily and designated it as *ccKir2.5* (EU182584).

ORFs for *ccKir2.1*, *ccKir2.2* and *ccKir2.5* consisted of 1284, 1314 and 1269 bp, coding for 427, 437 and 422 amino acids, respectively (supplementary material Fig. S1). To establish evolutionary relationships between the ccKir2 channels and the known vertebrate Kir channels, we constructed a phylogenetic tree with members from all seven Kir subfamilies. Phylogenetic analysis showed that

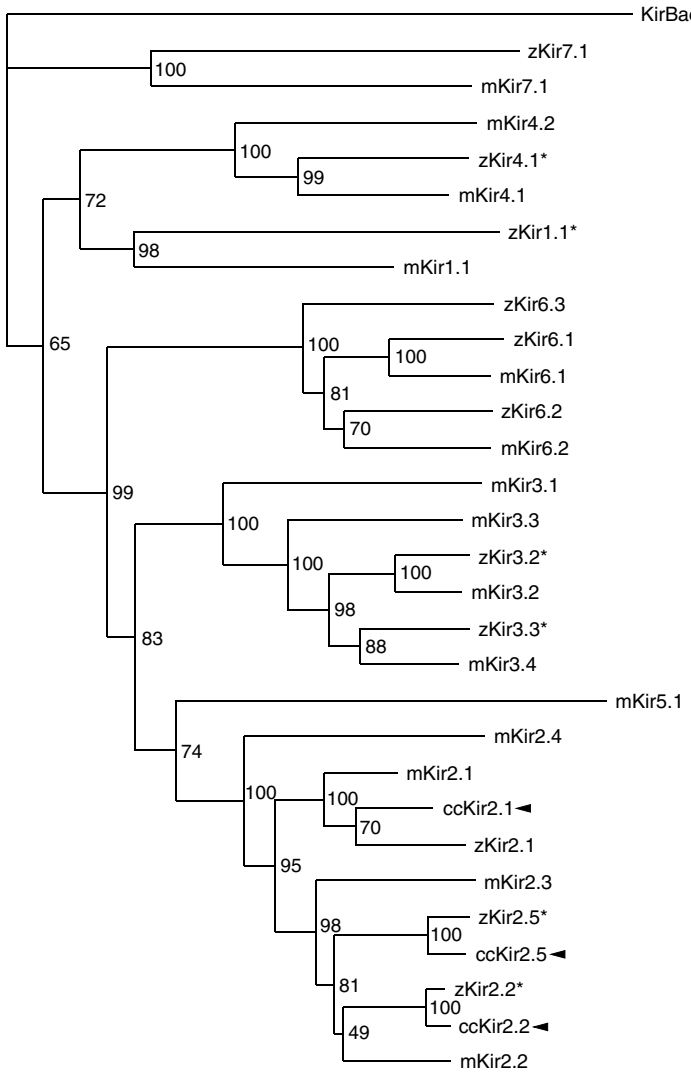


Fig. 2. A rooted phylogenetic tree of the vertebrate Kir channel family based on the amino acid sequences. KirBac3.1 was used as an outgroup. Numbers at the nodes indicate the bootstrap values for the clade of 1000 replications. Arrowheads indicate positions of the crucian carp Kir2 proteins in the tree. Predicted inward-rectifier potassium channels of the zebrafish are indicated with an asterisk. GenBank accession numbers for the mouse (m) Kir sequences are the same as in Fig. 1B and those for zebrafish (z) are as follows: Kir1.1, NM_001045169; Kir2.1, XM_687101; Kir2.2, XM_692512; Kir2.5, XM_001335914; Kir3.2, XM_692892; Kir3.3, XM_695527; Kir4.1, XM_001342957; Kir6.1, NM_001030153; Kir6.2, NM_001039827; Kir6.3, NM_001012387; and Kir7.1, NM_001045549.

ccKir2.1 and ccKir2.2 are, indeed, homologues of Kir2.1 and Kir2.2, respectively (Fig. 2). From the known Kir family members, the closest relative of ccKir2.5 was Kir2.2 with an 81% bootstrap value. Importantly, *ccKir2.5* shares a common ancestor with other *Kir2* genes, providing strong evidence that ccKir2.5 is a new member of the Kir2 subfamily. Taken together, crucian carp heart expresses ccKir2.1, ccKir2.2 and ccKir.2.5, but not a homologue of vertebrate Kir2.3.

