Original Article

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Experimental study on inhibition of rat ventricular I_{k1} by RNA interference targeting the *KCNJ2* gene

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Summary **The dominant-negative inhibition of *KCNJ2*-encoded inward rectifier potassium channels (Kir2) is currently considered the best approach to biological pacemakers. We hypothesized that inhibition of the inward rectifier potassium current (I_{K1}) in ventricular myocytes by RNA interference (RNAi) would convert ventricular myocytes into pacemaker cells. Five pieces of short hairpin RNA (shRNA) were designed to target the *KCNJ2* gene and then plasmids incorporating shRNA and green fluorescent protein (GFP) as a marker were constructed for transfection into rat ventricular myocytes. The levels of *KCNJ2* mRNA were analyzed with real-time quantitative RT-PCR to screen for pieces of shRNA that were effective at inhibiting the expression of the *KCNJ2* gene. The activity of potassium ionic channels was then studied in the transfected ventricular myocytes. In the recombinant plasmids, LYS2 transfection significantly inhibited the mRNA of the *KCNJ2* gene in comparison to other groups ($p < 0.05$), and the beating frequency of ventricular myocytes increased after LYS2 transfection. The open probability of I_{K1} potassium ion channels of cardiac myocytes transfected with the LYS2 plasmid was significantly down-regulated ($p < 0.05$) and the I_{K1} of ventricular myocytes was also significantly suppressed compared to the negative group ($p < 0.05$). Our study demonstrated that I_{K1} was clearly inhibited after the inhibition of *KCNJ2* gene expression by RNAi, and this may represent a new approach to the study of biological pacemakers.**

Keywords: RNA interference, *KCNJ2*, plasmid, biological pacemaker

1. Introduction

Since Swedish cardiac surgeon Ake Senning implanted the first built-in cardiac pacemaker in 1958 (*1*), the electrical cardiac pacemaker has become the gold standard for treatment of sinus bradycardia caused by sick sinus syndrome and a high-degree atrioventricular block. However, there are some complications that may be difficult to overcome with a built-in pacemaker, and such pacemakers are not suitable for patients with a high risk of infection or who are too young (*2*). Given cardiac physiological function and body adaptability, the biological pacemaker is expected to be the ideal pacemaker (*3*). At present, the study of biological pacemakers is focused mainly on three gene therapy strategies: i) Upregulation of β2-adrenergic receptors, which was found to increase the atrial rate in porcine hearts (*4*), ii) Overexpression of inward depolarizing current, which is coded for by hyperpolarization-activated, cyclic-nucleotide-gated (HCN) and is the primary pacemaker current in the sinoatrial node (*5*), and iii) Dominant-negative therapy to inhibit the inward rectifier potassium current (I_{K1}) (*6*). In the latter two strategies, the resting membrane potential (RMP) is disturbed to generate spontaneous slow diastolic depolarization. In addition to these gene therapy strategies, various cell therapy approaches such as stem cell therapy and an adult somatic cell-fusion approach have yielded some results (*7*).

The use of dominant-negative therapy to inhibit the inward rectifier I_{K1} is currently the most promising approach to biological pacemakers (*8*). I_{K1} is considered to serve as the primary conductance controlling the RMP and contributes significantly to repolarizing

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current during the terminal phase of the action potential (AP) in ventricular myocytes. Kir2.1 subunits, encoded by the *KCNJ2* gene, assemble to form tetrameric inward rectifier potassium channels in many cell types, including cardiac myocytes (*9,10*). Thus, Kir2.1 is essential to the generation of I_{K1}. The possibility that pacemaker activity is latent and is normally repressed by I_{K1} was investigated in ventricular myocytes. Ventricular myocytes will presumably be converted to pacemaker cells when I_{K1} is inhibited (*11*). Miake *et al.* (*6*) built a dominant negative construct by replacing three amino acid residues in the pore of Kir2.1, resulting in idioventricular pacemaker function in the guinea pig ventricle.

