**Abstract**—Previous studies have postulated an important role for the inwardly rectifying potassium current ($I_{K1}$) in controlling the dynamics of electrophysiological spiral waves responsible for ventricular tachycardia and fibrillation. In this study, we developed a novel tissue model of cultured neonatal rat ventricular myocytes (NRVMs) with uniform or heterogeneous Kir2.1 expression achieved by lentiviral transfer to elucidate the role of $I_{K1}$ in cardiac arrhythmogenesis. Kir2.1-overexpressed NRVMs showed increased $I_{K1}$ density, hyperpolarized resting membrane potential, and increased action potential upstroke velocity compared with green fluorescent protein–transduced NRVMs. Opposite results were observed in Kir2.1-suppressed NRVMs. Optical mapping of uniformly Kir2.1 gene-modified monolayers showed altered conduction velocity and action potential duration compared with nontransduced and empty vector-transduced monolayers, but functional reentrant waves could not be induced. In monolayers with an island of altered Kir2.1 expression, conduction velocity and action potential duration of the locally transduced and nontransduced regions were similar to those of the uniformly transduced and nontransduced monolayers, respectively, and functional reentrant waves could be induced. The waves were anchored to islands of Kir2.1 overexpression and remained stable but dropped in frequency and meandered away from islands of Kir2.1 suppression. In monolayers with an inverse pattern of Kir2.1 heterogeneity, stable high frequency spiral waves were present with $I_{K1}$ overexpression, whereas lower frequency, meandering spiral waves were observed with $I_{K1}$ suppression. Our study provides direct evidence for the contribution of $I_{K1}$ heterogeneity and level to the genesis and stability of spiral waves and highlights the potential importance of $I_{K1}$ as an antiarrhythmia target. (Circ Res. 2009;104:355-364.)

**Key Words:** Kir2.1 ■ inwardly rectifying potassium current ■ reentry ■ spiral waves ■ ventricular tachycardia ■ ventricular fibrillation

Ventricular fibrillation (VF) is the leading cause of cardiac arrest and sudden cardiac death in the industrialized world. Studies in the 1970s suggested that the heart could sustain electrical activity that rotated around a functional obstacle. These reentrant waves are believed to be the unitary components of fibrillation. Several other studies that focused on understanding the mechanisms of initiation and maintenance of VF concluded that the stability of spiral waves (functional form of reentrant waves) depends on the abbreviation of action potential duration, as well as the reduction of wavefront–wavetail interactions, at fibrillation frequencies. In addition, ionic heterogeneity may be a key factor in the initiation of spiral waves and their transition to the irregular spatiotemporal pattern seen in VF. Numerous studies pioneered mainly by Jalife and colleagues have indicated that $I_{K1}$ plays an important role in determining cardiac excitability and arrhythmogenesis and that $I_{K1}$ block has a significant effect on VF dynamics. Also, genetic mutations in Kir2.1, the molecular correlate of $I_{K1}$, can cause short QT syndrome SQT3 and Andersen syndrome with accompanying ventricular arrhythmias. Although previous studies in transgenic mice have concluded that $I_{K1}$ upregulation stabilizes high-frequency rotors (organizing center of spiral waves), Kir2.1 expression in such transgenic hearts can be heterogeneous, and the effects of such heterogeneity on arrhythmogenesis have not been explored. We therefore investigated the effects of altered Kir2.1 expression on spiral wave dynamics in a novel, in vitro model of cultured neonatal rat ventricular myocytes (NRVMs) in which regional variations of Kir2.1 were engineered by tissue engineering and somatic gene transfer approaches. In NRVM monolayers with distinct regions of Kir2.1 overexpression or dominant-negative suppression, the role of $I_{K1}$ heterogeneity in initiating and maintaining a high-frequency spiral wave was stud-
ied. Also, the secondary effects of $I_{K1}$ modulation on cardiac single-cell and tissue electrophysiological properties, such as resting membrane potential (RMP), action potential (AP) phenotype, maximum upstroke velocity ($dV/dt_{\text{max}}$), action potential duration (APD), conduction velocity (CV), and maximum capture rate were studied. The results of our study demonstrate that uniform upregulation of $I_{K1}$ does not necessarily create a medium that is prone to arrhythmogenesis and suggest that CV and APD differences arising from heterogeneous overexpression of Kir2.1 contribute to the genesis and stability of reentrant spiral waves that can underlie life-threatening cardiac arrhythmias.

Materials and Methods

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org. In brief, freshly isolated, nontransduced NRVMs and NRVMs transduced by empty lentiviral vectors (LV-Empty) or lentiviral vectors (LVs) encoding Kir2.1 (LV-Kir2.1) or Kir2.1AAA (LV-Kir2.1AAA) were plated on 12-mm fibronectin-coated glass (for patch clamping) or 21-mm fibronectin-coated plastic (for optical mapping) coverslips as previously described. A modified form of a stenciling technique, involving polydimethylsiloxane stamps, was used to spatially localize transduced NRVMs in a coculture model with nontransduced NRVMs. Six-day-old monolayers were characterized by immunostaining for cardiac troponin (cTnI), actin, Kir2.1, and connexin (Cx)43. The levels of Kir2.1 and Cx43 were also characterized by Western blot. The electrophysiological single-cell properties of Kir2.1 gene-modified NRVMs were characterized by standard microelectrode whole-cell patch clamp techniques. Optical mapping over a 17-mm-diameter field of view was performed on 6-day-old, 21-mm-diameter isotropic monolayers and monolayers with heterogeneous Kir2.1 expression to study the electrophysiological tissue properties. Bipolar line stimulation via platinum electrodes was applied just above one edge of the monolayer, and cells were stimulated with monophasic, 10-ms pulses at 2 Hz to determine CV and APD at 80% repolarization (APD$_{80}$). In monolayers with $I_{K1}$ heterogeneity, reentrant spiral waves were initiated by rapid pacing, and their inducibility, stability, and frequency were determined. All data are expressed as mean±SD and compared using the paired Student’s t test. A probability value of <0.05 was considered statistically significant.

