Functional Characterization of Inward Rectifier Potassium Ion Channel in Murine Fetal Ventricular Cardiomyocytes

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Key Words

Cardiogenesis • Inward rectifier potassium current • Ventricular cardiomyocytes • Excitability • Action potential

Abstract

Aims: Previous studies have shown the dramatic changes in electrical properties of murine fetal cardiomyocytes, while details on inward rectifier potassium current (IK1) are still seldom discussed. Thus we aimed to characterize the functional expression and functional role of IK1 in murine fetal ventricular cardiomyocytes. **Methods:** Whole cell patch clamp was applied to investigate the electrophysiological properties of IK1. Quantitative real-time PCR, western blotting and double-label immunofluorescence were further utilized to find out the molecular basis of IK1. **Results:** Compared to early developmental stage (EDS), IK1 at late developmental stage (LDS) displayed higher current density, stronger rectifier property and faster activation kinetics. It was paralleled with the downregulation of Kir2.3 and the upregulation of Kir2.1/Kir2.2. IK1 contributed to maintain the maximum diastolic potential (MDP), late repolarization phase (LRP) as well as the action potential duration (APD). However, the contribution

to MDP and velocity of LRP did not change significantly with maturation. **Conclusions:** During fetal development, the switch of IK1 subtypes from Kir2.1/Kir2.3 to Kir2.1 resulted in the dramatic changes in IK1 electrophysiological properties.

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Introduction

The inward rectifier potassium current (IK1) stabilizes the resting membrane potential and plays a major role during the final phase of action potential (AP) repolarization in the cardiomyocytes [1]. Abnormality of IK1 could lead to Anderson's syndrome [2-3], short QT syndrome [3-4], ventricular fibrillation [3, 5-6] and alterations of cardiac excitability [3, 7-9], etc.

IK1 channels in the heart are believed to be homo- and/or heterotetramers of Kir2.1, Kir2.2 and Kir2.3 subunits from the Kir2 family of inward rectifier potassium channels [10-13]. Previous studies have shown that the species-dependent [1] and tissue-specific expression [14] of different IK1 channels subunits may contribute to its variability [11]. In mouse, Kir2.1^{-/-} cardiomyocytes lack

detectable IK1 in whole-cell recordings, displaying significantly broader AP. About 50% reduction in IK1 currents is observed in Kir2.2^{-/-} mouse cardiomyocytes [15]. In neonatal rat cardiomyocytes the Kir2.3 knock-down leads to IK1 current densities decreased by about 80% [16]. Moreover, Kir2.1 and Kir2.3 are found to differentially distribute in the canine atrium and ventricle [17].

The embryonic cardiomyocytes display spontaneous occurring APs [18-19] and share some properties of the diseased cardiomyocytes which suffer from hypertrophy or cardiac failure [20-22]. Thus electrophysiological properties of fetal cardiomyocytes have recently been discussed intensively [23-26]. In some species, IK1 is one focus of interests [27-32]. During development, the current density of IK1 increases in the rat, rabbit, chick, and guinea-pig hearts [28-31]. Adult mouse hearts express a higher level of Kir2.1 than fetal hearts [33] and the subunits of IK1 channel switch from Kir2.1 to Kir2.2 in fetal rat [34]. However, more details of the electrophysiological properties and the species differences need to be further elucidated.

Therefore we aimed to investigate the contribution of different IK1 subunits in murine fetal cardiomyocytes with maturation. The data demonstrated the developmental changes of IK1 in the current densities, activation kinetics and inward rectifier properties. Accordingly, subunits of IK1 channel switched from Kir2.1/Kir2.3 to Kir2.1 with development.

Materials and Methods

Harvesting and isolation of embryonic ventricular cardiomyocytes

As dramatic developmental changes occur during early cardiomyogenesis, six to eight-week old female mice (Kunming mice provided by the Center of Animal Experimentation of Tongji Medical College, Huazhong University of Science and Technology, China) were superovulated for precise timing of their pregnancy [35]. Female mice were sacrificed by cervical dislocation at different developmental stages of the embryos: E10.5 and E17.5 were taken as EDS and LDS, respectively. Atria and ventricles of the embryonic hearts were dissected, only ventricles were enzymatically dissociated to single cells as described [24]. Spontaneously beating cardiomyocytes were used for functional studies after 24-36 hours of cultivation.