RNA interference (RNAi) is a type of effective experimental technique in molecular biology that was recently developed (*12,13*). The current study constructed and screened out short hairpin RNA (shRNA) in response to the *KCNJ2* targeting gene and then transfected that shRNA into rat ventricular myocytes. The open probability (Po) of I_{K1} potassium ion channels in transfected recombinant plasmids should be significantly down-regulated and the I_{K1} of the ventricular myocytes should be significantly suppressed, providing a new idea for and approach to the study of biological pacemakers.

2. Materials and Methods

2.1. *Design and synthesis of coded shRNA oligonucleotide DNA segments*

In accordance with the mRNA nucleotide sequence of the rat gene *KCNJ2* (NM-017296), five pairs of shRNA sequences were designed depending on bases in the coding area: +109 ~ +117 AACGCAATGCCGGAGTTCATA, +361 ~ +379 AAGCGTGTGTGTCTGAGGTCA, +926 ~ +934 AAGTCCATACCCGACAACAGT, +1156 ~ +1174 AAGAGGAAGAGGACAGTGAGA, +1176 ~ +1194 AACGGAGTTCCAGAGAGCACA. The structure of the DNA primer that transcribes the shRNA is as follows: BamHI + Sense + Loop + Antisense + Terminal Signal + EcoRI/SacI/SalI/SacI/XbaI + HindIII (the enzyme digestion sites of EcoRI, SacI, SalI, SacI and XbaI were designed respectively in the inserted segments targeting the gene in question, and segments were inserted between the two enzyme digestion sites of BamHI and HindIII).

2.2. *Plasmid construction*

Double enzyme digestion of BamHI + HindIII was carried out on the plasmid Pgenesil-1 (Figure 1A) and then longer segments were collected on a 1% agarose gel. Synthesized single-chain targeting gene segments were synthesized with 50 μL of annealing buffer and then cooled to room temperature after a water bath at 94ºC. Annealed segments were ligated with linear Pgenesil-1 plasmid-expressing vectors. After cloning into *Escherichia coli* DH5a, clones were selected on LB plates. Plates were left overnight in a warm box at 37ºC, and then the plasmid was extracted. The recombinant plasmids were termed LYS1, LYS2, LYS3, LYS4, LYS5, and HE (negative control). Enzyme digestion and sequencing of the recombinant plasmids were done by Wuhan Genesil Biotechnology Co., Ltd. The DNA sequence of transcribed shRNA includes 47 bps, with two 19-bp reverse repetitive sequences on both ends and a 9-bp ringlike structure in the middle. The hairpin DNA sequence with the U6 promoter was transcribed into shRNA by internal RNA polymerase III.

2.3. *Separation and identification of ventricular myocytes in newborn rats*

Fifteen newborn Wistar rats (provided by the laboratory animal center of Shandong University) 1-2 days of age were sacrificed by decapitation and their cardiac ventricles were removed. Ventricular myocytes were purified twice through enzyme digestion and differential attachment in culture dishes. Myocytes were cultured in six-well tissue culture plates, and 5×10^5 ventricular myocytes that were cultured at 37ºC in 5% CO_2 were added to each well. Ventricular myocytes were identified by immunohistochemical analysis with anti-α-actin antibody and anti-myosin antibody.

2.4. *Plasmid transfection in ventricular myocytes*

Liposomes were used to facilitate transfection. Metafectene liposomes and plasmid were mixed at a ratio of 3:1 (*14*). The culture medium with serum and antibiotic was removed before transfection and washed with PBS three times, and then culture medium without serum and antibiotic was added. One hour later, the ventricular myocytes were transfected when the myocytes reached 50-60%. The mixture was placed in six-well culture plates and 0.8 mL of culture medium without serum and culture was added; plates were kept at 37ºC for 6-8 h. Then, 1 mL of culture medium with 20% fetal bovine serum was added in 5% CO_2 and cultured for another 48 h at 37ºC. The transfection rate was measured using flow cytometry.

