Thermal adaptation of the crucian carp (Carassius carassius) cardiac delayed rectifier current, $I_{\text{Ks}}$, by homomeric assembly of Kv7.1 subunits without MinK

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Hassinen M, Laulaja S, Paajanen V, Haverinen J, Vornanen M. Thermal adaptation of the crucian carp (Carassius carassius) cardiac delayed rectifier current, $I_{\text{Ks}}$, by homomeric assembly of Kv7.1 subunits without MinK. Am J Physiol Regul Integr Comp Physiol 301: R255–R265, 2011. First published April 20, 2011; doi:10.1152/ajpregu.00067.2011.—Ectothermic vertebrates experience acute and chronic temperature changes which affect cardiac excitability and may threaten electrical stability of the heart. Nevertheless, ectothemic hearts function over wide ranges of temperatures without cardiac arrhythmias, probably due to special molecular adaptations. We examine function and molecular basis of the slow delayed rectifier K$^+$ current ($I_{\text{Ks}}$) in cardiac myocytes of a eurythermic fish (Carassius carassius L.). $I_{\text{Ks}}$ is an important repolarizing current that prevents excessive prolongation of cardiac action potential, but it is extremely slowly activating when expressed in typical molecular composition of the endothermic animals. Comparison of the $I_{\text{Ks}}$ of the crucian carp atrial myocytes with the currents produced by homomeric Kv7.1 and heteromeric Kv7.1/MinK channels in Chinese hamster ovary cells indicates that activation kinetics and pharmacological properties of the $I_{\text{Ks}}$ are similar to those of the homomeric Kv7.1/MinK channels. Consistently with electrophysiological properties and homomeric Kv7.1 channel composition, atrial transcript expression of the MinK subunit is only 1.6–1.9% of the expression level of the Kv7.1 subunit. Since activation kinetics of the homomeric Kv7.1 channel assembly is an evolutionary thermal adaptation of ectothermic hearts and the heteromeric Kv7.1/MinK channels evolved later to adapt $I_{\text{Ks}}$ to high body temperatures of endotherms.

CONSIDERING THE HIGH SENSITIVITY of endothermic hearts to arrhythmias in hypothermia and fever, it is surprising that the hearts of ectothermic vertebrates function properly over a wide range of temperatures. For example, thermal tolerance of temperate fish species range from 0°C to 36°C with undisturbed cardiac function, and involving heart rates from a few beats over 140 beats per min and cardiac action potentials (AP) varying from a few hundred ms to almost 3 s in duration (2, 11, 23, 34). The robustness of cardiac function over extensive temperature ranges requires that ion channel function provides adequate membrane excitability in different thermal conditions, but still ensures electrical stability under acute and chronic temperature changes.

In fish hearts there are specific K$^+$ channels or channel isoforms which modify the shape of cardiac AP under chronic and acute temperature changes (8–10, 12, 23, 38). However, the role of the slow delayed rectifier K$^+$ current ($I_{\text{Ks}}$) in thermal responses of the fish heart has not been examined. In endotherms, the slowly activating delayed rectifier K$^+$ current ($I_{\text{Ks}}$) plays an important role in controlling repolarizing phase of the cardiac AP. $I_{\text{Ks}}$ functions as repolarization reserve that prevents excessive prolongation of cardiac AP when beta-adrenergic tone is high or when other K$^+$ channels fail (16). $I_{\text{Ks}}$ is also important in limiting the duration of cardiac AP at high heart rates due to its ability to accumulate at high frequencies (24).

In mammals the cardiac $I_{\text{Ks}}$ is carried by channels consisting of the Kv7.1 (encoded by KCNQ1 gene) pore-forming alpha subunits and the MinK (encoded by KCN1E gene) regulatory subunits (25). The MinK (also called IsK) is a small protein of about 130 amino acids with a single membrane spanning sequence and short C- and N-terminal tails. The Kv7.1 comprises 6 transmembrane sequences and as a homotetramer produces functional K$^+$ ion channels. Though Kv7.1 alone can generate a K$^+$ current, association with the MinK is required to recapitulate the native mammalian cardiac $I_{\text{Ks}}$. When Kv7.1 is coexpressed with the ancillary MinK subunit, the behavior of the channel dramatically changes. MinK affects drug binding which is expressed as higher sensitivity of the Kv7.1/MinK channels to chromanol compared with the Kv7.1 channels (4). MinK is also necessary for beta-adrenergic enhancement of the $I_{\text{Ks}}$ and frequency-dependent modulation of the $I_{\text{Ks}}$ (14, 17). Finally, the Kv7.1/MinK channels and the native cardiac $I_{\text{Ks}}$ channels display extremely slow activation kinetics and strong temperature dependence (31, 32). Indeed, $I_{\text{Ks}}$ would be nonfunctional at low body temperatures of many ectothermic animals if endowed with kinetic properties of the mammalian $I_{\text{Ks}}$.