Sequence structure of the *ccKir2* genes

All vertebrate Kir proteins consist of a conserved pore-forming region (P) flanked by two transmembrane alpha helices (M1 and M2). These functional domains were identified from all cloned ccKir2 proteins and were more conserved than the cytoplasmic N- and C-termini (Fig. 1A, supplementary material Fig. S2). In mammalian Kir2.1, Mg^{2+} binds to serine S165 (numbering according to mouse Kir2.1) (Fujiwara and Kubo, 2002), whereas glutamates E224 and E299 function as an intermediate binding site for polyamines before entering to the pore-blocking site D172 (Xie et al., 2003). All these residues also exist in ccKir2.5, ccKir2.2 and ccKir2.1, suggesting a similar inward-rectification mechanism for mammalian and fish Kir2 channels. In contrast, some variability appeared in amino acids important for Ba^{2+} binding. In mammalian Kir2.1, glutamate E125 between M1 and P binds Ba^{2+} and facilitates its movement to the plugging site threonine T141 of the narrow pore region (Alagem et al., 2001). Threonine T141 is conserved in all mammalian and fish cardiac Kir2 channels. In contrast, glutamate E125 exists in mammalian Kir2.1, but not in mammalian Kir2.2 and Kir2.3 channels. In crucian carp, the glutamate E125 is replaced by other amino acids, not only in ccKir2.1, but also in ccKir2.5 and ccKir2.2. Yet, the cloned ccKir2.5 and ccKir2.2 channels were about 10 times more sensitive to Ba^{2+} than ccKir2.1. While this agrees with the finding that mammalian Kir2.2 is 5–10 times more sensitive to Ba^{2+} than Kir2.1 (Liu et al., 2001; Preisig-Müller et al., 2002), it strongly suggests that other residues in addition to E125 are important for Ba^{2+} binding.

Tissue distribution of ccKir2.5 mRNA

Transcript abundance of ccKir2 subunits was determined by quantitative PCR in seven tissues of the cold-acclimated crucian carp. All three *ccKir2* genes were expressed to some extent in heart, brain, gill, kidney, liver and skeletal muscle (Fig. 3). ccKir2.5 was a major ccKir2 channel component in atrium (59.1±2.1%) and ventricle (65.6±3.2%) of the heart and in the skeletal muscle (88.6±3.4%), while in other tissues it was weakly expressed (<25%), suggesting that it is a muscle-specific isoform.

Electrophysiological characteristics of ccKir2.5

The majority (~90%) of COS-1 cells transfected with *ccKir2* genes and a separate eGFP vector had a large inward-rectifying current

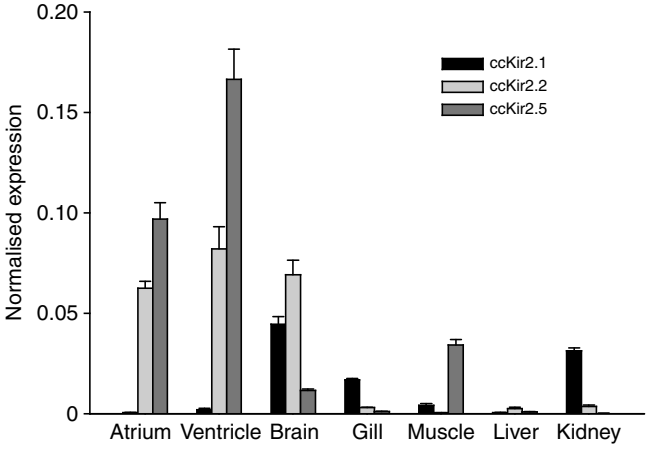


Fig. 3. Expression levels of ccKir2.1, ccKir2.2 and ccKir2.5 mRNAs in different tissues of the cold-acclimated crucian carp. The amount of ccKir2 mRNAs was normalised to the DnaJA2 expression level. Values are means ± s.e.m. of three pooled samples.