2.5. *Establishment of a method of real-time PCR detection of KCNJ2 mRNA*

Total RNA from rat ventricular myocytes was extracted, reverse transcription was used to construct cDNA, and *KCNJ2* and β-actin segments were amplified. Specific primers were designed in accordance with the mRNA sequence of the *KCNJ2* gene and the *KCNJ2* and β-actin segments were amplified. The primers were provided by

Shanghai Bioengineering Co., Ltd. Primer for the *KCNJ2* gene: upstream 5'- TGCCCGATTGCTGTTTTC-3', downstream 5'- GGCTGTCTTCGTCTATTT-3'(amplified segment 373 bp; Primer for β-actin: Upstream 5'-AACCCTAAGGCCAACCGTGAAA-3', downstream 5'- TCATGAGGTAGTCTGTCAGGTC-3'(amplified segment 241 bp). The RT-PCR reaction system for the *KCNJ2* gene and β-actin was as follows: reaction conditions: 94ºC for 10 min, 94ºC for 30 sec, 56ºC for 30 sec, 72ºC for 30 sec, 40 cycles; 72ºC for 2 min. The segments were cloned into plasmids as usual. Conditions for real-time quantitative RT-PCR were varied. The results were analyzed by calculating the Ct values for *KCNJ2* and β-actin in samples. PCR products were quantitatively analyzed given a standard quantitative curve.

Cultured ventricular myocytes from newborn Wistar rats were divided into three groups: (1) Experimental group (positive group): transfected with vector plasmid. (2) Control group (negative group): transfected with empty vector plasmid, and (3) Blank group: No treatment. The relative rate of *KCNJ2* mRNA expression in the three groups was analyzed *via* real-time quantitative RT-PCR, and the rate of suppression of mRNA was calculated.

2.6. *Western blot analysis*

KCNJ2 expression was examined in ventricular myocytes transfected with plasmid vectors using Western blotting. Cells were harvested in a lysis buffer (2% SDS, 50 mM Tris, pH 7.4/1 mM EDTA/ protease inhibitor mixture) 96 h after transfection and homogenized by sonification. Equal amounts of protein (40 μg) were separated by sodium dodecyl sulfate/ polyacrylamide gel electrophoresis (SDS-PAGE) on 8% gels, blotted on nitrocellulose, probed with anti-Kir2.1 goat polyclonal IgG (Santa Cruz Biotechnology) and subsequently with rabbit-anti-goat (HRP) (Beijing Zhong Shan Golden Bridge Biological Technology), and detected by chemiluminescence (Roche). Antibodies against β-actin (Sigma-Aldrich) were used to measure protein loading.

2.7. *Recording of single-channel current*

The activity of inward potassium channels was recorded using the whole-cell patch-clamp technique at 37°C with an Axopatch 200B amplifier while sampling at 10 kHz (for currents) or 2 kHz (for voltage recordings) and filtering at 2 kHz. Pipettes had a tip resistance of 2-4 MΩ when filled with the internal recording solution.

Cells were superfused with a physiological saline solution containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM glucose, 1 mM $MgCl_2$, and 10 mM HEPES; pH was adjusted to 7.4 with NaOH. The pipette solution consisted of 130 mM K-glutamate, 19 mM KCl, 10 mM Na-HEPES, 2 mM EGTA, 5 mM Mg-ATP, and 1 mM $MgCl_2$; pH was adjusted to 7.2 with KOH. For I_{K1} recording, $CaCl_2$ was reduced to 100 μM, $CdCl_2$ (200 μM) was added to block $I_{Ca,L}$, and I_{Na} was steady-state inactivated by using a holding potential of –40 mV. To obtain I_{K1} as a Ba^{2+}-sensitive current, currents recorded before and after the addition of Ba^{2+} (500 μM) were subtracted. Data were recorded with pClamp9.0 software.

2.8. *Analysis of single-channel current*

Single-channel data were analyzed with QUB single-channel activity analyzers in order to obtain the current of single channels, channel close probability (Pc), and the number of channels in the membrane. The normalized value of 5 sec was chosen for each channel analyzed. The following formula was used to calculate the open probability (Po) of a certain channel: $Po = 1 - Pc^{1/N}$ (N refers to the number of channels in the membrane, and Pc refers to the probability that N ionic channels were closed at the same time) (*10*).

2.9. *Statistical analysis*

The SPSS 11.0 for WINDOWS statistical software package was used to analyze one-way variance in basic data that were expressed as the mean ± S.E.M. A *t*-test was used for comparison among the groups, and $p < 0.05$ represented a significant difference. One-way ANOVA was used to compare the effects stretch-induced channel activity. $p < 0.05$ indicated a significant difference, and $p < 0.01$ indicated a highly significant difference.