Considering the importance of $I_{\text{Ks}}$ in AP repolarization under various physiologically situations, we hypothesized that $I_{\text{Ks}}$ would be valuable in stabilizing the electrical activity of the ectothermic hearts. To this end, we examined (1) the presence, (2) electrophysiological properties and (3) molecular background of the $I_{\text{Ks}}$ current in cardiomyocytes of the crucian carp (Carassius carassius L.), a teleost fish species with a wide thermal tolerance range (13). The properties of atrial $I_{\text{Ks}}$ were compared with the currents produced by the Kv7.1 channels alone and the coassembly of Kv7.1 and MinK channels of the crucian carp in Chinese hamster ovary (CHO) cells. It is shown that the atrial $I_{\text{Ks}}$ of the crucian carp heart is similar to the current generated by the homotetrameric Kv7.1 channels in regard to activation kinetics, voltage-dependence of activation and sensitivity to chromanol. These findings suggest that unlike endothermic hearts, the $I_{\text{Ks}}$ of the crucian carp heart is
mainly composed of homotetrameric K\(_{7.1}\) channels that produce fast activating currents at low body temperatures of the ectothermic crucian carp.

**MATERIALS AND METHODS**

**Animals.** Crucian carp (*Carassius carassius* L.) caught in summer and winter were directly placed at acclimation temperatures of 18\(^\circ\)C (warm-acclimation, WA) and 4\(^\circ\)C (cold-acclimation, CA), respectively. Four weeks were allowed for thermal acclimation under a 15 h:9 h light:dark photoperiod in the lab before experiments. WA crucian carp were fed three times a week with aquarium fish food (Tetra, Melle, Germany), while no food was given to CA crucian carp since winter-acclimatized crucian carp do not forage. The body masses of WA and CA fish were 58±12 g (n = 22) and 46±8 g (n = 25) (P > 0.05), respectively. All experiments were conducted with the consent of the national committee for animal experimentation (permission STTH2/52A).

**Cloning and sequencing of crucian carp KCNQ1 and KCNE1.** Total RNA of the heart was used to clone KCNQ1 and KCNE1 genes. RNA was extracted with TriZOL Reagent (Invitrogen, Carlsbad, CA) from atrium and ventricle powdered under liquid nitrogen. RNA was treated with DNase to avoid genomic DNA contamination. First strand cDNA synthesis was performed with M-MuLV RT H

**Table 1. Primer pairs used in cloning**

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primers for 1st PCR</th>
<th>Primers for 2nd PCR</th>
<th>Amplified Region</th>
</tr>
</thead>
</table>
| ccKCNQ1    | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | 191–442 |
| ccKCNQ1    | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | 205–760 |
| ccKCNQ1    | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | 967–1605 |
| ccKCNQ1    | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | 1354–1803 |
| ccKCNQ1    | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | 1–1956 |
| ccKCNQ1    | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | 1–239 |
| ccKCNQ1    | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | 96–133 |
| ccKCNQ1    | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | 1–396 |
| ccKCNQ1    | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | 1–1956 |
| ccKCNQ1    | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | 1–239 |
| ccKCNQ1    | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | 96–133 |
| ccKCNQ1    | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | 1–396 |
| ccKCNQ1    | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | 1–1956 |
| ccKCNQ1    | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | 1–239 |
| ccKCNQ1    | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | 96–133 |
| ccKCNQ1    | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | 1–396 |

**The primers are in 5’-3’ direction. The upper sequence shows the forward and the lower sequence of the reverse primer.**

was sequenced using ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Finally, the sequences were confirmed by sequencing the PCR-amplified ORFs straight from the PCR product.

Partial sequences were assembled and analyzed with AutoAssembler software. Transeq software was used to convert cDNA-sequences into amino acid sequences, and the predicted phosphorylation and glycosylation sites were sought using Prosite. Similarities of the crucian carp KCNQ1 and KCNE1 sequences with orthologous genes of other species were determined using Stretch and ClustalW softwares. For the phylogenetic analysis, protein sequences of the KCNQ and KCNE family members were searched from Ensemble-database. Fish sequences of the KCNQ-family (KCNQ1–5) were aligned with the putative crucian carp KCNQ1 using ClustalX. Because only a few KCNQ sequences were available for fish, the mouse orthologues (KCNE1 and KCNE4–1) were included in the alignment. The phylogenetic trees of KCNQ and KCNE gene families were rooted using Caenorhabditis elegans KCNQ-like gene KQT-1 and KCNE-like gene MPS-1, respectively. ClustalX and TreeView programs were used to construct and draw the neighbor-joining (NJ) tree. In the NJ analysis, Phylip distance measure and 1000 bootstrap replicas were used.