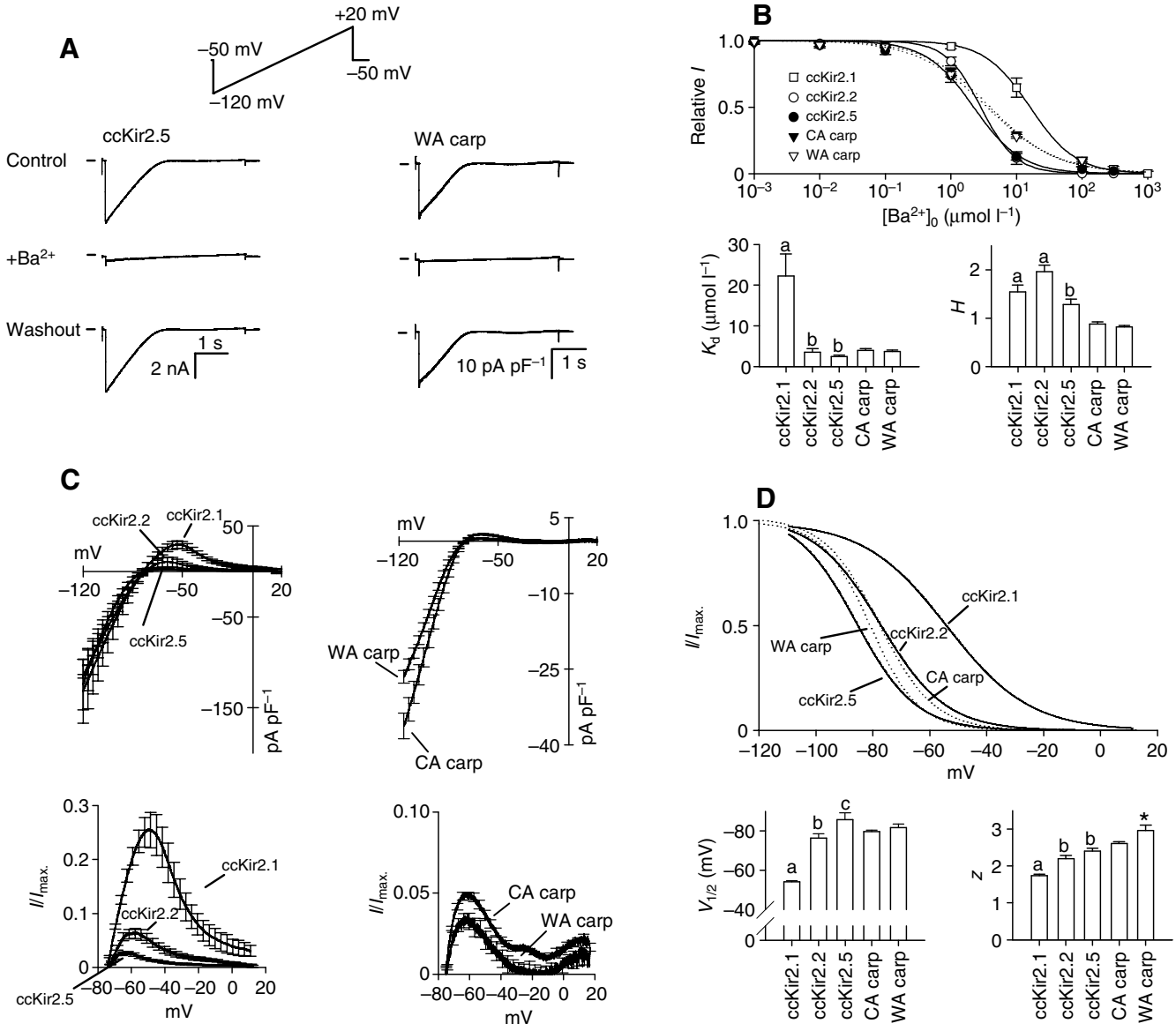


Fig. 4. Characterisation of the cloned ccKir2 channels in COS-1 cells and native I_{K1} of the crucian carp ventricular myocytes by Ba^{2+} sensitivity and inward rectification. (A) Representative whole-cell recordings of the inward-rectifying K^+ current through the cloned ccKir2.5 channel and I_{K1} of the warm-acclimated (WA) crucian carp myocyte, demonstrating a reversible block by 0.3 mmol l^{-1} external Ba^{2+}. On the basis of current density, expression levels of the three ccKir2 proteins were similar (P>0.8). (B) Dose–response relationship of Ba^{2+} block of the cloned ccKir2 proteins and endogenous I_{K1} of the ventricular myocytes. The upper panel shows dose–response curves and the lower panels give mean values of K_d (left) and Hill coefficient (H, right), respectively. CA, cold-acclimated carp. Different letters indicate statistically significant differences between the cloned ccKir2 channels. Number of tested cells is 7–11. (C) Current–voltage relationship of cloned ccKir2 channels (left) and I_{K1} of warm- and cold-acclimated ventricular myocytes (right). The lower panels show the relative outward current for the same recordings. Note the higher inward and outward current density of cold-acclimated myocytes in comparison to warm-acclimated myocytes. (D) Boltzmann fits (top) and half-voltage ($V_{1/2}$) and effective valency (z) of the inward rectification (bottom). Different letters indicate a statistically significant difference between cloned ccKir2 channels and an asterisk indicates a statistically significant difference between acclimation groups. Number of tested cells is 7–13.