Results

Characterization of Nontransduced and Kir2.1 Gene-Modified Cultures

Immunohistochemistry against Kir2.1, cTnI, actin, and Cx43 and Hoechst nuclear staining were performed to characterize the morphology, composition, and levels of Kir2.1 and Cx43 expression in 6-day-old nontransduced, LV-Empty–transduced, LV-Kir2.1–transduced, and LV-Kir2.1AAA–transduced monolayers. Immunohistochemistry against Kir2.1 showed that whereas nontransduced (Figure 1A) and LV-Empty–transduced (Figure 1B) NRVMs had low levels of native Kir2.1 protein, Kir2.1-transduced (Figure 1C), and Kir2.1AAA-transduced (Figure 1D) NRVMs had increased levels of wild-type Kir2.1 and dominant-negative mutant,
Kir2.1AAA, respectively. Immunostain images of cTnI, actin, and Cx43 in nontransduced and transduced monolayers (Figure I in the online data supplement) confirmed that in a given field of view, both nontransduced and transduced cultures were morphologically similar and had similar levels and distributions of gap junctional protein expression. Western blot (Figure 1E) and integrated pixel density analysis (Figure 1F) showed similar levels of tubulin and Cx43 expression in all groups and greatly increased (up to 17-fold) levels of Kir2.1 (wild-type and dominant-negative mutant in LV-Kir2.1 and LV-Kir2.1AAA, respectively) expression in Kir2.1 gene-modified groups compared with the nontransduced and LV-Empty–transduced groups.

Characterization of Single-Cell Electrophysiological Properties of Kir2.1-Transduced NRVMs

We performed whole-cell patch clamp on 6-day-old enhanced green fluorescent protein (eGFP)-transduced and Kir2.1 gene-modified NRVMs to assess their single-cell electrophysiological properties. Transduced NRVMs exhibited enhancement or suppression of $I_{K1}$ with overexpression or suppression of Kir2.1, respectively. When compared with eGFP-transduced NRVMs ($-41.7 \pm 2.6$ pA/pF; $n=6$; Figure 2A), the average $I_{K1}$ density at $-100$ mV was significantly larger in Kir2.1-overexpressed NRVMs ($-432.5 \pm 12.7$ pA/pF; $n=6$; $P=8.2 \times 10^{-11}$; Figure 2B) and significantly smaller in Kir2.1-suppressed NRVMs ($-5 \pm 1.4$ pA/pF; $n=6$; $P=2.3 \times 10^{-8}$; Figure 2C). Spontaneous APs were absent in Kir2.1-overexpressed NRVMs, and single APs could be triggered by a short depolarizing current stimulus. As reported by previous studies, with a greater and almost complete suppression of $I_{K1}$, Kir2.1AAA-transduced NRVMs fired spontaneous APs resembling those of genuine pacemaker cells. Spontaneous APs were also observed in eGFP-transduced NRVMs. Representative APs observed in eGFP-transduced, Kir2.1-transduced, and Kir2.1AAA-transduced NRVMs are shown in supplemental Figure I. Changes in $I_{K1}$ also significantly shortened APD at 90% repolarization (APD$_{90}$) in the Kir2.1-overexpressed group ($29.9 \pm 9.1$ ms; $n=6$; $P=1.2 \times 10^{-8}$) and significantly prolonged APD$_{90}$ in the Kir2.1-suppressed group ($183.9 \pm 7.2$ ms; $n=6$; $P=1.2 \times 10^{-8}$) compared with the eGFP-transduced control group ($116.3 \pm 6.8$ ms; $n=6$). The current/voltage (I-V) relationship of Kir2.1 gene-modified and eGFP-transduced NRVMs is shown in Figure 2D. As shown in the inset, overexpression of Kir2.1 also boosted outward $I_{K1}$, whereas suppression of Kir2.1 reduced outward $I_{K1}$ when compared with the eGFP-transduced control group (at $+30$ mV, $76.4 \pm 5.3$ pA/pF; $P=1.2 \times 10^{-7}$; $n=6$; versus $0.8 \pm 0.3$ pA/pF; $P=1.2 \times 10^{-6}$; $n=6$; versus $6.8 \pm 0.9$ pA/pF; $n=6$; in overexpressed, suppressed, and control groups, respectively). As expected, Kir2.1-overexpressed NRVMs showed a significantly hyperpolarized RMP ($-79.3 \pm 0.7$ mV; $n=3$; $P=0.0013$; Figure 2E), whereas Kir2.1AAA-overexpressed NRVMs had a depolarized maximum diastolic potential ($-65 \pm 2.9$ mV; $n=3$; $P=0.0381$) compared with eGFP-transduced NRVMs ($-73.2 \pm 0.3$ mV; $n=3$). The $dV/dt_{max}$ for APs of Kir2.1-overexpressed NRVMs was significantly higher ($260 \pm 27$ V/sec; $n=3$; $P=0.001$) and that of Kir2.1-suppressed NRVMs was significantly lower ($23.5 \pm 9$ V/sec; $n=3$; $P=0.001$).
Characterization of Tissue Electrophysiological Properties of Kir2.1-Transduced Monolayers