**Transcript abundance.** Five separate samples were prepared for extraction of the total RNA. Depending on the size of fish each sample represents a pooled tissue of 3–10 atria or ventricles. RNA was treated with DNase to avoid genomic DNA contamination. First strand cDNA synthesis was performed with M-MuLV RT H

**Table 2. Primer pairs (in 5’-3’ direction) used for quantitative RT–PCR**

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer Sequences</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
</table>
| ccKCNQ1     | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | 101 |
| ccKCNQ1     | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | 102 |
| ccKCNQ1     | F:GGCCACGGCTCGAATGACTAGC  
R:GGCCACGGCTCGAATGACTAGC | 93 |

**Data analysis.** The primer sequences were used to construct and draw the neighbor-joining (NJ) tree. In the NJ analysis, Phylip distance measure and 1000 bootstrap replicas were used.
conditions (9). No-template control and RT-control reactions were run for quality assessment in every run. The specificity of the PCR reaction was monitored by melting curve analysis. Dnad homolog subfamily A member 2 (DnajA2) was used as an endogenous reference gene, and its expression level was used to normalize mRNA levels of the ion channel genes. In thermal acclimation, fish DnajA2 is more stably expressed than many conventionally used reference genes making it a suitable reference gene (37).

**Heterologous expression of KCNQ1 and KCNE1.** Crucian carp KCNQ1 and KCNE1 were expressed in Chinese hamster ovary (CHO) cells (18). Open reading frames (ORF) for KCNQ1 and KCNE1 were digested from pGEM-T Easy plasmids and ligated to cohesively digested pcDNA3.1(zeo+). CHO cells were cultured in Ham’s Nutrient Mixture F-12 (EuroClone) with 4% fetal bovine serum at 37°C. CHO cells, cultured on glass cover slips, were transfected for 10–16 h in 3.5 ml dishes containing 15 μl Effectene Transfection Reagent (Qiagen), 0.3 μg pEGFP-N1 and 0.9 μg KCNQ1 or KCNE1 in pcDNA3.1(zeo+). The pEGFP-N1 plasmid produces green fluorescent protein and was used to visualize transfection of the cells. For cotransfection of KCNQ1 and KCNE1, 0.3 μg of each plasmid was used. Cells were used in patch clamp experiments 48–72 h after the transfection.

**Electrophysiology.** Atrial myocytes were used for electrophysiological experiments because $I_{Ks}$ is much larger in atrial than ventricular myocytes of the crucian carp, and therefore easier to record at low temperatures.

**Results.** Atrial myocytes were used for electrophysiological experiments because $I_{Ks}$ is much larger in atrial than ventricular myocytes of the crucian carp, and therefore easier to record at low temperatures. Also the interfering inward rectifier current, $I_{K1}$, is smaller in atrial cardiomyocytes (11). Atrial myocytes of the crucian carp heart were isolated with enzymatic digestion and were used within 8 h from isolation (35). Whole-cell voltage clamp experiments were performed using an Axopatch 1-D (Axon Instruments, Union City, CA) or an EPC-9 (HEKA Instruments, Lambrecht/Pfalz, Germany) amplifier (8). The experiments with CA myocytes were conducted at 4°C and 11°C and those with WA myocytes at 11°C and 18°C. Hence results were obtained both at the physiological body temperatures of the animals and at the common experimental temperature of 11°C, the latter enabling direct comparison of results between the acclimation groups. Temperature was adjusted to the desired value by Peltier devices (TC-10 or HCC-100A, Dagan, Minneapolis, USA) and was continuously monitored by thermistors positioned close to the myocyte. Patch pipettes were pulled from borosilicate glass (Garner, Claremont, Calif., USA) using a vertical two-stage puller (Narishige, PC-10). In each figure the results are at least from 3 different fish individuals, while n indicates the number of myocytes.

During the experiments myocytes were superfused with an external saline solution at the rate of 1.5–2.0 ml min$^{-1}$. The solution contained (mmol l$^{-1}$): 150 NaCl, 5.4 KCl, 1.8 CaCl$_2$, 1.2 MgCl$_2$, 10 glucose and 10 HEPES (pH adjusted to 7.7 with NaOH). When working on cardiac myocytes, tetrodotoxin (0.5μM, Tocris Cookson, UK), nifedipine (10 μM, Sigma), glibenclamide (10μM, Sigma) and E-4031 (10 μM, Tocris) were added to the solution to block Na$^+$, Ca$^{2+}$, ATP-sensitive K$^+$ current and rapid component of the delayed rectifier (Ik1), respectively. The pipette solution contained (mmol l$^{-1}$): 140 KCl, 1 MgCl$_2$, 5 EGTA, 4 MgATP and 10 HEPES (pH adjusted to 7.2 with KOH).

Voltage dependence of steady-state (SS) activation was obtained by a voltage protocol, where membrane potential was clamped from the holding potential of $-50$ mV to test potentials between $-40$ and $+60$ mV in 10 mV increments to activate the channels. Normalized currents ($I_{\text{norm}}$) were plotted as a function of membrane voltage and fit to the Boltzmann equation: $y = \frac{1}{1 + \exp \left( \frac{V - V_{0.5}}{S} \right)}$, where $V$ is membrane potential, $V_{0.5}$ is the midpoint and $S$ is the slope of the curve.