with a slope conductance of 100±15 nS, which reversed direction near the equilibrium potential of K^+ ions (–73.3±1.0 mV). The current was reversibly blocked by 0.1–0.3 mmol l^{-1} $[Ba^{2+}]_o$ (Fig. 4A), indicating that the cloned genes encoded functional inward-rectifying K^+ channels of the Kir2 family.

When expressed in COS-1 cells, ccKir2.5 was almost 10-times more sensitive to $[Ba^{2+}]_o$ (K_d, 2.43±0.37 μmol l^{-1}) than the ccKir2.1 channel (22.25±5.37 μmol l^{-1}; P<0.05), and it also rectified much more strongly (z=2.40±0.08, $V_{1/2}$=–85.62±3.50 mV) than ccKir2.1 (z=1.73±0.04, $V_{1/2}$=–54.20±0.57 mV; P<0.05; Fig. 4B–D). With regard to Ba^{2+} sensitivity, ccKir2.5 current was similar to the current generated by ccKir2.2 (3.48±0.90 μmol l^{-1}; P>0.05), but more strongly rectifying than ccKir2.1 ($V_{1/2}$=–76.29±2.21 mV; P<0.05). Accordingly, at the whole-cell level ccKir2.5 differs from the other ccKir2 channels with regard to either Ba^{2+} sensitivity or inward rectification, or both.

Even at the single-channel level, ccKir2.5 channels were functionally closer to ccKir2.2 than to ccKir2.1 channels (Fig. 5). Single-channel conductance of ccKir2.5 (44.19±1.89 pS) was 3 times as large as that of ccKir2.1 (14.62±1.95 pS; P<0.05), but only 1.6