Electrophysiological recordings

The whole-cell configuration of the patch-clamp technique was used throughout the study. The cells were placed in a temperature-controlled ((37±0.3)°C) recording chamber which was mounted on the stage of an inverted microscope

(Zeiss, Germany) and superfused with the normal Tyrode's solution containing (mM): NaCl 140, NaOH 2.3, KCl 5.4, CaCl₂ 1.8, MgCl₂ 1, HEPES 10, Glucose 10 (pH adjusted to 7.4 with NaOH). Patch pipettes with a resistance of 2-5 MΩ were used and filled with the internal solution (mM): Kaspertate 90, KCl 40, MgCl₂ 1.0, MgATP 3.0, EGTA 10, HEPES 10, NaCl 10 (pH adjusted to 7.2-7.4 with KOH). Due to the relative low expression of IK1 in embryonic cardiomyocytes [33, 36], and the increased extracellular K⁺ concentration increases potassium conductance [3, 12, 37-39], the normal Tyrode's solution was modified as following to record IK1 (mM): NaCl 45, KCl 100, MgCl₂ 1.0, EGTA 1.0, CaCl₂ 1.8, HEPES 10, Glucose 10, CdCl₂ 0.5, 4-aminopyridine 2 (pH adjusted to 7.2-7.4 with NaOH). IK1 was identified as the 2 mM BaCl₂ (Sigma Chemical Co. American) sensitive current.

Steady-state IK1 currents were recorded at a holding potential of -40 mV to inactivate the voltage gated Na⁺ channels. I-V relations were determined by subtracting the currents recorded in the presence of 2 mM BaCl₂ from the currents recorded in absence of 2 mM BaCl₂ in response to voltage steps (from -120 mV to 60 mV with 10 mV increments, 500 ms, holding potential -40 mV). Data were acquired at a sampling rate of 10 kHz, filtered at 2 kHz, stored on hard disk and analyzed off-line using the Clampfit analysis software (Axon Instruments, CA, USA). Cell capacitances were determined by analog measurement with the Axopatch 200A amplifier (Axon Instruments, CA, USA). In order to better display the activation kinetics, IK1 currents were also displayed with 20 mV increments, and fitted by a standard exponential function. Time constant of activation kinetics of IK1 was given as τ_{act} .

Quantitative real-time PCR

Total RNA was isolated from E10.5 or E17.5 ventricles using the RNeasy Mini Kit (Qiagen GmbH, Germany) followed by standard protocols for further processing [40]. Single-stranded cDNA synthesis was performed with total RNA using oligo d(T)₁₈ primer and ReverseTra Ace Transcriptase (Toyobo, Japan) following the manufacturer's instructions. First we performed classical PCR. For quantitative analysis of the mRNA of IK1 channel subunits, real-time PCR was performed on MxPRO3000 equipment (Stratagene Technology company, USA) in a total reaction volume of 20 µl containing 10 µl SYBR Green Real-time PCR Master Mix (Toyobo, Japan), 0.4 µM of each forward and reverse primer and 2 µl cDNA templates. The PCR processing consisted of 40 cycles of 10 s denaturation at 95°C, 20 s annealing at 60°C, and 15 s extension at 72°C. The primers are listed in Table 1. The transcript expression levels were quantified by using the threshold cycle (Ct) value method, where values were normalized to GAPDH as an internal control in the same sample. The specificity of the PCR products amplification was confirmed by the melting curves.