Because KCNQ1 and $I_{Ks}$ currents often show complex (sigmoidal) activation kinetics, time to half maximal current was used to describe time course of activation and was plotted against the voltage of depolarizing pulses. Temperature dependence of $I_{Ks}$ kinetics was expressed as a $Q_{10}$-value and was obtained from the equation where $T_1$ and $T_2$ are the temperatures that produce the kinetic rates of $R_1$ and $R_2$, respectively: $Q_{10} = \left( \frac{R_2}{R_1} \right)^{\frac{10}{T_2-T_1}}$.

Chromanol 293B (Tocris) block of the $I_{Ks}$ of atrial myocytes and the currents generated by homomeric KCNQ1 and heteromeric KCNQ1/KCNE1 channels were examined at 1, 10 and 100 μM concentrations. Chromanol was dissolved in ethanol as a 20 mM stock solution and diluted in the external saline solution to give the desired concentrations. Currents were elicited by depolarizing pulses from $-60$ mV to $+70$ mV for 4 s and the cells were exposed to each concentration until steady-state block was achieved.

For AP measurements sinoatrial preparations were gently fixed with insect pins on Sylgard-coated bottom of the 10-ml recording chamber filled with oxygenated (100% O$_2$) physiological saline (36). The preparation was allowed to equilibrate for about 1 h to reach a stable beating rate before APs were recorded with sharp microelectrodes. Pipettes had a resistance of about 22 MΩs when filled with 3 M KCl. Analog signals were amplified by a high-impedance amplifier (KS-700, WPI, UK) and digitized (Digitigata-1200 AD/DA board, Axon) with sampling rate of 2 kHz before storing on the computer with the aid of Axotape (Axon) acquisition software. AP characteristics were analyzed with Clampfit software (Axon).

**RESULTS.**

**Sequencing and ccKCNQ1 (K7.1) and ccKCNE1 (MinK).** For heterologous expression of the $I_{Ks}$, putative molecular components of the channel complex were cloned and sequenced from crucian carp. The cDNA sequences of the crucian carp KCNQ1 and KCNE1 genes are denoted as ccKCNQ1 (GenBank No. GU073448) (cc for Carassius carassius) and ccKCNE1 (GenBank no. GU073449), respectively. The ccKCNQ1 is composed of 191 bp 5’-UTR sequence, an ORF of 1,956 bp coding for 651 amino acid residues and 3’-UTR of 803 bp (Fig. 1A). The deduced amino acid sequence of the ccK7.1 shares 88.1% and 70% similarity with zebrafish and mammalian K7.1, respectively (Fig. 1D). In the phylogenetic comparison, the ccKCNQ1 gene grouped with KCNQ1 genes of other fish species, indicating that it is orthologous to other vertebrate KCNQ1s (Fig. 2A).

Sequences of P-loop and S6-transmembrane domain are important for K$^+$ ion selectivity, binding of the MinK beta subunit to the K7.1 and affinity of the channel complex to its blocker, chromanol. Both the P loop and the S6 domain are identical in the ccK7.1 and the mammalian K7.1. Thus, ccK7.1 includes all those amino acid residues that are...
regarded important for chromanol binding and interaction with the MinK (Fig. 1A). The P-loop of the ccKv7.1 has the signal sequence (TxGYG) of the voltage-dependent K⁺ channels. Moreover, the ccKv7.1 contains 8 consensus sites for phosphorylation by the protein kinase C (PKC).

A partial cDNA of the ccKCNE1 forms an ORF of 396 bp coding for 131 amino acid residues and a 12 bp 3'UTR sequence (Fig. 1B). The 5'UTR sequence of the ccKCNE1 remained undetermined. The primary amino acid sequence of the ccMinK shows high similarity within the membrane
spanning domain of other species’ MinK. However, the N- and C-termini were highly variable between fish and mammalian genes, leading to low (about 40%) global similarity between crucian carp and mammalian MinK (Fig. 1D). The similarity between crucian carp and zebrafish MinK was much higher (82.4%), indicating that the cloned crucian carp gene is orthologous to the zebrafish MinK. This was further strengthened by the phylogenetic analysis of vertebrate KCNE gene family, which clearly showed that ccKCNQ1 groups with KCNE1 genes rather than with other members of the KCNE family (Fig. 2B). The deduced amino acid sequence of ccMinK contained three PKC phosphorylation sites at positions 79, 118 and 126, one of which (S79) was similar with the phosphorylation site of the mammalian MinK (Fig. 1B and C).