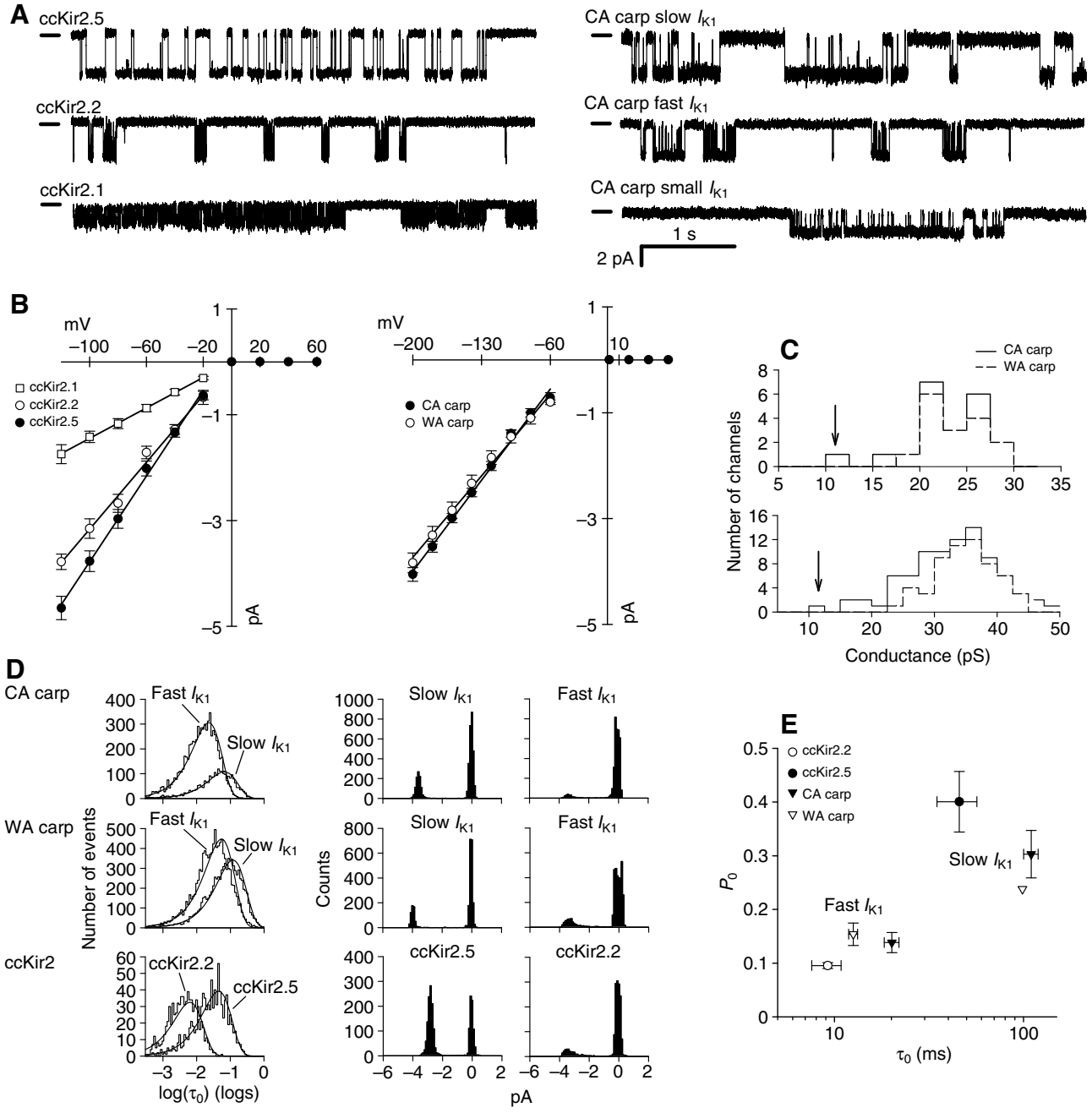


Fig. 5. Single-channel characteristics of the cloned ccKir2 channels from COS-1 cells and endogenous Kir2 channels of the crucian carp ventricular myocytes. (A) Comparison of inside-out recordings of the cloned ccKir2 proteins (left) and cell-attached recordings of the endogenous I_{K1} currents (right) demonstrating three distinct channel types on the basis of kinetics and conductance. Horizontal lines indicate the zero current level. (B) Current–voltage relationships of the ccKir2.1, ccKir2.2 and ccKir2.5 proteins in inside-out recordings from COS-1 cells (left) and cell-attached recordings of the endogenous I_{K1} in ventricular myocytes (right). Endogenous currents are means of all inward-rectifier channel types present in ventricular myocytes. (C) Conductance variation of endogenous ccKir2 currents of crucian carp ventricular myocytes in cell-attached (top) and inside-out (bottom) recordings. Distinct populations of small conductance (10–14 pS) channels are indicated with arrows. (D) Comparison of open time distribution (left) and open probability (right) of the cloned ccKir2.5 and ccKir2.2 channels and the endogenous fast and slow I_{K1} of ventricular myocytes. Open time distributions of the endogenous channels of temperature-acclimated fish include all recordings of the particular current type (slow I_{K1} or fast I_{K1}): good fits to a monoexponential function suggest the presence of two separate channel types with different kinetics. Note also the different open time distributions of the cloned ccKir2.2 and ccKi2.5 channels. (E) Comparison of heterologously expressed ccKir2.5 and ccKir2.2 channels and endogenous slow and fast I_{K1} on the basis of mean open time (τ_o) and open probability (P_o).

times as large as the conductance of the ccKir2.2 (27.54±5.3 pS; Fig. 5B). The three channels also had variable single-channel kinetics. The mean open time at –100 mV was 45.73±10.89, 22.93±9.11 and 9.21±1.63 ms for ccKir2.5, ccKir2.2 and Kir2.1, respectively. Furthermore, ccKir2.5 had 4.17 times as high an open probability as ccKir2.2 (0.401±0.056 *vs* 0.096±0.005; $P<0.05$; Fig. 5E), and is therefore likely to contribute more to the whole-cell I_{K1} than ccKir2.2. Taken together, the functional properties of