Western blotting

To investigate the expression of Kir2.1 and Kir2.3 at protein levels, protein extracts of ventricular samples at EDS and LDS were prepared in RIPA buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 1 mM phenylmethylsulfonyl

Target sequence	Accession no. *	Primer	Sequence (5'→3')	Amplicon length (bp)
Kir2.1	NM008425	Sense	GGAATGGCAAGAGTAAAGTCCA	232
		Antisense	AGGGCTATCAACCAAAACACA	
Kir2.2	NM008426	Sense	CGCCAACCTCTTTCTGCTATG	168
		Antisense	ATCTCC GACTCCCGTCTGT	
Kir2.3	NM008427	Sense	CCAGACGACCATTTGGCTAC	195
		Antisense	CGAACGGAGATGACAGCAT	
GAPDH	NM008084	Sense	AAGCCCATCACCATCTTCCAGGAG	308
		Antisense	AGCCCTTCCACAATGCCAAAG	

Table 1. Sequences of PCR Primers. * In GenBank release

Fig. 1. Developmental changes in IK1 current obtained from ventricular cardiomyocytes. (A) The representative whole cell recordings of IK1 currents in the presence (middle panel) or absence (left panel) of 2 mM BaCl₂ at EDS and LDS. The current (right panel) was obtained by subtracting the current in middle panel from the current in the left panel. The voltage pulse protocol was showed on the top. (B) Averaged current–voltage relationships of IK1 currents at EDS (n=9) and LDS (n=6). (C) Normalized I–V curves of IK1 current from ventricular cardiomyocytes at EDS (n=6) and LDS (n=6). Current at each voltage is normalized to that at -120 mV.

fluoride) according to the standard methods [17]. Protein concentration was determined using the BCA protein assay kit (Pierce, Rockland, USA). 30 µg of total protein per lane was resolved by 10% SDS-PAGE and transferred to PVDF membrane. The following antibodies were used for normalization: rabbit polyclonal anti-Kir2.1 (Santa Cruz Biotechnology, USA), goat polyclonal anti-Kir2.3 antibody (Santa Cruz Biotechnology, USA) and mouse monoclonal anti-GAPDH antibody (Proteintech Group, USA). The primary antibodies were diluted at 1:100 and detected using horseradish peroxidase-conjugated anti-rabbit, anti-goat or anti-mouse IgG, and the enhanced chemiluminescent reagent, ECL (Pierce Biotechnology, USA). Immunoreactive bands were detected using Kodak BioMax ML film. The results were characteristic of at least three independent experiments.

Double-label immunofluorescence

Cardiomyocytes at EDS or LDS were subjected to immunostaining after 24-hour cultivation. After the process consisting of 10 min of fixation with methanol, 5 min of permeabilization with 1% Triton X-100, and 1 hour of blocking with 5% BSA at room temperature, the cells were incubated with the primary antibodies rabbit anti-Kir2.1 (1:50, Santa Cruz Biotechnology, USA) or goat anti-Kir2.3 (1:50, Santa Cruz Biotechnology, USA) overnight at 4°C, followed by a further incubation of the secondary antibody donkey anti-rabbit IgG-FITC (Proteintech Group, USA) or donkey anti-goat IgG-Cy3 (Proteintech Group, USA) for 60 min at room temperature. The cells were then mounted onto glass slides and the pictures were captured using Immuno Floure (F-601-AF camera, Nikon, Japan).

Statistical analysis

All experimental results were presented as mean±SEM. The significance of differences between the means was evaluated by Student's paired or unpaired t-test if applicable. A value of $p < 0.05$ was used as the criterion for statistical significance.

Results

Developmental changes in electrophysiological properties of IK1 currents

The Ba²⁺-sensitive currents at EDS and LDS showed a pronounced IK1 with significant inward currents at voltages more negative than the reversal potential and small outward currents at voltages more positive than the reversal potential (Fig. 1). There was no significant shift of the reversal potentials between EDS (-11.55 ± 4.55 mV, $n=9$) and LDS (-18.41 ± 8.68 mV, $n=10$) ($p > 0.05$ EDS vs. LDS).