Transcript expression of ccKCNQ1 and ccKCNE1. Transcripts of ccKCNQ1 and ccKCNE1 were expressed in both atrium and ventricle of the crucian carp heart (Fig. 3). However, the ccKCNE1 transcript abundance was only 1.9 ± 0.1 and 1.6 ± 0.3% of the ccKCNQ1 expression in the CA and WA carp atrium, respectively. In the ventricle, the abundance of ccKCNE1 compared with the ccKCNQ1 expression was a little higher, 5.0 ± 0.5 and 3.1 ± 0.4% in CA and WA carp, respectively. The higher relative expression of the ccKCNE1 in ventricular tissue is due to lower ccKCNQ1 level in the ventricle (P < 0.05): expression in the atrium was 2.3 ± 0.3 and 2.6 ± 0.1 fold as high as in the ventricle of CA and WA crucian carp, respectively. There were no differences (P > 0.05) in the expression levels of ccKCNE1 and ccKCNQ1 between the acclimation groups.

\(I_{\text{Ks}}\) of the crucian carp atrial myocytes. The maximum density of \(I_{\text{Ks}}\) was 6.98 ± 0.68 and 1.77 ± 0.23 pA/pF (P < 0.05) in WA and CA myocytes, respectively, when measured at the acclimation temperatures of the fish (18°C and 4°C) (Fig. 4C). At 11°C, the current density did not differ between acclimation groups (4.80 ± 0.64 and 4.37 ± 0.39 pA/pF for CA and WA fish, respectively). Noticeably, current density increased between 4°C and 11°C with Q10 of 4.15 and between 11°C and 18°C with a value of 1.96. The voltage dependence of activation was also similar in both acclimation groups, and was characterized by a sigmoidal shape with half-activation voltages of 6.61 ± 3.62 and 4.78 ± 2.15 mV for WA and CA fish, respectively (P > 0.05) (Fig. 4B). Collectively, these findings indicate that thermal acclimation...
Tail currents of the crucian carp atrial \( I_{Ks} \) displayed an initial transient increase before the slow deactivation, producing a "hooked" current tail, in particular following depolarization to strongly positive voltages (Fig. 4A). The hook is attributed to channel gating where recovery from inactivation occurs faster than deactivation (31). Hence, the transient increase of the tail current suggests that the current partly inactivates during depolarization and causes subsequently a small and rapid recovery from inactivation. Partial inactivation of the K\(_{7.1}\) current is typical to the homotetrameric K\(_{7.1}\) channels, but not the currents produced by heteromeric K\(_{7.1}/\)MinK channels. The hooked tail current in atrial myocytes of both WA and CA crucian carp suggests therefore that channels responsible for the atrial \( I_{Ks} \) are mainly formed by the homotetrameric ccK\(_{7.1}\) channels without the ancillary ccMinK subunit.

Comparison of cloned channels and atrial \( I_{Ks} \). To compare whether the native \( I_{Ks} \) resembles more the pure ccK\(_{7.1}\) currents than currents generated by coexpression of ccK\(_{7.1}\) and ccMinK subunits, ccK\(_{7.1}\) was expressed separately and concurrently with the ccMinK in CHO cells (Fig. 5). Consistent with the previous findings from other vertebrate species, ccK\(_{7.1}\) currents activated much faster (\( t_{0.5} \) = 0.14 ± 0.01 s at +70 mV) than currents generated by coexpression of the ccK\(_{7.1}\) and the ccMinK, which were extremely slow to activate (\( t_{0.5} \) = 20.36 ± 4.32 s at +70 mV) \((P < 0.05)\) (compare Fig. 4A to Fig. 5A). The homomeric ccK\(_{7.1}\) current differs from the atrial \( I_{Ks} \) in that there is prominent inactivation during the depolarizing pulses, in particular, at strongly positive voltages (Fig. 5A).

Although the real SS-activation of the ccK\(_{7.1}/\)ccMinK currents couldn’t be determined due to very slow activation of the current, it is evident that ccMinK has a drastic effect of voltage-dependence of SS-activation. The presence of ccMinK in the channel complex shifted SS-activation curve to positive

![Fig. 3. Expression of ccKCNQ1 and ccKCNE1 transcripts relative to the DnaJA2 mRNA (means ± SE; \( n = 5 \)) in atrial and ventricular tissue of cold acclimation (CA) and warm acclimated (WA) crucian carp. Statistically significant difference (*\( P < 0.05 \)) between atrial and ventricular preparations by Student’s \( t \)-test for paired data.](http://ajpregu.physiology.org/)

![Fig. 4. Characterization of \( I_{Ks} \) in atrial myocytes of thermally acclimated crucian carp. A: voltage protocol (left) and representative current tracings of \( I_{Ks} \) activation in CA and WA atrial myocytes. Tail currents are shown on extended time scale (oblique arrows indicate the hook). B: voltage dependence of \( I_{Ks} \) activation at 4°C and 18°C for CA and WA fish, respectively. C: density of \( I_{Ks} \) at +60 mV at the acclimation temperature of the fish and at 11°C. D: time course of \( I_{Ks} \) activation as a function of membrane potential at the acclimation temperature of the fish. Means were compared using Student’s \( t \)-test for unpaired data or after logarithm conversion of the data using nonparametric Mann-Whitney \( U \)-test. Significant differences (*\( P < 0.05 \)) between WA and CA fish (B and D) or between the values at 11°C and acclimation temperatures (4°C or 18°C) (C). The results are means ± SE of 5–12 cells.](http://ajpregu.physiology.org/)
voltage-dependent activation of the pure ccKv7.1 channels with IC50 values of 23.24 ± 4.99 and 106.07 ± 39.62 μM, respectively (P < 0.01) (Fig. 6).