The tissue electrophysiological properties of uniformly Kir2.1 gene-modified monolayers significantly differed from those of nontransduced and LV-Empty–transduced monolayers. Representative isochrone maps for impulse propagation at 2-Hz pacing rate (Figure 3A) showed that APs propagated through nontransduced and LV-Empty–transduced monolayers with similar CV values of 19.1±0.5 cm/sec (n=7) and 18.9±0.7 cm/sec (n=7; P=0.07; compared with nontransduced control), respectively. However, APs propagated with increased CV (27.9±1.1 cm/sec; n=7; P=2×10⁻⁵; compared with nontransduced control) in Kir2.1-overexpressed monolayers and decreased CV (12.2±0.7 cm/sec; n=7; P=5×10⁻⁶; compared with nontransduced control) in Kir2.1-suppressed monolayers. Our findings of CV in Kir2.1-modified NRVM monolayers are consistent with our previous study¹⁷ and with our findings of increased dV/dt_{max} with Kir2.1 overexpression (Figure 2F). The suggestion of increased sodium channel availability and enhanced excitability with f_{K1} upregulation has also been previously reported.¹³ AP waveforms obtained from mapping (Figure 3B) showed normal APD_{80} values in nontransduced (174±6 ms; n=7)
and LV-Empty–transduced (169 ± 7 ms; n = 7; P = 0.27; compared with nontransduced control) monolayers, significantly abbreviated APD_{80} values in LV-Kir2.1–transduced monolayers (70 ± 5 ms; n = 7; P = 9 × 10^{-7}; compared with nontransduced control) and significantly prolonged APD_{80} values in LV-Kir2.1AAA–transduced monolayers (200 ± 9 ms; n = 7; P = 2.8 × 10^{-5}; compared with nontransduced control). Our findings of APD_{80} changes in Kir2.1-modified NRVM monolayers are consistent with previous findings^{20} and confirm that I_K1 regulates the duration of the AP in these cells. Average CV and APD_{80} values for each group are summarized in Figure 3C and 3D. The average normalized upstroke velocity of propagating APs at a 2-Hz pacing rate in LV-Empty–transduced, Kir2.1-overexpressed, and Kir2.1-suppressed monolayers (n = 7 each) were 3.23 ± 0.42 (P = 0.72), 5.36 ± 0.43 (P = 2.1 × 10^{-8}), and 1.93 ± 0.47 (P = 2.4 × 10^{-7}), respectively, when compared with that of nontransduced monolayers (3.11 ± 0.62; n = 7). Finally, in nontransduced and LV-Empty–transduced monolayers, successful 1:1 capture occurred up to a pacing rate of 4.5 ± 0.3 Hz (n = 7), whereas Kir2.1-overexpressed and Kir2.1-suppressed monolayers had a maximum capture rate of 9 ± 0.5 Hz (n = 7) and 3 ± 0.3 Hz (n = 7), respectively (Figure 3E). Importantly, reentry could not be induced in nontransduced and uniformly transduced monolayers even at a very high pacing rate (for example, 9 Hz for Kir2.1-overexpressed monolayers).

Development of an In Vitro Tissue Model of NRVMs With Heterogeneous I_K1 Expression

Transduced NRVMs from a first day of cell isolation were spatially localized within a clear boundary defined by the stenciling technique (Figure 4A). Nontransduced NRVMs from a successive day of cell isolation were added to the whole coverslip and grown under normal culture conditions for an additional 5-day period (total of 6 days for original NRVMs) to develop an in vitro model of cardiac myocytes with a spatially localized functional heterogeneity (Figure 4B). Over a prolonged culture period of 6 days and beyond, nontransduced and transduced NRVMs microscopically coupled well without any discernible structural heterogeneities (Figure 4C). To assess the percentage of nontransduced NRVMs in the transduced central region, we transduced NRVMs from the first day of isolation with LV-eGFP and labeled nontransduced NRVMs from the second day of isolation with CellTracker Red CMTPX. Fluorescent images of the interface region showed that eGFP-transduced NRVMs were sharply confined within the circular boundary initially set by the stenciling procedure (Figure 4D). The nontransduced NRVMs attached to the outside of the gene-modified region and formed a monolayer, although some could be found in the transduced region. Collectively, the green and red channel images of the interface region confirmed the ability of the stenciling technique to create a confluent monolayer of nontransduced NRVMs with a central region of transduced heterogeneity (Figure 4E). Images from inside the gene-modified region (Figure 4F) showed that the central island consisted mostly of transduced NRVMs (80.9 ± 1.8% of cell-covered area as measured from 3 monolayers with 1 field of view per monolayer).

**Impulse Propagation in Monolayers With Regions of Kir2.1 Gene Modification**

In monolayers with LV-Empty–transduced islands, at 2-Hz pacing rate, APs propagated through the islands with CV values similar to those of the rest of the monolayer (19.3 ± 0.5 cm/sec in islands; n = 9; P = 0.32 and 19.6 ± 0.4 cm/sec in the surrounding nontransduced region; n = 9), as seen with an unperturbed linear wavefront in the direction of impulse propagation (Figure 5A, left column). As expected, APs propagated with increased CV (25.6 ± 1.8 cm/sec; n = 9; P = 5.3 × 10^{-14}; compared with 19.4 ± 0.4 cm/sec in the nontransduced region; n = 9) in Kir2.1-overexpressed islands and decreased CV (12.7 ± 2.3 cm/sec; n = 9; P = 4.3 × 10^{-10}; compared with 19.3 ± 0.6 cm/sec in the nontransduced region; n = 9) in Kir2.1-suppressed islands, as seen with the respective convex and concave curvature of the propagating wavefronts (Figure 5B and 5C, left column). Representative isopotential maps of AP propagation in monolayers with LV-Empty–transduced or Kir2.1 gene-modified islands are shown in supplemental Figure III. In LV-Empty–transduced islands, the APD_{80} values were similar to those of nontransduced regions (169 ± 8 ms; n = 9; P = 0.12 and 175 ± 8 ms; n = 9, respectively). However, abbreviated APD_{80} (71 ± 6 ms; n = 9; P = 6.6 × 10^{-14}; compared with 168 ± 7 ms in the nontransduced region; n = 9) and prolonged APD_{80} (210 ± 7 ms; n = 9; P = 4.1 × 10^{-6}; compared with 172 ± 4 ms in the nontransduced region; n = 9) were seen in Kir2.1-overexpressed islands.
and Kir2.1-suppressed islands, respectively (Figure 5A through 5C, right column). The CV and APD_{50} values of LV-Empty, Kir2.1-overexpressed, Kir2.1-suppressed, and nontransduced regions were not statistically different (P > 0.05 for all cases) from those observed for these groups in uniformly transduced monolayers.

**Dynamics of Induced Reentrant Spiral Waves in Monolayers With Heterogeneous I_{K1} Expression**

We next studied the dynamics of an induced spiral wave in monolayers with altered I_{K1}. Rapid pacing failed to induce reentrant waves in monolayers with nontransduced and LV-Empty–transduced NRVM islands (n = 9 each). However, reentry was successfully initiated in the 2 groups of monolayers with islands of heterogeneous expression of I_{K1}. But the dynamics of reentry varied greatly between the 2 groups. Reentrant waves were anchored to islands of Kir2.1 overexpression (Figure 6A), remained stable for a prolonged period of time (more than 2 hours), and had a frequency of 9 ± 1 Hz (n = 9), as shown by the optical recordings from a representative recording site (Figure 6B). When the tip of the induced spiral wave was tracked over 3 successive cycles, it maintained an almost circular trajectory inside the region of Kir2.1 overexpression (Figure 6C). The reentrant wave propagated with a CV of 6.4 ± 0.2 cm/sec (n = 9) in the transduced region at a distance of 2 mm from the wavetip and a CV of 16.3 ± 0.4 cm/sec (n = 9) in the nontransduced region at a distance of 6 mm from the wavetip. After 1 minute of superfusion (includes wash-in time and time allowed for the solution to mix in the chamber) with Tyrode’s solution containing 50 μmol/L Ba^{2+}, which selectively blocks I_{K1} currents, the frequency of reentry gradually decreased to 7 ± 1 Hz (n = 9).

