Comparative Biochemistry and Physiology, Part C xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Molecular basis and drug sensitivity of the delayed rectifier (I_{Kr}) in the fish heart

Minna Hassinen *, Jaakko Haverinen, Matti Vornanen

University of Eastern Finland, Department of Biology, P.O. Box 111, 80101 Joensuu, Finland

ARTICLE INFO

Article history:
Received 20 May 2015
Received in revised form 15 July 2015
Accepted 17 July 2015
Available online xxxx

Keywords:
Delayed rectifier potassium current
Cardioactive drugs
Erg channels
Fish heart

ABSTRACT

Fishes are increasingly used as models for human cardiac diseases, creating a need for a better understanding of the molecular basis of ion channel function in fish hearts. To this end we cloned and sequenced KCNH6 K^+ channel of the crucian carp (*Carassius carassius*) that produces the rapid component of the delayed rectifier K^+ current (I_{Kr}), the main repolarising current of the fish heart. KCNH6 (ccErg2) was the main isoform of the K_v11 potassium channel family with relative transcript levels of 98.9% and 99.6% in crucian carp atrium and ventricle, respectively. Only small amounts (1.1% in atrium and 0.4% in ventricle) of the KCNH2 (ccErg1) channel, an orthologue to human cardiac Erg (Herg) channel, was expressed in the crucian carp heart. The native atrial I_{Kr} and the cloned ccErg2 were inhibited by similar concentrations of verapamil, terfenadine and KB-R7943 ($P > 0.05$), while the atrial I_{Kr} was about an order of magnitude more sensitive to E-4031 than ccErg2 ($P < 0.05$). This suggests that the atrial I_{Kr} channel may involve some accessory β-subunits or alternatively spliced Erg isoforms in addition to the ccErg2. Sensitivity of the crucian carp atrial I_{Kr} to E-4031, terfenadine and KB-R7943 was similar to what has been reported for the Herg channel. In contrast, the sensitivity of the crucian carp I_{Kr} to verapamil was approximately 30 times higher than the previously reported values for the Herg current. In conclusion, the cardiac I_{Kr} is produced by non-orthologous gene products in fish (Erg2) and mammalian hearts (Erg1) and there are also some marked differences in drug sensitivity between fish and mammalian Erg1/2 which need to be taken into account when using fish heart as a model for human heart.

1. Introduction

Potassium currents are vital for the excitability of the heart by maintaining the negative resting membrane potential (RMP) of atrial and ventricular myocytes and regulating repolarization of cardiac action potential (AP) in all cardiac compartments (Nerbonne and Kass, 2005; Schmitt et al., 2014). Cardiac K^+ currents are flexible entities which change in various physiological and pathological states of the cardiovascular system. The density of cardiac K^+ currents is modified as an outcome of physical training and in cardiac ischemia, coronary artery disease, cardiac hypertrophy, heart failure and other disease states of the heart (Carmeliet, 1999; Liu et al., 2010; Wang and Hill, 2010; Yang et al., 2010). Furthermore, the composition of the cardiac K^+ currents markedly differs between species partly because heart rate (HR), and therefore duration of AP, strongly varies depending on the body mass of the animal (Vornanen, 1989; Rosati and McKinnon, 2009). Small mammals with high HRs and short APs, in particular the murine hearts, express K^+ currents that activate and inactivate quickly (transient outward current, I_{to}), while in hearts of larger mammals kinetically slower K^+ currents (fast and slow components of the delayed rectifiers, I_{Kr} and I_{Ks}) are often prevailing. Due to the peculiar AP shape and special K^+ current composition, murine hearts are not in all respects satisfactory models for human cardiac electrophysiology (Nerbonne, 2004; Milan and MacRae, 2005). Although basic features of electrophysiological remodeling may be similar in murine and human hearts under disease states (e.g. prolongation of AP in heart failure), different molecular basis and associated differences in biophysical properties of ion currents make extrapolation of findings from murine models to human heart problematic.