3. Results

3.1. *Identification of plasmid Pgenesil-1 and enzyme digestion of recombinant plasmids*

BamHI and HindIII enzyme digestion was carried out on the plasmid Pgenesil-1. After enzyme digestion, ringlike plasmids were converted into linear plasmids (Figure 1B). The multiple cloning sites (MCS) of the plasmid Pgenesil-1 were as follows: –HindIII–insertDNA–BamHI–U6 Promoter–EcoRI–SalI–XbaI–DraIII–. Analysis of enzyme digestion indicated that the plasmids LYS1-1, LYS2-4, LYS3-8, LYS4-22, and LYS5-9 all met the design requirements (Figures 1C and 1D).

3.2. *Sequencing of recombinant plasmids*

The transfected plasmid bacterium solution was used to sequence the plasmid. The shRNA-encoding sequences of six recombinant plasmids were identical to those of the designed segments, indicating that the recombinant plasmids were correctly constructed.

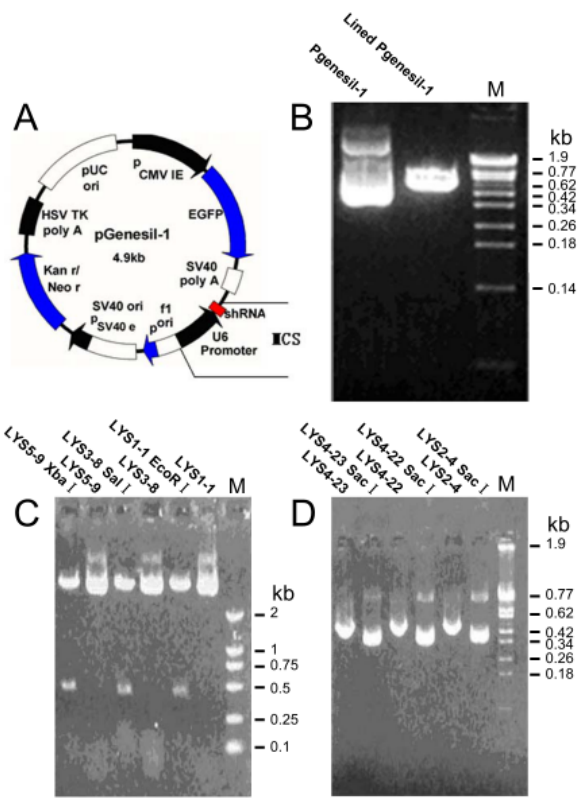

Figure 1. Restriction map of the Pgenesil-1 plasmid and recombinant plasmids.

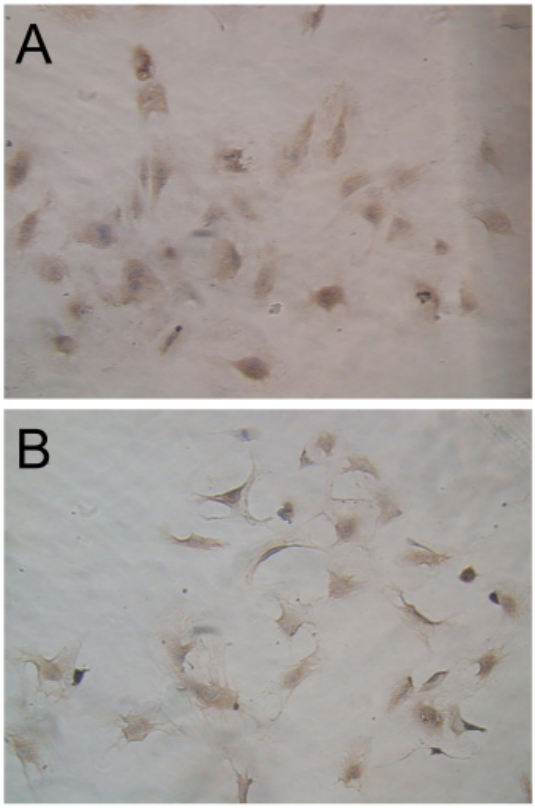

Figure 2. Identification of ventricular myocytes. Anti-actin **(A)** and anti-myosin **(B)** immunohistochemistry(inverted microscope, 200×): the cytoplasm of ventricular myocytes was stained brownish-yellow.