The stability of the spiral wave was also disturbed, as reflected by a transition from a circular wavetip pattern to a meandering pattern of almost the same size. The previously measured reentry CVs in the transduced and nontransduced regions of the monolayer decreased to 5.1 ± 0.2 cm/sec (n = 9) and 14.1 ± 0.5 cm/sec (n = 9), respectively. The changes in the dynamics of the reentry after 1 minute of Ba^{2+} superfusion are summarized in supplemental Figure IV. After 3 minutes of Ba^{2+} superfusion, the stability of the reentry was heavily lost (Figure 6D), and the rotation frequency decreased further to 5 ± 1 Hz (n = 9; Figure 6E). The trajectory of the spiral wavetip also followed a meandering pattern much larger in size than before (Figure 6F). The reentry CV in the transduced and nontransduced regions decreased further to 3.2 ± 0.2 cm/sec (n = 9) and 11.7 ± 0.2 cm/sec (n = 9), respectively. On continued Ba^{2+} superfusion, the reentry terminated within the next 2 to 4 minutes.

Similar to a previous report, dominant-negative suppression of Kir2.1 gave rise to spontaneous activity (6 of 9 monolayers). This activity emerged from inside the transduced region to the rest of the monolayer (Figure 7A) at a frequency of 1.5 ± 0.5 Hz (n = 6). Rapid pacing of monolayers with islands of Kir2.1 suppression successfully initiated reentrant waves (Figure 7B), which were quasi-stable. The spiral wave moved around the island with a frequency of 3 ± 1 Hz (n = 9). Shortly after initiation (within 3 to 5 minutes), it detached from the island and terminated (3 of 9 monolayers), transitioned into a sustained complex of coupled spiral waves (4 of 9 monolayers; Figure 7C), or transitioned into a 2-armed spiral wave (2 of 9 monolayers; Figure 7D) that rearranged itself into a figure-of-eight reentry (Figure 7E), which drifted away from the island and terminated. Thus, Kir2.1-suppressed NRVM islands stabilized reentry but with a lower frequency and shorter duration compared with Kir2.1-overexpressed NRVM islands.

In a final set of experiments, monolayers with an inverse pattern of I_{K1} heterogeneity were developed with nontransduced NRVM islands in the center and Kir2.1 gene-modified NRVMs on the outside. Nontransduced NRVM islands with LV-Empty–transduced NRVMs on the outside were used as controls. Although reentry could not be induced in control cultures, it was successfully initiated in monolayers with an
inverse pattern of Kir2.1 overexpression or suppression. However, the dynamics of the reentry varied greatly between the 2 groups. With Kir2.1 overexpression, reentrant spiral waves were stable and persisted for more than 2 hours (Figure 8A) and had a frequency of $7 \pm 0.5$ Hz ($n=9$). The frequency of the reentry was slower than the $9 \pm 1$ Hz seen in monolayers with central islands of Kir2.1 overexpression. The tip of the spiral wave followed an almost circular pattern in the region of Kir2.1 overexpression over the course of 3 successive cycles (Figure 8B). In an opposite fashion, with Kir2.1 suppression, the induced spiral wave was unstable and terminated within 5 minutes after initiation (Figure 8C). The reentry had a frequency of $3 \pm 0.5$ Hz ($n=9$) similar to the $3 \pm 1$ Hz seen in monolayers with central islands of Kir2.1 suppression, and its tip followed a meandering pattern (Figure 8D). Thus, in the case of inverse heterogeneity, altered $I_{K_1}$ expression also enhanced the genesis of spiral waves compared with uniformly transduced monolayers, with greater stability for $I_{K_1}$ overexpression compared with $I_{K_1}$ suppression.

Thus, with heterogeneous Kir2.1 expression, spiral waves are consistently observed to be more stable with Kir2.1 overexpression than with Kir2.1 suppression, whether altered Kir2.1 expression occurs in the island or in the surrounding region (for the inverse pattern). These observations support the notion that decreased head–tail interaction secondary to shortened APD contributes to the stability of reentrant waves.

**Discussion**

Studies have previously suggested the importance of $I_{K_1}$ in VF dynamics by relating the stability of high-frequency rotors to the levels of outward component of $I_{K_1}$. Increased $I_{K_1}$ during atrial fibrillation at hyperpolarizing potentials is also considered to be an important factor for the maintenance of the arrhythmia. In transgenic animals, the role of $I_{K_1}$ upregulation in the frequency and stability of rotors has been studied. However, in such transgenic hearts, there was reported to be cell-to-cell variability in the expression of $I_{K_1}$, and the effects of such heterogeneities on arrhythmia dynamics are unknown. Hence, in the present study, using a combination of tissue engineering, somatic gene transfer, immunohistochemistry, Western blot, whole-cell patch clamp, and optical voltage-mapping techniques, we devel-
oped a novel, in vitro model of cardiac tissue with a spatially localized $I_{K1}$ heterogeneity and characterized the electrophysiology and dynamics of reentrant spiral waves in this tissue model. In comparing such a tissue model against transgenic animals, there is the advantage (and disadvantage) of not having compensatory mechanisms coming into play for the gain or loss of function of proteins of interest. Our study demonstrates the capacity of $I_{K1}$, when heterogeneously expressed, to initiate and maintain a high-frequency spiral wave in cultured NRVM monolayers. Hence, it may not be simply the increase in $I_{K1}$ that contributes to increased stability of spiral waves$^{13}$ but also the heterogeneity in $I_{K1}$ expression and the subsequently large $I_{K1}$ differences. The faster rotation rates and persistence of the rotor have been previously attributed to the increased outward component of $I_{K1}$. Our study provides new insight on how spiral wave anchoring by $I_{K1}$ can predispose the heart to fatal cardiac arrhythmias. Hence, altering Kir2.1 expression levels and modulating $I_{K1}$ currents may lead to alternative and potentially effective antiarrhythmic approaches.