Fishes, in particular the zebrafish, are increasingly used as a model for human cardiac electrophysiology, cardiovascular drug screening and cardiac diseases (Langheinrich et al., 2003; Leong et al., 2014). This is based on the similarity of HR and AP shape between human and zebrafish hearts, which makes conclusions between species at phenomenological level (changes in HR and AP duration) fairly straightforward. Although the ion current systems of fish and mammalian hearts are in several respects similar, significant differences exist in biophysical properties, molecular composition and drug sensitivity of the currents (Baker et al., 1997; Vornanen, 1997; Vornanen et al., 2002; Paajanen and Vornanen, 2004; Haverinen et al., 2007; Nemtsas

Abbreviations: AP, action potential; CHO cells, Chinese hamster ovary cells; HR, heart rate; RMP, resting membrane potential; UTR, untranslated region.

* Corresponding author at: University of Eastern Finland, Department of Biology, P.O. Box 111, 80101 Joensuu, Finland.
E-mail address: Minna.Hassinen@uef.fi (M. Hassinen).

http://dx.doi.org/10.1016/j.cbpc.2015.07.005
1532-0456/

et al., 2010; Hassinen et al., 2011; Abramochkin et al., 2014). Inasmuch as the molecular basis of fish cardiac ion currents remains incompletely known, the possibility exists that the similarities in electrophysiological phenotypes of fish and mammalian hearts are produced by different molecular entities, and therefore the fish cardiac model may pose similar problems as the murine model. Longer evolutionary distance between mammals and fish and the whole genome duplication in the teleost fish lineage increase the likelihood of genetic differences between the two vertebrate groups (Volff, 2005).

On the other hand, the enormous diversity of fish species could offer natural models for human cardiac diseases, since evolution has produced physiological adaptations to various stresses (Albertson et al., 2009; Schartl, 2014). Comparing evolutionary solutions to specific stresses with the responses of human heart to similar problems may help to understand beneficial and detrimental gene functions of the human heart under the stress. Due to their exceptional tolerance to oxygen deprivation, the crucian carp (*Carassius carassius*) could be an interesting model to examine cellular and molecular mechanisms, which enable survival and function of the vertebrate heart under severe oxygen deprivation (Vornanen et al., 2009). However, successful modeling of human cardiac problems requires that it is known which fish genes are orthologous to the human genes (Howe et al., 2013). To enable the use of crucian carp as a hypoxia tolerant model of cardiac excitation, we have analyzed molecular genetic background of various cardiac ion channels of the crucian carp (Hassinen et al., 2008; Hassinen et al., 2011; Vornanen et al., 2011). The current study examines molecular background and drug sensitivity of the crucian carp cardiac delayed rectifier potassium current (I_{Kr}). I_{Kr} is one of the most important repolarizing ion currents in cardiac myocytes of human and fish heart (Vornanen et al., 2002; Sanguinetti and Tristani-Firouzi, 2006). I_{Kr} is generated by the voltage-gated potassium channels ERG1 or ERG2, which are tetrameric assemblies of the α-subunits encoded by KCNH2 and KCNH6 genes, respectively. Inherited mutations of the human KCNH2 gene and blockade of the ERG1 channel by diverse medicinal drugs can lead to life-threatening arrhythmias. The importance of ERG1 channel as a putative target in treating cardiac arrhythmias and as an unintentional target of drugs developed for treating non-cardiac diseases has led to extensive study of human ERG channel and attempts to find suitable animal models for those studies. In the present study, the Erg channel was cloned and sequenced from crucian carp and expressed in the mammalian cell line. Properties of the native atrial I_{Kr} of crucian carp heart and the cloned Erg channel in Chinese hamster ovary (CHO) cells were compared.

2. Materials and methods

2.1. Animals

Crucian carp (*C. carassius*) (4 males and 21 females) were caught from the wild and reared in the animal facilities of the University of Eastern Finland, Joensuu campus for the minimum of 4 weeks before used in the experiments. Fishes were maintained in 500 L metal tanks under a 12:12-h light-dark photoperiod and constant recirculation of aerated tap water at +18 °C. Food was provided *ad libitum* 5 times per week. All experiments were conducted with the consent of the national committee for animal experimentation (permission PH472A).

2.2. Molecular studies

2.2.1. RNA extraction

Total RNA was extracted with TriZOL Reagent (Invitrogen, Carlsbad, USA) from atrium and ventricle of the heart, brain and skeletal muscle which were powdered under liquid nitrogen. Integrity and quantity of RNA was assessed by agarose gel electrophoresis and NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), respectively.