However, marked differences in other electrophysiological properties at EDS and LDS were detected (Fig. 1, Fig. 2). Peak inward current density (I_p) measured at -120 mV was significantly greater at LDS (EDS: -6.72 ± 1.02 pA/pF, $n=9$; LDS: -92.29 ± 3.03 pA/pF, $n=10$, $p<0.01$) (Fig. 2A-B). Outward current peaked at voltage of 22.58 ± 2.42 mV ($n=9$) for EDS and shifted to 18.32 ± 0.79 mV ($n=10$) for LDS ($p<0.05$). At LDS, the averaged rectification ratio (RR) (-13.39 ± 1.9) was 4-fold as much as that at EDS (-3.36 ± 0.92) (Table 2), indicating a stronger rectification at LDS. The I-V relations (Fig. 1C), after normalization to the peak current at -120 mV, further illustrated the stronger inward rectification properties of IK1 channels at LDS than that at EDS, which again displayed a much stronger averaged rectification ratio (RR) (-12.22 ± 1.84 , $n=10$) at LDS than that at EDS (-3.79 ± 0.87 , $n=6$, $p<0.01$).

More interesting developmental changes were observed in activation kinetics. After the channel displayed a pseudoinstantaneous components of activation (not fit) [11], a much slower monoexponential current (fit) increase was followed with faster activation kinetics at LDS than EDS (at hyperpolarized potentials) (Fig. 2C). The activation Tau at -120 mV (Fig. 2D) demonstrated a similar activation kinetics at EDS to those of Kir2.1/Kir2.3 concatemer in tested HEK293 cells, while the kinetics of IK1 activation at LDS were similar to those of Kir2.1-mono in tested HEK293 cells [11]. Additionally, inward IK1 of EDS myocytes displayed pronounced inactivation at far hyperpolarized potentials, which was almost absent in LDS myocytes (Fig. 2C).

Developmental changes in expression of IK1 channel subunits at mRNA level

To reveal the molecular basis of the developmental changes in special electrophysiological properties, the mRNA expression levels of IK1 channels subunits at EDS and LDS were further studied. At EDS, Kir2.1 and Kir2.3 were expressed at a comparable level, however, toward LDS Kir2.1 was upregulated ($n=4$, $p<0.05$ EDS vs. LDS) and Kir2.3 was downregulated ($n=3$, $p<0.05$ EDS vs. LDS) (Fig. 3). At LDS, Kir2.1 became the predominant subunit. Kir2.2 was detected at low level throughout the fetal development and upregulated ($n=5$, $p<0.01$ EDS vs. LDS).

Developmental changes in expression of Kir2.1 and Kir2.3 subunits at protein level

Since the functional expression of Kir2.1 and Kir2.3 determined the electrical characterization of IK1 channels

Fig. 2. Developmental changes in IK1 current density and activation kinetics of IK1 current. (A) The representative IK1 current traces recorded with 100 mM K⁺ in the extracellular solution. The currents were elicited from a holding potential of -40 mV to -120 mV. (B) Summarized data of IK1 current density at -120 mV. Filled bar: EDS ($n=10$); Open bar: LDS ($n=10$). (C) The representative whole cell recordings of IK1 currents at EDS and LDS. Currents were recorded in the presence of 100 mM extracellular K⁺ in response to voltage steps from -40 mV holding potential to -120 mV with 20 mV increments. Currents were fit with a two-exponential function to account for the slow inactivation of IK1 at far negative potentials. Six traces selected starting at -120 mV down to depolarized potentials using 20 mV increments were shown. (D) Activation Tau of IK1 currents at EDS ($n=10$) and LDS ($n=10$) obtained from currents recorded at -120 mV. *, $p<0.05$ EDS vs. LDS.

Parameters	EDS ($n=9$)	LDS ($n=10$)
IK1(-40 mV) (pA/pF)	-2.16 ± 0.24	-24.03 ± 1.07 **
IK1(10 mV) (pA/pF)	0.61 ± 0.32	2.99 ± 0.96 **
RR	-3.36 ± 0.92	-13.39 ± 1.9 **

Table 2. Parameters of IK1 currents in the ventricular cardiomyocytes at EDS and LDS. Inward and outward current amplitudes (pA/pF) were measured at potentials 30 mV negative and 20 mV positive to the respective IK1 reversal potentials, which approximately correspond to -40 mV and +10 mV, respectively. RR (rectification rate) = IK1 (-40 mV)/IK1 (10 mV). Statistics were obtained by student's t-test. **, $p<0.01$ LDS vs. EDS. Values are means $\pm$ SEM; IK1, inward rectifier potassium current; RR, rectification ratio.

