Published in final edited form as:

Pflugers Arch. 2018 April ; 470(4): 599–611. doi:10.1007/s00424-017-2099-3.

Kir2.1 channels set two levels of resting membrane potential with inward rectification

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Abstract

Strong inward rectifier K⁺ channels (Kir2.1) mediate background K⁺ currents primarily responsible for maintenance of resting membrane potential. Multiple types of cells exhibit two levels of resting membrane potential. Kir2.1 and K2P1 currents counterbalance, partially accounting for the phenomenon of human cardiomyocytes in sub-physiological extracellular K⁺ concentrations or pathological hypokalemic conditions. The mechanism of how Kir2.1 channels contribute to the two levels of resting membrane potential in different types of cells is not well understood. Here we test the hypothesis that Kir2.1 channels set two levels of resting membrane potential with inward rectification. Under hypokalemic conditions, Kir2.1 currents counterbalance HCN2 or HCN4 cation currents in CHO cells that heterologously express both channels, generating N-shaped current-voltage relationships that cross the voltage axis three times and reconstituting two levels of resting membrane potential. Blockade of HCN channels eliminated the phenomenon in K2P1-deficient Kir2.1-expressing human cardiomyocytes derived from induced pluripotent stem cells or CHO cells expressing both Kir2.1 and HCN2 channels. Weakly inward rectifier Kir4.1 or inward rectification-deficient Kir2.1•E224G mutant channels do not set such two levels of resting membrane potential when co-expressed with HCN2 in CHO cells or when over-expressed in human cardiomyocytes derived from induced pluripotent stem cells. These findings demonstrate a common mechanism that Kir2 channels set two levels of resting membrane potential with inward rectification by balancing inward cation currents through different cation channels such as hyperpolarization-activated HCN channels or hypokalemia-induced K2P1 leak channels.

Keywords

Kir2.1 channel; inward rectification; resting membrane potential; HCN channel; cardiomyocyte

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Author contributions

HC, DZ, KC, and ZL designed research. HC, DZ, and KC analyzed data. DZ and KC performed research. HC, DZ, and KC wrote the paper.

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Conflict of interest

The authors declare no competing financial interests.

Introduction

Inward rectifier K⁺ channels (Kir2.1) mediate the I_{K1} background K⁺ current that is primarily responsible for maintenance of resting membrane potential and for regulation of excitability. Kir2.1 channels contribute to the final repolarization stages of the action potential of cardiomyocytes [(14)]. Kir2.1 channels exhibit unique strong inward rectification, but the physiological roles of Kir2.1 inward rectification are not well understood.

Multiple types of cells show two levels of resting membrane potential in pathophysiological or physiologically ionic conditions. Sub-physiological extracellular K⁺ concentrations ($[K^+]_e$) occur in pathological hypokalemia during which blood K⁺ concentrations decrease from normal levels of 3.5 to 5.0 mM to 1.5 to 3.0 mM [(26)]. Under hypokalemic conditions, human cardiomyocytes [(23)], cardiac Purkinje fibers [(4, 8, 18, 30)], and skeletal muscle cells [(12, 16, 31, 33)] spontaneously shift from hyperpolarization at a value close to the K⁺ equilibrium potential of approximately -90 mV to depolarization at a much more positive value than predicted by the Nernst equation and display two stable levels of resting membrane potential. Such unusual electrical behaviors cause cardiac arrhythmia [(37)] and periodic paralysis [(3)].

In physiological $[K^+]_e$, macrophages and osteoclasts exhibit two levels of resting membrane potential [(10, 11, 27, 32)]. Cardiac Purkinje fibers and osteoclasts show N-shaped current-voltage ($I-V$) relationships that intersect the voltage axis three times. The first and third zero-current potentials determine the two levels of resting membrane potential [(8, 32)]. Kir2.1 channels are critical for such N-shaped $I-V$ relationships as Kir2.1 conductance is strongly inward rectifying [(8, 14)]. The types of cells that have two levels of resting membrane potential conduct significant Kir2 currents [(8, 10, 32, 33)].

We recently provided evidence supporting that Kir2.1 channels contribute to the two levels of resting membrane potential of human cardiomyocytes derived from induced pluripotent stem cells (iPSC) [(39)]. Human iPSC-derived cardiomyocytes conduct small Kir2 currents and have a resting membrane potential of around -60 mV, which is significantly depolarized compared to the approximately -80 mV of human adult cardiomyocytes [(6, 15, 19)]. Thus, human iPSC-derived cardiomyocytes are not mature in electrical activity, compared to human adult cardiomyocytes. Enhanced expression of Kir2.1 in human iPSC-derived cardiomyocytes restores mature action potential of human cardiomyocytes [(6, 15, 19)]. Under pathological hypokalemic conditions, human iPSC-derived cardiomyocytes with enhanced Kir2.1 expression can exhibit two levels of resting membrane potential. In approximately 75% of the Kir2.1-expressing human iPSC-derived cardiomyocytes that show the phenomenon of the two levels of resting membrane potential Kir2.1 currents counterbalance low $[K^+]_e$ -induced K2P1 leak cation currents, accounting for the phenomenon. We found that 18 of 24 of the iPSC-derived cardiomyocytes that showed the phenomenon had relatively large K2P1-like inward Na⁺ currents in 0 mM $[K^+]_e$; the other six cells did not conduct K2P1-like inward Na⁺ currents. We also demonstrated that

inhibition of *K2P1* expression reduced the percentage of the cardiomyocytes that had two levels of resting potential in 2 mM $[K^+]_e$ from 35% to 8.8% [(39)].