Chromanol sensitivity of the native IKS of the crucian carp heart is mainly carried by the homotetrameric ccKv7.1 subunits. The MinK subunit is known to increase the binding of chromanol to the K7.1 channels, thereby decreasing the drug concentration needed to inhibit the IKS. The ccKv7.1/ccMinK channels were about 10 times more sensitive to chromanol than the pure ccKv7.1 channels with IC50 values of 23.24 ± 4.99 and 106.07 ± 39.62 μM, respectively (P < 0.01) (Fig. 6).

Chromanol sensitivity of the native IKS (at 18°C) was similar to the homotetrameric ccKv7.1 channels with an IC50 value of 233.25 ± 55.43 μM. These findings strongly suggest that the atrial IKS of the crucian carp heart is mainly carried by the homotetrameric ccKv7.1 subunits.

Effects of forskolin on the IKS. Density of IKS in the mammalian heart is upregulated by PKA-dependent phosphorylation of the K7.1 subunit. Furthermore, the response is critically dependent on the presence of the MinK, although the accessory subunit itself is not phosphorylated. Activation of the PKA pathway with 10 μM forskolin did not increase IKS of the crucian carp atrial myocytes (Fig. 7). Similar results were obtained when using 1 μM isoprenaline (data not shown). Thus, IKS of the crucian carp heart is not increased by activation of the PKA.

Frequency response of the IKS. One of the characteristics of the cardiac IKS is its accumulation at high heart rates due to incomplete deactivation, while the pure K7.1 current does not build up. Experiments on the cloned crucian carp channels in CHO cells are consistent with this: current density of the pure ccKv7.1 current remains stable at pacing frequencies of 40, 50 and 60 times per min, while the ccKv7.1/ccMinK channels show prominent increase in current density with higher frequencies (Fig. 8). The IKS of crucian carp atrial myocytes shows a very weak frequency response, fairly similar (P > 0.05) to that of the homomeric ccKv7.1 channels.

Significance of IKS in the regulation of AP duration. Contribution of IKS to AP duration of crucian carp atrial myocytes was tested in intact atrial preparations of WA fish (Fig. 9). 100 μM chromanol, which blocks about 30% of the IKS, caused 12% increase in duration of AP (APD50) from 209 ± 24 ms to 233 ± 26 ms (P < 0.05).

DISCUSSION

This is the first report of IKS and its molecular background in the heart of an ectothermic vertebrate. It is shown that genes of both the alpha subunit (ccKv7.1) and the ancillary beta subunit (ccMinK) of the slow delayed rectifier channel are expressed in the crucian carp heart. However, both transcript expression of the subunits and electrophysiological properties of the atrial IKS suggest that the crucian carp cardiac IKS is mainly formed by the homomeric ccKv7.1 channels instead of the heteromeric ccKv7.1/ccMinK complex. This molecular arrangement produces a very weak frequency response, fairly similar (P > 0.05) to that of the homomeric ccKv7.1 channels.
logically relevant for this species considering the blunted beta-adrenergic responsiveness of the crucian carp heart (36).

Sequence structures of ccKCNQ1 ccKCNE1. In mammals, the cardiac $I_{Ks}$ current is produced by the coassembly of the pore-forming $K_{v}7.1$ channel and the accessory $\beta$-subunit, MinK (1, 25). The primary amino acid sequence of the ccKv7.1 shows high similarity (about 70%) to its mammalian orthologues, whereas the sequences of the mammalian MinK and the ccMinK differ considerably (similarity about 40%) from each other. The low sequence similarity of fish and mammalian MinK subunits is due to highly different N- and C-termini. Mutations associated with the long QT syndrome in the C-terminus of the MinK often accelerate deactivation of the $I_{Ks}$, diminish single channel conductance and reduce open probability of the $K_{v}7.1$/MinK channels (6, 27). Differences in the C-termini of fish and mammalian MinK are probably not only due to the evolutionary distance of the two vertebrate classes, but may be indicative of species-specific differences in function of the accessory subunit (although not necessarily in $I_{Ks}$).