3.3. *Identification of ventricular myocytes*

The purity of the ventricular myocytes was 95.1% (according to positivity for anti-α-actin) and 94.8% (according to positivity for anti-myosin) (Figure 2).

3.4. *Inhibition of mRNA of the KCNJ2 gene after LYS2 transfection*

A logarithmic chart of the corresponding concentrations according to the cycle threshold value (Ct) of β-actin and *KCNJ2* resulted in two straight lines. The relevant coefficients of β-actin and *KCNJ2*, *i.e.*, straight lines indicated by real-time quantitative measurement, were –0.993 and –0.999, respectively, and the gradients were –3.24. According to the formula E = 10-1/S-1, the amplification efficiency of the two genes was 100%. The Ct value for plasmid LYS2 was higher in the intervention group than in the blank group, and the rate of inhibition after correction was 86%, which was significantly greater than other suppression rates ($p < 0.05$). Plasmid LYS2 was thus used to transfect cells.

3.5. *Inhibition of Kir2.1 protein expression after LYS2 transfection*

Western blot analysis indicated that the experimental group had low levels of expression of the Kir2.1 protein

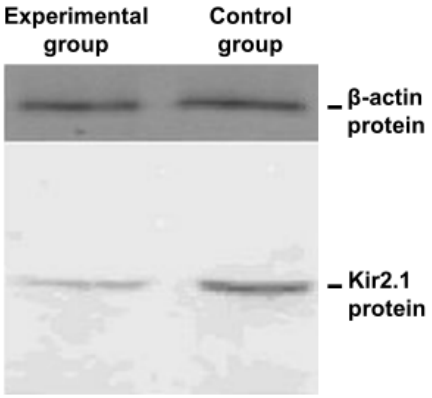

Figure 3. Western blotting analysis of LYS2-transfected ventricular myocytes and non-transfected ventricular myocytes (left-to-right). The experimental group had a lighter Kir2.1 protein blot than the control group, indicating that Kir2.1 protein expression was suppressed by RNAi in the experimental group.

while the control group had high levels of expression of that protein. There were no significant differences in β-actin protein expression in the two groups (Figure 3).

3.6. *Increase in the beating frequency of ventricular myocytes after RNAi*

Observed under a microscope, adjacent ventricular myocytes extended pseudopods to interlace and connect

as a syncytium with the same beating frequency, which is designated here as a cell cluster. After 24 h (transfected LYS2), the experimental group and negative plasmid control group had significantly fewer beating cell clusters than the blank group, and their beating frequency was lower than that in the blank group ($p < 0.01$). After 48 h, the number of beating cell clusters in the experimental group and the negative plasmid control group increased, and the beating frequency increased compared to the beating frequency at 24 h. The increase in clusters was more obvious in the experimental group, and the beating frequency was more obvious in the experimental group, and the beating frequency was greater than that in the two control groups ($p < 0.01$). There were no significant differences between the two control groups. After 72 h, the beating frequency increased in the experimental group compared to the beating frequency at 48 h. There were no significant changes in the two control groups. The experimental group had a significant faster beating frequency than did the two control groups ($p < 0.01$). There were no significant changes in the beating frequency from 72 h to 96 h for any of the groups, indicating that the RNAi effect was stable (Figure 4A).

3.7. *Identification of I_{K1} potassium ionic channels in rat ventricular myocytes*

Ca^{2+} current was blocked by adding 0.3 mM of CdCl to Tyrode's solution. The membrane voltage was hyperpolarized to –120 mV and then depolarized to +30 mV in steps. I_{K1} current was induced for 300 msec with a stimulation frequency of 0.5 Hz and command voltage of 10 mV at each step (Figures 4B-4D).