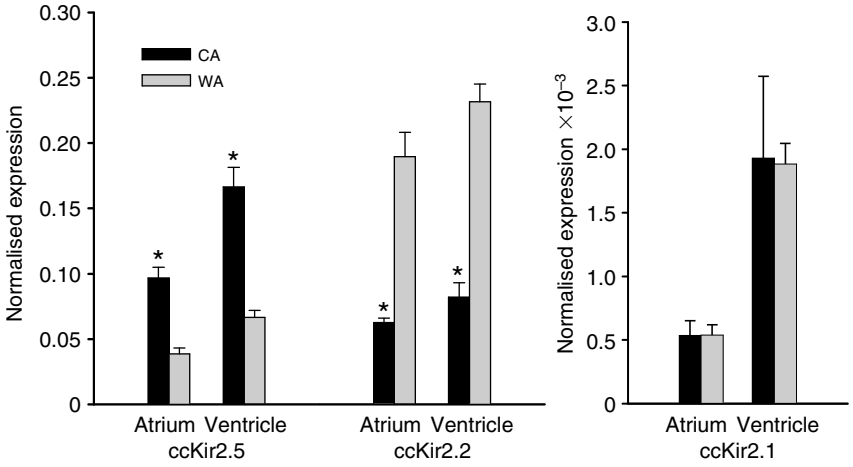


Fig. 6. Transcript abundance of ccKir2.1, ccKir2.2 and ccKir2.5 in atrium and ventricle of cold- and warm-acclimated crucian carp. A statistically significant difference (P<0.05) between acclimation groups is indicated by an asterisk.

ccKir2.5 are strikingly different from those of ccKir2.1 and closer to, although still distinct from, those of the ccKir2.2.

Effect of thermal acclimation on the I_{K1} of the crucian carp heart

Adjustment of cardiac ion channel function to changing temperature conditions is probably vital for proper excitability of ectothermic hearts. In crucian carp ventricular myocytes, acclimation to cold (4°C) increased the slope conductance of I_{K1} (707±49 *vs* 1001±59 pS pF^{-1}, between –120 and –80 mV; P<0.05) and the density of both inward and outward I_{K1} (Fig. 4C), indicating positive thermal compensation of the ccKir2 channel system. To resolve this change at the molecular level, transcript expression and electrophysiological properties of the cardiac ccKir2 channels were examined.

ccKir2.5 and ccKir2.2 were clearly the main ccKir2 subunits in the crucian carp heart, forming together over 99% of all ccKir2 transcripts. ccKir2.1 accounted for less than 0.8% of the transcripts, which was considered to be physiologically insignificant. Thermal acclimation for 4 weeks had a striking impact on ccKir2 mRNA expression. In cold-acclimated fish, ccKir2.5 was the dominating isoform representing 59.1±2.1% and 65.6±3.2% of the total ccKir2 transcripts in atrium and ventricle, respectively. In contrast, in warm-acclimated fish hearts ccKir2.2 was the main component, accounting for 83.6±1.6% of atrial and 77.7±1.7% of ventricular Kir2 mRNA, respectively (Fig. 6). These findings indicate that in cold-acclimated carp the major part of the ccKir2.2 transcripts is replaced by ccKir2.5, suggesting that ccKir2.5 is the cold-adapted isoform and is probably important for acclimation of the heart to low temperatures. Interestingly, the total amount of ccKir2 transcripts was slightly higher in warm- than cold-acclimated crucian carp heart, both in atrium and ventricle (P<0.05).

Ba^{2+} sensitivity of I_{K1} was similar in cold- and warm-acclimated fish, which is not unexpected considering the similar Ba^{2+} sensitivities of ccKir2.5 and ccKir2.2 (Fig. 4B). The voltage dependence of inward rectification of I_{K1} was also similar in cold- and warm-acclimated carp ventricular myocytes (–79.60±0.85 *vs* –81.74±1.72), but I_{K1} had a slightly shallower slope of rectification (z=2.5±0.1 *vs* 3.0±0.1; P<0.05) in cold- than warm-acclimated fish. The rectification properties of I_{K1} from cold- and warm-acclimated fish do not conform well to the rectification characteristics of the cloned ccKir2 channels and the transcript levels of ccKir2.5 and ccKir2.2 in ventricular myocytes, suggesting that temperature acclimation must also modify the regulation of Kir2 channel function.