$I_{K1}$ Regulates Excitability and Electrophysiological Properties of Cardiac Tissue

In this study, we demonstrated that overexpression of Kir2.1 significantly increased $I_{K1}$ density, whereas dominant-negative suppression of Kir2.1 significantly decreased $I_{K1}$. Also, upregulation of $I_{K1}$ significantly hyperpolarized RMP, increased dV/dt$_{max}$ and shortened duration of the AP. These findings are consistent with the role of $I_{K1}$ in regulating sodium channel availability and excitability of cardiac tissue.$^{13}$ As expected, suppression of $I_{K1}$ significantly depolarized RMP, decreased dV/dt$_{max}$, and prolonged duration of the AP, indicative of reduced channel availability.

The secondary effects of modulation of $I_{K1}$ on cardiac electrophysiological tissue properties, namely APD and CV, were then characterized. We chose bipolar line stimulation because with flat excitation wavefronts, the effects of wavefront curvature on CV can be eliminated. Furthermore, the bipolar electrodes concentrate the stimulation current in the gap between the electrodes and hence avoid distant stimulation. In contrast, the convex excitation wavefronts produced by a point stimulus propagate slower than flat excitation wavefronts and facilitate the formation of reentrant waves. We observed increased CV in monolayers with uniform Kir2.1 overexpression and decreased CV in monolayers with Kir2.1 suppression. Because CV depends on gap junction coupling and excitability, the absence of changes in Cx43 levels between nontransduced, LV-Empty–transduced and Kir2.1 gene-modified monolayers together with the observed changes in dV/dt$_{max}$ support the notion that CV changes following Kir2.1 gene modification are mediated by associated changes in RMP and sodium channel availability. Another factor that may alter upstroke velocity and CV in the case of $I_{K1}$ modification is suppression or exaggeration of phase IV pacemaker depolarization as a consequence of altered outward currents in the threshold voltage range. As expected, Kir2.1 overexpression abbreviated APD$_{80}$, whereas Kir2.1 suppression prolonged APD$_{80}$. Similar to a previous report,$^{20}$ $I_{K1}$ suppression resulted in prolonged APD and spontaneous activity in islands of Kir2.1AAA-transduced NRVMs.

In Kir2.1AAA-transduced monolayers, APD, and thus the effective refractory period, is significantly prolonged with a much shorter excitable gap, suggesting there could be incom-
complete recovery of the sodium channel at high stimulation rates, in addition to the reduction in sodium channel availability owing to the reduction of RMP.

**I\textsubscript{K1} Regulates Cardiac Reentry Dynamics**

Sustained arrhythmias have been previously induced in cultured monolayers of NRVMs using a rapid pacing protocol that relies on preexisting heterogeneity in cellular or tissue properties (eg, excitability, refractory period, or anisotropy) to cause the formation of a wavebreak. In this study, the uniformity of tissue properties was such that rapid pacing failed to induce stable reentry in nontransduced monolayers. Furthermore, rapid pacing failed to induce reentry in uniformly transduced monolayers and in monolayers with islands of nontransduced NRVMs. On the other hand, the presence of sharp CV and APD differences in monolayers with islands of Kir2.1 overexpression or suppression, or with the inverse patterns, promoted reentry initiation. Interestingly, whereas reentry could be induced at pacing rates of $\approx 5$ Hz in monolayers with islands of Kir2.1 overexpression, and at 5.6 Hz in monolayers with the inverse pattern, no reentry could be induced even at 9 Hz in monolayers with uniform overexpression of Kir2.1. Similarly, reentry could be induced in monolayers with heterogeneous but not uniform Kir2.1 suppression, although the reentrant waves were less stable than those observed with heterogeneous Kir2.1 overexpression. The findings of our study demonstrate that the uniform overexpression of Kir2.1 does not necessarily create a medium that is prone to reentry formation and that it is $I_{K1}$ heterogeneity that enhances the genesis of stable spiral waves. Following Ba$^{2+}$ perfusion of monolayers containing islands of heterogeneous Kir2.1 expression, $I_{K1}$ is blocked in both the transduced and nontransduced regions of the monolayer, lowering the level of $I_{K1}$, abolishing $I_{K1}$ heterogeneity, and transforming the monolayer into a homogeneous medium. Hence, the spiral wave meanders, loses stability, and eventually terminates by drifting off the edge of the monolayer.

In our study, both the differences and level of $I_{K1}$ density seem sufficient to account for many aspects of VF dynamics. Nevertheless, we cannot discount the involvement of other outward currents in the manifestation of cardiac arrhythmias. Recently, increased $I_{Ks}$ has been shown to enhance conduction block and wavebreak formation by means of postpolarization refractoriness. However, simulations have predicted that $I_{K1}$ has a greater effect on rotor frequency than other potassium currents such as $I_{ss}$, $I_{to}$, and $I_{Kslow}$. Thus, $I_{K1}$ is an important regulator of spiral wave frequency and stability because of its role in governing the excitation threshold, as well as the terminal phase of repolarization.

Our results from perturbations of $I_{K1}$ in the central island indicate that changes in rotation rate of the spiral wave can occur from perturbations in $I_{K1}$ expression solely near the spiral tip without changes in the arms of the spiral wave, unlike the case with transgenic animal experiments. Also, more stable spiral waves are consistently observed with Kir2.1 overexpression than with Kir2.1 suppression, whether altered Kir2.1 expression occurs in the island or in the

---

**Figure 8.** Dynamics of induced reentrant spiral waves in 21-mm-diameter monolayers with reverse pattern of $I_{K1}$ heterogeneity. The mapping region is 17 mm in diameter. A, An induced spiral wave remained stable in the region of Kir2.1 overexpression for a prolonged period of time. The central nontransduced NRVM island is shown by the white circle. B, The tip of the spiral wave followed an almost circular pattern in the region of Kir2.1 overexpression over the course of 3 successive cycles. The region of nontransduced NRVMs is shown with the black circle. The $x$ and $y$ axes are position on monolayers in mm. C, With Kir2.1 suppression, the induced spiral wave was unstable and terminated within 5 minutes after initiation. D, The tip of the spiral wave followed a meandering pattern over the course of 3 successive cycles.
surrounding region. In conclusion, our study provides new experimental evidence for the contribution of $I_{K1}$ to cardiac arrhythmogenesis and emphasizes the potential importance of $I_{K1}$ as an antiarrhythmic target.