In mammals, $I_{Ks}$ is regulated by serine-threonine (30, 33, 39, 42) and tyrosine kinases (21). The ccKv7.1 of the crucian carp heart contains 8 putative PKC phosphorylation sites. Four of them are conserved in both mammals and crucian carp (T217, S361, S376, and S559), two are conserved among fish species (S348 and S491) and two are specific for the ccKv7.1 (T50 and S361, S376, and S559), two are conserved among fish species (S348 and S491) and two are specific for the ccKv7.1 (T50 and S559). Interestingly, the ccKv7.1 does not have any consensus sequence for PKA phosphorylation site. In the mammalian $K_{v}7.1$ (T50 and S426). Interestingly, the ccKv7.1 does not have any consensus sequence for PKA phosphorylation site. In the mammalian $K_{v}7.1$, PKA-dependent phosphorylation of the S27 mediates the activating effect of the sympathetic nervous system on $I_{Ks}$ (20). Moreover, the ccMinK contains three putative cytoplasmic phosphorylation sites at positions S79, S118 and S126, one of which (S79) is conserved in the mammalian MinK. Thus, sequence analysis suggests that the crucian carp $I_{Ks}$ could be regulated by PKC but not by PKA. Indeed, activation of the PKA pathway with forskolin did not increase $I_{Ks}$ of the crucian carp atrial myocytes. Since PKA-mediated regulation of the $I_{Ks}$ requires coexpression of $K_{v}7.1$ with MinK (17), the $I_{Ks}$ of the crucian carp atrial myocytes would probably be insensitive to PKA-stimulation even if the phosphorylation site existed in the ccKv7.1.

Crucian carp $I_{Ks}$ is mainly produced by the homomeric ccKv7.1 channels. In mammals, the slow delayed rectifier is formed by coassembly of four $K_{v}7.1$ and two MinK subunits (5, 22, 40). According to this molecular arrangement the transcript abundance of the ccKCNE1 should be about half that of the ccKCNQ1. However, the transcript level of the ccKCNE1 was only 1.6–1.9% of the ccKCNQ1 mRNAs. Therefore, no more than 3.2–3.8% of the atrial $I_{Ks}$ channels are expected to be heteromers of ccKv7.1 and ccMinK channels. Even if the protein level were not directly proportional to gene transcripts, the low transcript expression suggests that the quantity of the ccMinK subunits is not sufficient to produce large numbers of the heteromeric ccKv7.1/ccMinK channels, i.e., majority of the functional delayed rectifiers in crucian carp atrial myocytes does not have the ancillary ccMinK subunit. Indeed, the transcript expression of ccKCNE1 in the crucian carp atrium is much less than e.g., in the human atria, where KCNE1 transcripts are about 50% and 100% of the KCNQ1 expression in left and right atrium, respectively (19). However, a small amount of ccKCNE1 expression may be needed for trafficking of the ccKv7.1 to the membrane and a minor population of ccKv7.1/ccMinK channels might explain the small deviations in current characteristics between $I_{Ks}$ and ccKv7.1.

In mammalian channels, the coassembly of MinK with $K_{v}7.1$ changes electrophysiological properties of the $I_{Ks}$ lead-
Fig. 9. Effect of chromanol (100 μM) on action potential (AP) duration in intact atrium of WA crucian carp. Left: original recordings of atrial AP in the absence and presence of chromanol. Right: Effects of chromanol on AP duration at 50% repolarization level are shown. Values are expressed as means ± SE. Statistically significant difference between control and chromanol treatment by Student’s *t*-test for paired data (*P* < 0.05). Number of examined hearts (*n*) was 5.

...ing to increased current amplitude, slower activation kinetics, abolition of inactivation and shift of SS-activation to more positive membrane potentials (31, 41). Furthermore, the presence of the MinK in the channel complex changes pharmacological properties of the *I* _K_s, which appears e.g., as increased sensitivity to the blocking effect of chromanol (4, 32).

Comparison of the properties of the atrial *I*_K_s with the currents produced by homomeric ccKv7.1 and heteromeric ccKv7.1/ccMinK channels expressed in the CHO cells strengthens the assumption that *I*_K_s of the crucian carp atrial myocytes is mainly formed by the homomeric ccKv7.1 channels rather than by the heteromeric ccKv7.1/ccMinK coassembly. Fast activation kinetics and more negative position of the SS-activation curve of the crucian carp atrial *I*_K_s current are closer to those of the homomeric ccKv7.1 channels. The *I*_K_s of the atrial myocytes was about ten times less sensitive to chromanol block than the heteromeric K_v7.1/MinK channels but similar to the sensitivity of the homomeric K_v7.1 channels. Moreover, the tail current of the atrial *I*_K_s had a “hook” indicating that the current inactivates. Taken together, activation kinetics, voltage-dependence of activation, inactivation of the current and reduced sensitivity to chromanol block are indicative that the ancillary ccMinK subunit is not involved in the formation of crucian carp native *I*_K_s.

**Frequency-dependence of the *I*_K_s.** *I*_K_s has a special function as repolarization reserve in situations where AP tends to prolong due to high beta-adrenergic tone or failure of other repolarizing K⁺ currents (15). This property stems from time- and frequency-dependence of the *I*_K_s, due to relatively slow activation kinetics the current increases with increased duration of depolarization, while the slow deactivation leaves some of the delayed rectifier channels in open state at short diastolic intervals and therefore immediately conductive when the driving force is restored by the next depolarization (14, 24, 29). These characteristics of the *I*_K_s are dependent on the heteromeric coassembly of the K_v7.1 and MinK subunits and absent in the homomeric K_v7.1 channels.