Single-channel currents with similar characteristics to the currents of cloned ccKir2 channels were observed in crucian carp ventricular myocytes (Fig. 5) (Paajanen and Vornanen, 2003). Channels with ccKir2.5 characteristics (the slow I_{K1}) were found in 8 out of 19 and 1 out of 16 cell-attached patches from cold- and warm-acclimated fish, respectively, whereas ccKir2.2-type channels (the fast I_{K1}) were found in 9 out of 19 and 15 out of 16 cell-attached patches from cold- and warm-acclimated fish, respectively. These frequencies are in line with mRNA expression levels of ccKir2.5 and ccKir2.2 in warm- and cold-acclimated fish. Single-channel amplitudes and mean open times of slow I_{K1} and fast I_{K1} channels of ventricular myocytes correspond well with the values of cloned ccKir2.5 and ccKir2.2 channels (Fig. 5D,E), suggesting that they are composed of ccKir2.5 and ccKir2.2 subunits, respectively. Although Kir2.5 channels were more frequent in cold- than warm-acclimated fish hearts, the mean conductance of the endogenous Kir2 channels did not differ between acclimation groups (P>0.05; Fig. 5B). A small amplitude (~14 pS) current (small I_{K1}) was found only in a few cell-attached patches (Fig. 5C). Similarly, the probability of finding a small amplitude channel was low in inside-out patches (in 6 out of 100 and 5 out of 52 patches from cold- and warm-acclimated fish, respectively). These findings suggest that the small ccKir2.1-like channels have low expression levels in ventricular myocytes of cold- and warm-acclimated crucian carp.

DISCUSSION

The background I_{K1} current, formed by Kir2 channels, is involved in stabilisation of resting membrane potential and in late phase-3 repolarisation of action potential in cardiac myocytes (Shimoni et al., 1992; Lopatin and Nichols, 2001). In mammalian hearts, this task is accomplished by Kir2.1, Kir2.2 and Kir2.3 channels, Kir2.1 being the dominant isoform (Lopatin and Nichols, 2001; Liu et al., 2001; Wang et al., 1998). We have shown here that in crucian carp ventricular myocytes too, three inward-rectifier channels are involved in the formation of I_{K1}, two of them being homologues of the known mammalian cardiac Kir2.1 and Kir2.2 channels. The third gene of the crucian carp cardiac Kir assembly seems to belong to the *Kir2* subfamily, even though it is not clearly homologous to any of the known *Kir2* genes, and accordingly is proposed to be a new, fifth member for the vertebrate *Kir2* subfamily, *ccKir2.5*. Indeed, the phylogenetic analysis showed that *ccKir2.5* has a common ancestor with all four known *Kir2* genes (*Kir2.1*, *Kir2.2*, *Kir2.3*, *Kir2.4*), thus providing firm evidence that it belongs to the *Kir2* subfamily. Interestingly, a predicted protein (XM_001335914) sharing 98.3% similarity with ccKir2.5 was found from the zebrafish sequence database, suggesting the Kir2.5 channels might be more generally expressed in different fish species. In contrast, no homologues of *ccKir2.5* were found in mammalian genomes. Thus, it is likely that the novel *ccKir2.5* is unique to ectothermic vertebrates.

All vertebrate Kir2 channels rectify strongly due to a high-affinity voltage-dependent block by free polyamines and Mg^{2+}, and thus in a given cell type the magnitude of the physiologically important outward current is primarily determined by the sensitivity of Kir2 channels to polyamines (Dhamoon et al., 2004; Panama and Lopatin, 2006). Although all three cardiac ccKir2 channels of the crucian carp heart have a distinct negative slope conductance and they completely rectify at 0 mV, notable differences exist between the channel isoforms in terms of inward rectification. ccKir2.5 is clearly the strongest inward rectifier followed by ccKir2.2 and ccKir2.1. Among the mammalian cardiac Kir2 channels, Kir2.1 is the weakest and Kir2.2 the strongest inward rectifier (Dhamoon et al., 2004; Panama and Lopatin, 2006). Kir2.3 is intermediate between Kir2.1 and Kir2.2, but it rectifies incompletely. Evidently, ccKir2.5 is the strongest inward-rectifier K^+ channel of the vertebrate heart and passes little outward current. Consequently, its membrane potential stabilising and repolarising effects are relatively weak.