Fig. 3. Developmental changes in expression of Kir2.1, Kir2.2 and Kir2.3 at mRNA levels in fetal ventricles. (A) mRNA expression of Kir2.1, Kir2.2 and Kir2.3 at EDS and LDS. (B) Quantitative analysis of Kir2.1, Kir2.2 and Kir2.3 transcripts (normalized to GAPDH) at EDS (filled bar) and LDS (open bar). Data were obtained from 3-6 independent RNA preparations. *, $p < 0.05$, EDS vs. LDS.

Fig. 4. Developmental changes in expression of Kir2.1 and Kir2.3 at protein levels in fetal ventricles and immunolocalization of Kir2.1/Kir2.3. (A) Western blotting data to detect protein expression of Kir2.1 and Kir2.3. (B) Normalized expression of Kir2.1 and Kir2.3 (normalized to GAPDH) in EDS (filled bar) and LDS (open bar) murine embryonic ventricular tissue. Values at LDS were normalized by that at EDS. Data were obtained from 3-6 independent protein preparations. *, $p < 0.05$, EDS vs. LDS. (C) Double-label immunofluorescence to detect co-localization of Kir2.1 and Kir2.3. Kir2.1 (left) and Kir2.3 (middle) in the same ventricular myocytes at EDS (upper) and LDS (lower).

[1, 12], we investigated the expression of Kir2.1 and Kir2.3 at protein level. Both Kir2.1 and Kir2.3 proteins were expressed in the murine ventricles at EDS and LDS (Fig. 4A). The developmental changes were in good agreement with the above observation at mRNA level. Kir2.1 and Kir2.3 were predominant at EDS, while Kir2.1 was upregulated and Kir2.3 was downregulated toward LDS ($n=3$) (Fig. 4B). The double-label immunofluorescence analysis revealed the co-localization of Kir2.1 and Kir2.3 (Fig. 4C) in both EDS and LDS ventricular cardiomyocytes.

Functional contribution of IK1 to ventricular cardiac excitability during development

The above data suggests that the different properties of IK1 channels may lead to different contribution of IK1

to AP configurations at EDS and LDS. Consistent with increased IK1 density with maturation, action potential duration (APD) was substantially decreased at LDS, and the maximum diastolic potential MDP shifted to a more negative level (Fig. 5A). More details were listed in Table 3. Moreover, Ba^{2+} application led to a prolongation of APD, depolarization of MDP, and a decrease in velocity of the late repolarization phase (V_{lrp}) at both EDS ($n=7$, $p < 0.05$) and LDS ($n=9$, $p < 0.05$) (Fig. 5B-D), implying the virtual contribution of IK1 to AP configuration regardless of different current densities at EDS and LDS. As compared to EDS, the Ba^{2+} effects on APD₉₀ and APD₅₀ at LDS were much stronger ($p < 0.05$ EDS vs. LDS). Interestingly, there was no significant difference between the Ba^{2+} effects on MDP or V_{lrp} ($p > 0.05$ EDS vs. LDS).

Discussion

In the present study we have shown that during murine fetal development, the total amplitude of IK1 current in ventricular cardiomyocytes increased, the activation kinetics increased, and the IK1 inward rectifier property became stronger. Molecular biological data revealed the molecular basis underlying such developmental changes: with maturation, Kir2.1 and Kir2.2 were upregulated, while Kir2.3 was downregulated. Double-label immunofluorescence illustrated the co-localization of Kir2.1 and Kir2.3 in the embryonic cardiomyocytes. Parallely, the contribution of IK1 to ventricular cardiac excitability also underwent a developmental change. These data provide a novel insight into the properties of IK1 channels, which shape the cardiac AP and play a role in the spontaneous AP in fetal ventricular myocytes.