2.2.2. Cloning and sequencing

Total RNA from skeletal muscle and heart was used to clone partial sequences for crucian carp KCNH2 (ccErg1) and KCNH6 (ccErg2) genes, respectively. DNase treated RNA was reverse-transcribed using random primers (Promega, Madison, WI, USA) and M-MuLV Reverse Transcriptase RNase H- (Thermo Scientific, Waltham, Massachusetts, USA). The resulting cDNA was PCR amplified using degenerative primers for KCNH2 and KCNH6 (Table 1), DynaZyme EXT DNA-Polymerase (Thermo Scientific) and PCR conditions described earlier (Hassinen et al., 2007). PCR products were run on a 0.8% agarose gel and if no products were observed, 0.5 μl of the PCR-product was reamplified with the same or nested primers as described earlier (Hassinen et al., 2007). 5′-RACE and 3′-RACE kits (Invitrogen) were used to clone the sequences for 5′ and 3′ untranslated region (UTR) of the KCNH6 gene, respectively. PCR products were ligated to the pGEM-T Easy cloning vector (Promega) and transformed to DH5α cells. At least one of the obtained clones was sequenced using ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). Finally, the sequences were confirmed using Phusion High-Fidelity DNA-polymerase (see below). Partial sequences were assembled with AutoAssembler software and the cDNA-sequence was converted into deduced amino acid sequence using Transeq software. ClustalW was used to align the ccKCNH6 sequence with other vertebrate KCNH genes.

2.2.3. Quantitative RT-PCR

Transcripts of ccKCNH2 and ccKCNH6 were quantified from atrium, ventricle, brain and skeletal muscle of crucian carp ($n = 6$) to determine their expression profiles. Total RNA was extracted as described above and treated with RNase free RQ1 DNase (Promega) to avoid genomic DNA contamination. RNA was then reverse transcribed and qPCR-experiments conducted using DyNAmoHS SYBR Green 2-step qPCR-kit (Thermo Scientific). Gene expression was quantified using species-specific primers (5′-CAACCTTCGAGATGCAGACA-3′ and 5′-GCTGTAAG GGGTTCCTCCTC-3′ for ccKCNH6; 5′-CCTGTATCTGGTTCGCCATC-3′ and 5′-GATGCTGCTGTTGTGTGGTT-3′ for ccKCNH2) and PCR conditions described previously (Hassinen et al., 2007). No-template and -RT controls (RNA treated like in cDNA synthesis, but without RT enzyme) were included in every run for each sample to detect possible DNA contamination. DnaJA2 was used as a reference gene as previously described (Hassinen et al., 2008).

2.2.4. Heterologous expression of ccErg2

The open reading frame (ORF) for *ccKCNH6* was PCR-amplified in two pieces (nucleotides 338–2667 and 1722–3895) with Phusion High-Fidelity DNA polymerase (Thermo Scientific) according to the following protocol: initial denaturation at 98 °C for 30 s, 35 cycles at 98 °C for 10 s, 64 °C for 20 s and 72 °C for 1.5 min and final extension at 72 °C for 5 min. Overhang A was added to the 3′-ends of these PCR products and ligated into the pGEM-T Easy vector and sequenced. The resulting plasmids were digested with XcmI and SacI and ligated to form the whole protein coding sequence for ccErg2. The resulting ORF for ccErg2 was digested from pGEM-T Easy plasmid with NotI and BcuI (Fermentas), and ligated to cohesively digested pcDNA3.1/Zeo(+) (Invitrogen).

CHO cells (ECACC) were cultured in Ham's Nutrient Mixture F-12 (EuroClone, Milan, Italy) with 10% FBS (EU Approved FBS, EuroClone) and 100 U/ml penicillin-streptomycin (EuroClone) at 37 °C (5% CO_2). CHO cells cultured on glass coverslips were co-transfected with 0.9 μg ccERG2-pcDNA3.1 and 0.4 μg pEGFP-N1 using Effectene transfection reagent (Qiagen) according to manufacturer's instructions. The pEGFP-N1 plasmid producing green fluorescent protein was used to visualize the transfected cells. Cells were used in patch clamp experiments 24–56 h after transfection.

Please cite this article as: Hassinen, M., et al., Molecular basis and drug sensitivity of the delayed rectifier (I_{Kr}) in the fish heart, Comp. Biochem. Physiol., C (2015), http://dx.doi.org/10.1016/j.cbpc.2015.07.005