The present findings indicate that under chronic thermal stress the phenotype of crucian carp cardiac I_{K1} is changed by a compensatory increase in the density of I_{K1} in the cold. In the chronic cold, ccKir2.5 transcripts were strongly upregulated, suggesting that the ccKir2.5 isoform might be important in producing the cold-acclimated phenotype of the cardiac I_{K1}. Concomitantly with the increased ccKir2.5 expression, transcripts of ccKir2.2 were strongly suppressed, suggesting the possibility that the cold-induced increase in the cardiac I_{K1} was obtained by an isoform shift from ccKir2.2 towards ccKir2.5. But how is the cold-induced increase in I_{K1} achieved by the strongly rectifying ccKir2.5 channels, especially when the total amount of ccKir2 transcripts is simultaneously reduced? Two single-channel properties of ccKir2.5 could be contributing. First, open probability and mean open time of ccKir2.5 are 4.17 and 4.96 times, respectively, as large as those of ccKir2.2. Second, ccKir2.5 has a larger single-channel conductance than ccKir2.2. Thus, in spite of the strong voltage-dependent block by polyamines, ccKir2.5 channels might allow more outward current than ccKir2.2 channels, mainly because they stay longer in the open state and because they have a larger conductance than ccKir2.2 channels. While the larger slope conductance of I_{K1} in cold-acclimated crucian carp is consistent with this, the rectification of the endogenous I_{K1} does not match the rectification properties of the cloned ccKir2.2 and ccKir2.5 channels and the expression levels of these channels in cold- and warm-acclimated fish. Rectification of I_{K1} in cold-acclimated fish is weaker than would be assumed on the basis of the high ccKir2.5 expression level. Therefore, other mechanisms in addition to ccKir2 isoform change must be involved in temperature-dependent regulation of the crucian carp cardiac I_{K1}. The present results do not provide an explanation for the divergence in rectification properties between cloned and endogenous currents, but it can be speculated that in cardiac myocytes ccKir2.2 and ccKir2.5 channels are located in different membrane compartments and therefore might face different free polyamine concentrations and/or compositions. A different distribution of Kir2.1 and Kir2.3 channels between cholesterol-rich and cholesterol-poor membrane domains has recently been suggested (Tikku et al., 2007), raising the possibly that regulation of Kir2 isoforms may differ depending on their subcellular location.

Although isoform change of ccKir2 channels does not alone explain temperature-induced changes in the I_{K1} of the crucian carp heart, it probably has a central role in thermal modification of the I_{K1}. Recently, we showed that Kir2.2 is upregulated by warm acclimation in another fish species, the rainbow trout (Hassinen et al., 2007). Together these studies suggest that temperature-induced changes in Kir2 isoforms may be a common way for temperature acclimation of the cardiac I_{K1} in fish. Evidently, ccKir2.2 is a warm-adapted and ccKir2.5 a cold-adapted isoform of the cardiac inward-rectifying K^+ channel and the cardiac phenotype of I_{K1} is determined by their relative abundance.

It is interesting that temperature compensation of I_{K1} density was produced by an isoform shift and not by parallel upregulation of all three Kir2 channel isoforms in the cold. The potential benefit of isoform switching in comparison to a simple increase in the number of the ccKir2 population remains unexplained, especially since the electrophysiological properties of ccKir2.2 and ccKir2.5 do not radically differ (with the exception of mean open time and open probability). It can be speculated that there might be some constraints on genome function that would prevent upregulation of ccKir2.2 and ccKir2.1 isoforms at low temperatures. In this regard it is interesting that rainbow trout heart, which responds to constant cold by a decrease in the density of the I_{K1}, does not express Kir2.5 (Vornanen et al., 2002; Hassinen at al., 2007). Thus, it is possible that cold-induced compensation of cardiac I_{K1} is dependent on the expression of the Kir2.5 isoform, and species that lack Kir2.5 or cannot express it in the heart are unable to upregulate I_{K1} in the cold. Future research should find out how widely the novel Kir2.5 isoform is distributed among fish species and other ectotherms, and to clarify its importance in thermal adaptation of heart and muscle tissues.

In conclusion, a new member for the Kir2 subfamily of the inward-rectifying K^+ channels has been isolated and characterised from the heart of crucian carp. Increased expression of the novel ccKir2.5 channel, at the expense of the ccKir2.2 isoform, is assumed to contribute to the cold-induced increase of atrial and ventricular I_{K1}, which partly compensates for the depressive effects of low temperature on the current that maintains negative resting membrane potential and accelerates the phase-3 repolarisation of the cardiac action potential. These electrophysiological effects may be necessary to limit action potential prolongation and stabilise membrane potential to prevent cardiac arrhythmias in the cold winter waters.

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