In line with previous studies [33], we have shown here that total current densities of IK1 at LDS were increased by about 81.6% compared to EDS. The quantitative PCR results demonstrated downregulation of Kir2.3 and upregulation of Kir2.1/Kir2.2 of IK1 during fetal development. Since the single channel conductances of exogenously expressed Kir2 including ~20–31 pS for Kir2.1 [3, 41–44], ~34–42 pS for Kir2.2 [3, 43, 45–46], and ~10–14 pS for Kir2.3 [3, 43, 47–49], we can speculate that the increased current density at LDS may be a result of the subtype switch from low conductance Kir2.3 to high conductance Kir2.1/Kir2.2 in embryonic murine ventricle.

The IK1 currents we recorded in fetal ventricular myocytes displayed a characteristic pseudoinstantaneous phase at hyperpolarized potentials followed by a much slower monoexponential current increase, as illustrated in adult mammalian hearts [11, 50–51]. Additionally, we observed that the native IK1 currents in EDS ventricular myocytes had slower activation kinetics, as shown in a previous study that the activation became slower as the number of Kir2.3 subunits increased in a Kir2.1/Kir2.3 concatemer in HEK293 cells [11].

Moreover, evidence for the role of Kir2.3 subunits in IK1 channels described that Kir2.3 isoform had smaller potassium conductance, slower activation kinetics, weaker rectification and more depolarized activated potential of maximum outward current than Kir2.1 in HEK293 cells [1, 3, 11–12, 47]. This was consistent with our findings that with higher expression of Kir2.3 at EDS, IK1 exhibited a smaller averaged rectification ratio (RR) and a weaker inward rectifier property. The outward

Fig. 5. Developmental changes in contribution of IK1 current to AP configuration. (A) APs recorded at EDS and LDS. (B) APs recorded in the presence and absence of Ba²⁺ at EDS. (C) APs recorded in the presence and absence of Ba²⁺ at LDS. (D) Comparison of the virtual contribution of IK1 currents to AP parameters between EDS and LDS. * $p < 0.05$ EDS vs. LDS.

Parameters	EDS (n=46)	LDS (n=22)
MDP (mV)	61.6±1.7	63.8±1.1
APD90 (ms)	144.5±7.6	80.3±4.1**
APD50 (ms)	106.3±5.6	60.8±3.8**
APD20 (ms)	59.9±4.5	29.6±3.0**
Vlrp	1.40±0.14	2.0±0.06**

Table 3. Parameters of action potential in the ventricular cardiomyocytes at EDS and LDS. MDP: maximum diastolic potential; APD90: action potential duration at 90% of repolarization; APD50: action potential duration at 50% of repolarization; APD20: action potential duration at 20% of repolarization; Vlrp: velocity of late repolarization phase. Values are means±SEM; **, $p < 0.01$ LDS vs. EDS.

current of IK1 peaked at a more positive potential compared to LDS. The double-label immunofluorescence data confirmed the co-localization of Kir2.1/Kir2.3 in fetal cardiomyocytes.

Based on the differences in the rectification profiles of the Kir2.x channels shown in HEK293 cells in previous studies [1, 3, 12, 51], downregulation of Kir2.3 at LDS

relatively weakened the functional contribution of IK1 by a decaying outward current which played a key role in maintaining the MDP and the late repolarization phase. Thus the contribution of IK1 to MDP and V_lrp did not match well with the significant increase in current density compared to EDS. Although questions remained regarding different electrophysiological properties of IK1 in fetal murine cardiomyocytes, the variability could potentially arise because of the importance of Kir2.2 [1, 3, 12, 43, 52] and different intracellular polyamine [50-51, 53].

This is the first paper that reveals the functional characterization of IK1 in murine fetal cardiomyocytes and its functional role in cardiac excitability, which clearly demonstrated the developmental changes in the IK1

subunits. The findings here may offer a new aspect for better understanding of cardiogenesis and heart disease such as myocardial hypertrophy and heart failure [20-22].

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