3.8. *Suppression of the I_{K1} of ventricular myocytes by RNAi*

Five membranes with little background channel activity were analyzed. At a voltage of +10 mV, the normal group had a significantly higher Po than did the transfected plasmid group ($p < 0.05$). At a voltage of −40 mV, the normal group had a higher Po than did the transfected plasmid group ($p < 0.01$) (Table 1). At different voltages, the transfected plasmid group had a significantly lower I_{K1} current in ventricular myocytes than did the control group ($p < 0.05$) (Figure 5).

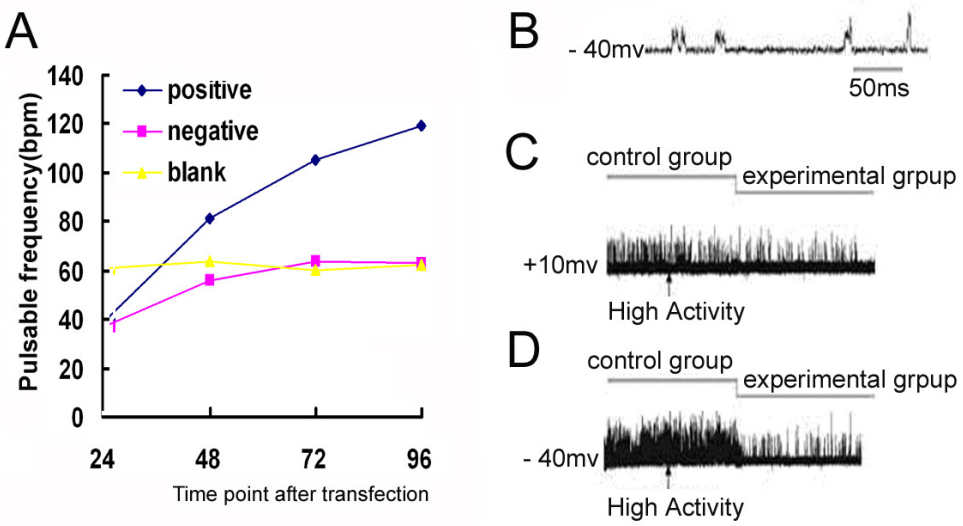

Figure 4. The I_{k1} activity of ventricular myocytes decreased and their beating frequency increased after RNAi. (A) Changes in the beating frequency of cardiomyocytes after transfection; **(B)** Activity of the normal inward rectifier K^+ channel current in rat ventricular myocytes (in a cell-attached state, −40 mV membrane potential control. Sustained recording for 1 min); **(C)** At a voltage of +10 mV, the LYS2-transfected group had less I_{k1} activity than the control group; **(D)** At a voltage of −40 mV, the LYS2-transfected group had much less I_{k1} activity than the control group.

Table 1. Real-time quantitative detection of *KCNJ2* mRNA expression after 72 h of RNAi

Group	*LYS1*	*LYS2*	*LYS3*	*LYS4*	*LYS5*	*HE*	Blank
ΔCt	−10.23	−10.17	−10.24	−10.28	−10.26	−10.26	−10.21
ΔΔCt	−0.02	−0.5	−0.03	−0.07	−0.05	−0.05	
Suppression rate	--	0.29	--	--	--	--	
Corrected suppression rate	--	0.86	--	--	--	--	

Note: ΔCt: The difference between β-actin Ct and *KCNJ2* Ct; ΔΔCt =ΔCtb − ΔCtf (ΔCtb: The difference between β-actin Ct and *KCNJ2* Ct in the transfected groups and negative group; ΔCtf: The difference between β-actin Ct and *KCNJ2* Ct in the blank control group); Suppression rate =$1-2^{-\Delta\Delta Ct}$; Corrected suppression rate = Suppression rate/transfection rate.

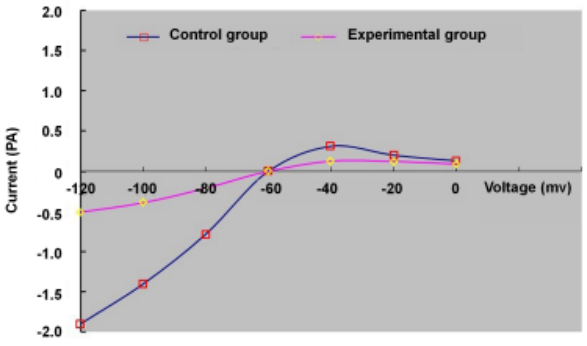

Figure 5. I-V line graph of the experimental group and control group. At different voltages, the ventricular myocytes in the transfected plasmid group (experimental group) had a significantly lower I_{K1} current than the control group ($p < 0.05$).