**Acknowledgments**

We thank Peihong Dong and Seth Weinberg for assistance with molecular biology and mapping data analysis, respectively.

**Sources of Funding**

This work was supported by American Heart Association Predoctoral Fellowship 0715212U (to R.B.S.); National Heart, Lung, and Blood Institute grant P0-1 HL77180 (to G.G.H.; D.A. Kass, principal investigator), Fondation Leducq (to E.M.), and NIH grant HL66239 (to L.T.).

**Disclosures**

None.

**References**

Heterogeneity Affects Genesis and Stability of Spiral Waves in Cardiac Myocyte Monolayers

Rajesh B. Sekar, Eddy Kizana, Hee C. Cho, Jared M. Molitoris, Geoffrey G. Hesketh, Brett P. Eaton, Eduardo Marbán and Leslie Tung

Circ Res. 2009;104:355-364; originally published online January 2, 2009;
doi: 10.1161/CIRCRESAHA.108.178335

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/104/3/355

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2009/01/02/CIRCRESAHA.108.178335.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Supplement Material

Expanded Materials and Methods

Plasmid construction and lentivirus preparation

Lentiviral vectors (LVs) based on the human immunodeficiency virus Type 1 were used throughout this study. The third-generation constructs used in this study include the self-inactivating (SIN) long terminal repeat (LTR), the central polypurine tract (cPPT) and the woodchuck hepatitis virus post-transcriptional regulatory element (Wpre). The four plasmids required for lentivirus vector (LV) production were kindly supplied by Professor Inder Verma from the Salk Institute, San Diego, USA. The cDNAs for mouse Kir2.1 or dominant-negative mutant, Kir2.1AAA, were subcloned into the LV plasmid, pRRLsin18.cPPT.CMV.eGFP.Wpre following the removal of eGFP. A control lentiviral plasmid devoid of a heterologous expression cassette was also constructed to produce empty LVs (LV-Empty). LVs encoding eGFP (LV-eGFP), Kir2.1 (LV-Kir2.1), dominant-negative mutant Kir2.1AAA (LV-Kir2.1AAA) and LV-Empty were produced by calcium-phosphate co-precipitation transfection of the four LV plasmids into human embryonic kidney (HEK) 293T cells as previously described. The supernatant from HEK cell flasks containing LVs was collected 48 and 72 hours after transfection, filter sterilized using 0.2 μm cellulose acetate filter units (Corning, Cambridge, MA) and concentrated by ultra-filtration (100,000 MWCO, Centricon Plus-70, Millipore, Milford, MA). Transduction titer was assigned on concentrated viral stock by assessing transgene expression in HEK 293T cells using a limiting dilution assay in the presence of 8 μg/mL of Polybrene (Sigma-Aldrich, St. Louis, MO) three days after transduction. For LV-
Empty, the titer was assigned by performing an enzyme-linked immunosorbent assay (ELISA) for HIV-1 p24 core antigen (PerkinElmer Life Sciences, Inc., Boston, MA).

**Cell culture**

NRVMs were enzymatically dissociated from the ventricles of 2-day-old Sprague-Dawley rats (Harlan, Indianapolis, IN) with the use of trypsin (Amersham Biosciences, Piscataway, NJ) and collagenase (Worthington Biochemical Corporation, Freehold, NJ).\(^2\) Freshly isolated NRVMs were resuspended in M199 culture medium (Gibco) supplemented with 10% FBS, glucose, 2 mM L-glutamine, penicillin, vitamin B12, HEPES buffer and MEM non-essential amino acids (Gibco). Two 90-minute pre-plating steps were performed to reduce fibroblasts and enrich cardiac myocyte content in the culture. The final cell suspension was collected, counted for NRVMs and diluted at the desired plating concentration. For patch-clamping studies, \(10^5\) cells were plated on 12-mm glass coverslips coated with fibronectin at a concentration of 25 μg/mL. For mapping studies, \(10^6\) cells were plated on 21-mm fibronectin-coated plastic coverslips. The time of plating is considered day 0 for the NRVM cultures. After 24 hours, the coverslips were washed with warm phosphate buffered saline (PBS) and fresh medium with 10% serum was added. Starting from day 2 (two days after plating), the cultures underwent media change on every second day and were maintained in medium containing 2% serum.

All animal experiments were performed in accordance with guidelines set by the Johns Hopkins Committee on Animal Care and Use and were in compliance with all federal and state laws and regulations. The animals, neonatal rat pups, were only used for harvesting cardiac tissue and were sacrificed thereafter.
Lentiviral transduction of NRVMs

For all transduction experiments, the concentrated LV stock was applied at the indicated multiplicity of infection (MOI; i.e., the number of active vector particles per target cell) in the presence of 8 μg/mL of Polybrene (Sigma-Aldrich) as previously described.²