In contrast to the heteromeric channels of the mammalian heart, the homomeric ccK_v7.1 channels of the crucian carp atrial myocytes produce a flat frequency response of the *I*_K_s. In this regard, it is important to note that the beta-adrenergic signaling has practically no effect on heart rate in crucian carp (36) (but compare (28)). In the absence of positive chronotropic response to the activation of the beta-adrenergic cascade, the weak frequency response of the *I*_K_s is completely appropriate for cardiac function in this species. The absence of phosphorylation site for PKA in the ccK_v7.1 and the consequent absence of beta-adrenergic stimulation of the *I*_K_s is also meaningful considering that the beta-adrenergic stimulation is totally absent in the sinoatrial tissue of crucian carp (36). Indeed, the parsimonious molecular composition of the cardiac *I*_K_s channel is a relevant and well adjusted channel construction that fulfills the requirements of cardiac function in crucian carp.

**Effects of temperature on *I*_K_s.** Electrophysiological properties of the crucian carp *I*_K_s are intimately associated with its homomeric ccK_v7.1 composition and can be seen as adaptation to low temperature. The homomeric ccK_v7.1 assembly provides a simple molecular solution for thermal compensation of ion channel function, since removal of the MinK subunit form the channel assembly makes the activation kinetics of the *I*_K_s much faster (41). The homomeric K_v7.1/MinK channels enable contribution of the *I*_K_s to repolarization of the crucian carp cardiac AP, which would be impossible for the Kv7.1/MinK channels of the endothermic hearts.

It has been shown that the inward rectifier (*I*_K_1) and the fast delayed rectifier (*I*_K_s) K⁺ current have an essential role in thermal acclimation of cardiac AP duration in fish (9, 10, 38). By analogy, it could be assumed that *I*_K_s is also involved in regulation of AP duration under chronic temperature changes. However, neither transcript expression of the channel subunits nor electrophysiological characteristics of the cardiac *I*_K_s displayed any differences between CA and WA crucian carp. Acute cooling decreases the density of *I*_K_s, but there is no compensatory increase of *I*_K_s that would counteract the acute temperature effect in CA fish. Therefore, the importance of the *I*_K_s in AP regulation is associated with acute temperature changes to accelerate/decelerate repolarization of cardiac AP when temperature and heart rate rises/drops. It has been previously shown that activation kinetics of the K_v7.1/MinK channels have stronger temperature dependence (Q_10 2.5) than that of the homomeric K_v7.1 channels (Q_10 1.5) (32). The weaker temperature dependence of the homomeric K_v7.1 channels is also adaptive, since it extends the thermal tolerance range of the functional *I*_K_s to lower temperatures.

**Limitations of the study.** Although electrophysiological properties of the *I*_K_s of the crucian carp myocytes are in several respects similar to those of the homomeric ccK_v7.1 channels, the two currents are not completely identical. This could be due to the presence of some heteromeric ccK_v7.1/ccMinK channels and/or assembly of the ccK_v7.1 channels with some other β-subunit. Mammalian heart is known to express five KCNE genes (KCNE1-KCNE5), which could produce heteromers with the KCNQ1 gene product (3). However, when coexpressed with KCNQ1, KCNE2 and KCNE3 produce currents that have instantaneous activation, while association of either KCNE4 or KCNE5 with KCNQ1 strongly suppresses density of the *I*_K_s (3, 7, 19, 26). Furthermore, in a complex where both KCN1 and KCNE4 are coexpressed with KCNQ1, KCNE1 seems to exert dominant effect as far as the current is practically identical to that of KCNQ1/KCN1 channels (3). Considering that the *I*_K_s of the crucian carp atrial myocytes is not instantaneous and its density is high, it seems unlikely that *I*_K_s would be significantly contributed by any of the known KCNQ1/KCNE2-KCNE5 heteromers. However, possible ef-
fects of different KCNE products on crucian carp *I*~ks~ cannot be completely excluded without characterizing all possible channel combinations of ccKCNQ1 with putative ccKCNE products in heterologous system and measuring expression levels of different ccKCNE genes in the crucian carp heart.

**Perspectives and Significance**

This is the first time that cardiac *I*~ks~ of a vertebrate species has been shown to be mainly produced by the homomorphic K~7.1~ channels rather than the heteromeric K~7.1~/MinK assemblies. The homomeric channel composition allows fast activation of the *I*~ks~ at temperatures that are 20–35°C lower than body temperatures of endothermic animals. To our knowledge, this is a novel molecular mechanism for evolutionary thermal compensation of ion channel function. These findings predict that similar homomeric molecular arrangement of the *I*~ks~ delayed rectifier channels would be present in the hearts of other cold-living ectotherms and that the heterotetrameric Kv7.1 channels rather than the heteromeric Kv7.1/MinK assistance.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

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