4. Discussion

The inward rectification of I_{K1} plays an important role in the ventricular myocardial activity (*15,16*). When the membrane potential is higher than the potential for K^+ equilibrium, an outward current is generated, speeding up the complex polarization of the AP. When the membrane potential is lower than the potential for K^+ equilibrium, an inward current is generated. This plays a leading role in maintaining the resting potential and in the potassium sensitivity of cells (*17*). At the end of complex polarization, most channels are inactivated and I_{K1} is activated, facilitating rapid complex polarization and inhibiting early after-depolarization (*18*). Adult myocardial cells have latent pacing ability but are inhibited by an inward rectifier current (I_{K1}) that stabilizes the resting potential at a negative level. I_{K1} is highly expressed in ventricular and atrial myocytes (*19*), so it inhibits automatic rhythmicity of the ventricle and atrium. Miake (*20*) provided direct proof-of-concept for biological pacemaking. Thus, the current study used an RNAi technique to suppress the *KCNJ2* gene in order to provide an approach for the development of biological pacemakers. RNAi has been widely used as a tool for targeted gene silencing (*21,22*). In the current study, five groups of shRNA recombinant plasmids specific to the *KCNJ2* gene were constructed based on the concept of RNAi. Analysis of enzyme digestion indicated that the constructed recombinant plasmid had the same size as expected. There were no base mutations according to sequencing analysis, indicating that construction was successful. For the five plasmids expressing shRNA in the current experiment, a LYS2 vector provided the optimal level of mRNA inhibition based on the results of quantitative mRNA detection and the corrected inhibition rate of 86%, which is significantly higher than that of other vectors.

Based on this experiment, I_{K1} was studied in rat ventricular myocytes using the membrane clamp technique. Membranes were attached to cells. When the K^+ ion concentration in the internal solution is 140 mM, the equilibrium potential (Ek) of K^+ ions approaches 0 mV (*23*). When the potential of the membrane is below 0 mV (hyperpolarization), a type of inward pulse of current is recorded in most membranes. Channels are randomly opened temporarily, and the most common pattern is clustered opening (*24*). The current increases with the increasing polarization of the membrane. When the potential of the membrane exceeds 0 mV, channel opening is difficult to record. Even if opening is recorded accidentally, the channel amplitude is smaller, *i.e.,* the channel has intense inward rectification. Its reverse potential is –60 mV, so when the potential is less than –60 mV the channel current reflects inward rectification. When the potential is more than –60 mV, the channel current reflects outward rectification. Potential was kept at –40 mV and electrical stimulation was supplied with 500-ms square-wave pulses. The potential was depolarized from –120 mV to +30 mV, and a step voltage of 10 mV was used. I_{K1} in ventricular myocytes is a stable current that does not change over time. It is part of the inward current during hyperpolarization and has a larger amplitude. It becomes part of the outward current during depolarization, indicating inward rectification.

Results indicated significant differences ($p < 0.05$) in the Po of channels in the control group and the LYS2-transfected group at voltages of +10, 0, and –40 mV. There were also significant differences ($p < 0.01$) in the Po of channels in the control group and LYS2-transfected group at voltages of –80, –100, and –120 mV. This experiment measured current at voltages of –120, –100, –80, –40, –20, and 0 mV, which are more sensitive to current activity. After transfection of LYS2, the channel current decreased but was not blocked completely, indicating that LYS2 transfection had interfered with I_{K1} potassium channels and significantly inhibited the I_{KI} in rat ventricular myocytes.

In conclusion, RNAi affected I_{K1} in rat ventricular myocytes, and the current amplitude of I_{K1} potassium channels clearly decreased after transfection of the plasmid LYS2. This RNAi technique has provided a new method for the study of cardiac pacemakers and has laid the foundation for clinical cardiac pacemaking.

Acknowledgements

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