Stenciling and preparation of monolayers with heterogeneous $I_{K_1}$ expression

Two populations of NRVMs isolated on two consecutive days were used to obtain confluent monolayers with regions of altered Kir2.1 expression. In this modified stenciling technique, 200-300 μL of fibronectin (Sigma-Aldrich) solution (50 μg/mL) in deionized water was carefully transferred to the surface of the PDMS stencils with a central hole 6 mm in diameter made by a punch. The stencils were left undisturbed for 1-2 h. After this period, stencils with adsorbed fibronectin were cast against UV-treated, circular, plastic coverslips (21 mm diameter) and fibronectin solution at a lower concentration (25 μg/mL) was added to the circular well region formed by the stencil and the coverslip. After about 1 hour, excess fibronectin solution was aspirated, and a mixture of freshly isolated NRVMs (from first day of isolation) and concentrated lentiviral vectors encoding target genes of interest (empty, Kir2.1, or Kir2.1AAA) were plated into the wells. After 20-24 h, the excess medium was aspirated, and PBS was added to wash off any unattached cells. Without much delay, the PDMS stencils were gently peeled off the coverslips, which were immediately left in culture plates containing warm Tyrodes solution. Fibronectin transferred to the plastic coverslips via the stencils was wetted by this procedure and enabled subsequent plating and attachment of non-transduced NRVMs (from second day of isolation). Non-transduced NRVMs were added to already adherent and transduced NRVMs to form an in vitro model of cardiac myocytes with spatially-localized functional heterogeneity.
After 20-24 h, the preparation was washed twice with warm PBS to remove unattached NRVMs. At this time, a well-defined monolayer of non-transduced NRVMs with a spatially (centrally) localized population of transduced NRVMs was obtained. To obtain a monolayer with an inverse pattern of heterogeneity, non-transduced NRVMs from first day of isolation were plated inside the stencil region. After 24 h, NRVMs from second day of isolation were transduced with LVs in suspension for 2-3 h, pelleted by centrifugation at 4°C and 750 rpm for 3 minutes and resuspended in fresh cell culture medium. The process of pelleting and resuspending was repeated three more times so that the final cell suspension was devoid of free LVs. This suspension was then added to already adherent and centrally localized non-transduced NRVMs from previous day to obtain confluent monolayers with inverse pattern of \( I_{K1} \) heterogeneity.

**CellTracker labeling**

CellTracker Red CMTPX (Invitrogen) was dissolved to 10 mM in DMSO (Sigma-Aldrich) and further diluted to 25 μM in serum-free medium. Freshly isolated non-transduced NRVMs were centrifuged to form a pellet, supernatant was aspirated, and cells were resuspended in staining solution pre-warmed to 37°C. The cells were incubated for 30 mins under normal growth conditions. Stained cells were pelleted, resuspended in culture medium containing 10% serum, plated on fibronectin-coated plastic coverslips and allowed to adhere over a period of 24 h.

**Immunohistochemistry**

Six-day old non-transduced, LV-Empty transduced and Kir2.1 gene-modified NRVMs were characterized thoroughly for their myocyte content, morphology, and levels of Kir2.1 and gap junction protein expression using an immunostaining assay with antibodies against cardiac
Troponin I (cTnI), actin, Kir2.1 and connexin43 (Cx43). Cultures were washed with PBS and fixed in 4% paraformaldehyde for 10 minutes at room temperature. Cells were washed again with Tris-buffered saline (TBS) and permeabilized using TBS with 0.1% Triton X-100 for 20 minutes. Cells were blocked overnight at 4°C with 5% non-fat dry milk in TBS with Tween-20 (TBS-T). Primary antibodies against cTnI (1:50, SC-15368, Santa Cruz Inc., Santa Cruz, CA), Kir2.1 (1:50, SC-28633, Santa Cruz Inc.) and Cx43 (1:200, C6219, Sigma-Aldrich, St. Louis, MO) were diluted in the blocking medium. Cells were incubated with primary antibodies for 1 h at room temperature then washed with blocking reagent. Cells were incubated with Alexa Fluor conjugated secondary antibodies (1:200, Invitrogen) for 1 h at room temperature and washed extensively with TBS-T. Actin was stained using Alexa Fluor 647 phalloidin (1:200, A22287, Invitrogen) and nuclei were stained using Hoechst (1:10000, Invitrogen). Coverslips were mounted with ProLong Gold antifade reagent (P36930, Invitrogen), sealed with nail polish, and imaged.

Fluorescence imaging

Confocal fluorescence imaging of immunostained 6-day old NRVM cultures was performed on a Zeiss LSM 510 META confocal laser scanning microscope (Carl Zeiss Inc., Thornwood, NY), and all images were processed with the Zeiss LSM software (Carl Zeiss Inc.) and ImageJ software (National Institutes of Health, Bethesda, MD).

Western Blot

Six-day old non-transduced and transduced cultures were characterized for their Kir2.1, Cx43 and tubulin levels using standard Western blotting techniques. Cultures were lysed with SDS-
PAGE sample buffer and boiled for 5 minutes. Proteins were separated with 4-12% bis-tris gels (Invitrogen) using MES running buffer and transferred onto 0.45 µm nitrocellulose membranes using an iBLOT apparatus (Invitrogen). Equal protein loads were confirmed by Ponceau-S stain. After transfer to nitrocellulose, membranes were blocked overnight at 4°C then incubated with primary antibodies against Kir2.1 (1:1000, Santa Cruz), Cx43 (1:1000, Sigma-Aldrich) and α-tubulin (1:1000, Abcam) for 1 hour at room temperature. Membranes were incubated with alkaline phosphatase–conjugated secondary antibodies (1:20000, Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature. Proteins were visualized with chemiluminescent substrate and x-ray film, and images were analyzed using ImageJ.

**Whole-cell patch clamping**

Experiments were carried out using standard microelectrode whole-cell patch-clamp techniques with an Axopatch 200B amplifier (Axon instruments, Union City, CA). Data was sampled at 20 kHz and low-pass Bessel-filtered at 5 kHz. All experiments were performed at room temperature. Six-day old cells were washed with a normal Tyrode’s solution containing (mM) NaCl 138, KCl 5, CaCl2 2, glucose 10, MgCl2 0.5, and HEPES 10; pH 7.4. The micropipette electrode solution was composed of (mM): K-glutamate 130, KCl 9, NaCl 8, MgCl2 0.5, HEPES 10, EGTA 2, and Mg-ATP 5; pH 7.2. For the measurement of $I_{K1}$ density, 138 mM Na$^+$ in the external bath solution was replaced with 140 mM K$^+$ (total). Microelectrodes had tip resistances of 2 to 4 MΩ when filled with the internal recording solution. Voltage-clamp recordings were obtained with an inter-episode interval of 2.5 seconds. Action potentials were either initiated by short depolarizing current pulses (Kir2.1-overexpressed NRVMs) or were spontaneous (eGFP-
transduced control and Kir2.1AAA-overexpressed myocytes). Data was corrected for the measured liquid junction potential of -14.3 mV.\textsuperscript{4,5}

**Optical mapping**

Optical mapping was performed on NRVM monolayers 6-7 days post-transduction as previously described.\textsuperscript{2} Bipolar line stimulation via platinum electrodes was applied just above one edge of the monolayer. To determine APD\textsubscript{80} and CV, cells were stimulated with monophasic, 10ms pulses at 2 Hz delivered by the stimulus electrode at twice diastolic threshold. A 3-s recording was taken after a ten beat drive train. To initiate reentry, a rapid pacing protocol (with a pacing frequency of 5 Hz or above) was used.\textsuperscript{6}

**Data analysis**

For patch clamping, the dV/dt\textsubscript{max} for eGFP-transduced and Kir2.1 gene-modified NRVMs was equal to the maximum positive value of the first derivative of the action potential. For optical mapping, the raw optical signals were detrended by subtracting a fitted second-order polynomial curve and then low-pass filtered with a fourth order elliptical filter. Isopotential and isochrone maps were generated from the processed signals using custom-written MATLAB (The MathWorks Inc., Natick, MA) scripts. APD\textsubscript{80} was defined as the interval from the activation time to the time in the repolarization phase where the AP amplitude dropped to 20\% of its maximum, and APD\textsubscript{80} values were averaged across all channels and over 3 to 4 APs. CV was defined as the inverse of the distance along a line normal to the wavefront that crossed isochrones spaced 10 ms apart for paced waves and 3 ms apart for reentrant waves. For paced waves, CV was measured along 4 to 5 manually selected paths and averaged spatially across these paths and temporally
over 3 to 4 APs. For reentrant waves, CV was measured at a fixed distance from the wave tip measured along the wave front. Paths were chosen to be sufficiently far away from the stimulus site so that latency delays associated with excitation could be neglected. MCR was defined as the maximum pacing rate at which each stimulus evoked a tissue response in at least 90% of recording sites. To track the spiral wave tip, we analyzed the system in phase space and tracked the phase singularity, the point at which wavefront and waveback meet.7

Expanded Results

Characterization of non-transduced and Kir2.1 gene-modified cultures

Immunostain images of cTnl (Online Figure I, first column) show NRVMs in non-transduced (Online Figure IA) and in LV-Empty (Online Figure ID), LV-Kir2.1 (Online Figure IG) and LV-Kir2.1AAA (Online Figure IJ) transduced cultures. Immunohistochemistry against actin (Online Figure I, second column) and Cx43 (Online Figure I, third column) confirmed that in a given field of view both non-transduced and transduced cultures were morphologically similar (Online Figure IB, IE, IH and IK for non-transduced, LV-Empty, LV-Kir2.1 and LV-Kir2.1AAA transduced, respectively) and had similar levels of gap junctional protein expression (Online Figure IC, IF, II and IL for non-transduced, LV-Empty, LV-Kir2.1 and LV-Kir2.1AAA transduced, respectively) that was primarily localized at cell-to-cell appositions. No striking differences in the levels and distribution of Cx43 expression were apparent among the cultures.

Characterization of single-cell electrophysiological properties of Kir2.1-transduced NRVMs
Spontaneous APs were observed in eGFP-transduced NRVMs (Online Figure IIA). While spontaneous APs were absent in Kir2.1-overexpressed NRVMs, single APs could be triggered by a short depolarizing current stimulus (Online Figure IIB). Kir2.1AAA-transduced NRVMs fired spontaneous APs resembling those of genuine pacemaker cells (Online Figure IIC).

**Impulse propagation in monolayers with islands of Kir2.1 gene-modification**

Representative isopotential maps of AP propagation in monolayers with islands of LV-Empty transduced, Kir2.1-overexpressed and Kir2.1-suppressed NRVMs are shown in Online Figure IIIA, IIIB and IIIC, respectively.

**Dynamics of induced reentrant spiral waves in monolayers with heterogeneous $I_{K1}$ expression**

After 1 minute of superfusion with Tyrode’s solution containing 50 μM Ba$^{2+}$, the frequency of reentry in a monolayer with a central island of Kir2.1 overexpression gradually decreased (Online Figure IVA) to 7±1 Hz (n=9; Online Figure IVB). The stability of the spiral wave was also disturbed, as reflected by a transition from a circular wavetip pattern (as seen in Figure 6C of the main text) to a meandering pattern of almost the same size (Online Figure IVC).

**References**


**Figure Legends**

**Online Figure 1.** Characterization of non-transduced and Kir2.1 gene-modified NRVM monolayers. Immunostain images of cTnI (first column), actin (second column) and Cx43 (third column) in non-transduced (A-C), LV-Empty transduced (D-F), LV-Kir2.1 transduced (G-I) and
LV-Kir2.1AAA transduced (J-L) monolayers. Hoechst (blue) was used to label the nuclei in Cx43 immunostain images. All scale bars, 50 µm.

**Online Figure II.** Representative action potentials obtained by whole-cell current clamp of Kir2.1-transduced NRVMs. (A) Spontaneous APs in eGFP-transduced control myocytes. (B) Electrically stimulated APs in $I_{K1}$-overexpressing myocytes. (C) Spontaneous APs in $I_{K1}$-suppressed NRVMs.

**Online Figure III.** Impulse propagation in monolayers with LV-Empty transduced or Kir2.1 gene-modified NRVM islands. Representative isopotential maps of AP propagation in monolayers with a central island of LV-Empty transduced (A), Kir2.1-overexpressed (B) or Kir2.1-suppressed (C) NRVMs. Representative sites in the transduced and non-transduced regions of the monolayer are indicated by “x” and “+” symbols, respectively. The direction of impulse propagation is indicated by black arrows, and islands are shown by white circles.

**Online Figure IV.** Dynamics of induced reentrant spiral waves in monolayers with a central island of Kir2.1 overexpression. (A) Spiral wave after 1 min of superfusion with Tyrode’s solution containing 50 µM Ba$^{2+}$. (B) Five sec and 1 sec acquisitions of the spiral wave shown in (A) from site 224 marked by “x” in A. (C) Tip trajectory of the spiral wave after 1 min of Ba$^{2+}$ superfusion. The X- and Y- axes are position on monolayer in mm.
ONLINE FIGURE III

A  Monolayer with LV-Empty transduced island

B  Monolayer with island of Kir2.1-overexpression

C  Monolayer with island of Kir2.1-suppression