In this report, we sought to understand how Kir2.1 channels contribute to the two levels of resting membrane potential in multiple types of cells that may conduct inward background cation currents through various types of cation channels. We tested the hypothesis that Kir2.1 channels set the resting membrane potential with inward rectification through a common mechanism: Kir2.1 currents counterbalance inward cation currents through various types of channels, generating the N-shaped *I-V* relationships with three zero-current potentials. We investigated how Kir2.1 currents counterbalance the inward cation currents rather than low $[K^+]_e$ -induced K2P1 leak cation currents and set resting membrane potential. We still focused on inward background cation currents in cardiac Purkinje fibers and human cardiomyocytes, which are influenced by reduction of $[K^+]_e$ during pathological hypokalemia. Hyperpolarization-activated cyclic nucleotide-gated (HCN) nonselective cation channels are not background cation channels, but they conduct inward cation currents in normal resting membrane potential [(25)]. HCN2 and HCN4 channels are highly expressed in cardiac Purkinje fibers and detected in cardiomyocytes [(13, 28)]. Reduction of normal $[K^+]_e$ results in a large decrease in HCN current amplitude [(2)].

We found that in sub-physiological $[K^+]_e$ Kir2.1 currents counterbalance HCN2 or HCN4 cation currents, yielding the N-shaped *I-V* relationships with three zero-current potentials and setting two levels of resting membrane potential in Chinese hamster ovary (CHO) cells that heterologously express both Kir2.1 and HCN channels. Blockade of HCN channels eliminated the phenomenon of the two levels of resting membrane potential of K2P1-deleted, Kir2.1-expressing human iPSC-derived cardiomyocytes or CHO cells expressing both Kir2.1 and HCN channels. Weak inward rectifier Kir4.1 or inward rectification-deficient Kir2.1•E224G mutant channels do not set two levels of resting membrane potential when either co-expressed with HCN2 channels in CHO cells or over-expressed in human iPSC-derived cardiomyocytes. These findings support the hypothesis that Kir2.1 channels set two levels of resting membrane potential with strong inward rectification through the same molecular mechanism in various cell types. Kir2.1 currents are able to counterbalance inward cation currents through various types of cation channels that are gated by different mechanisms, such as hyperpolarization-activated HCN cation currents and low $[K^+]_e$ -induced K2P1 leak cation currents, and set the two levels of resting membrane potential with strong inward rectification.

Materials and methods

Constructs and adenoviruses

Human HCN2 or HCN4 in pcDNA3 vectors were provided by Dr. Juliane Stieber, University of Erlangen, Germany. Rat Kir4.1 in pcDNA6 vectors were provided by Dr. Stephen Tucker, University of Oxford, UK. Adenovirus construction, purification, and titration were performed by Vector Biolabs. Viral particles of Ad-mCherry-hKir2.1 and Ad-GFP-hK2P1 shRNA #1 were prepared as described previously [(39)]. Kir2.1•E224G mutant was generated using a QuikChange kit (Stratagene). The inserts encoding hKir2.1•E224G sequences were then subcloned into pDUAL-CCM(–) shuttle vector (Vector BioLabs)

through XhoI and BamHI sites. The DNA was then cloned into the pAd vectors in which expression of the *GFP* reporter gene is driven by a CMV promoter. All constructs were confirmed by automated DNA sequencing. Viral particles of Ad-mCherry-hKir2.1, Ad-mCherry-hKir2.1•E224G, and Ad-GFP-hK2P1 shRNA #1 were obtained at concentrations on the order of 10^{10} plaque-forming units (PFU)/ml. mCherry and GFP allowed identification of transduced cells. Viral particles were diluted 100 fold with DMEM media, aliquoted, and stored at -80°C .

Cell culture, transduction, and transfection

CHO cells were cultured as previously reported [(22)]. Briefly, CHO cells were maintained in DMEM supplemented with 10% fetal calf serum in a 5% CO_2 incubator. CHO cells were seeded on glass cover slips in 35-mm dishes 24 h before transduction or transfection. Cells at 60 to 80% confluence were transfected with Lipfectamine 2000 (Invitrogen). 1 μg of human HCN2 plasmid alone, human HCN4 plasmid alone, rat Kir4.1 plasmid alone, both Kir2.1 and HCN2 plasmids, both Kir2.1 and HCN4 plasmids, both Kir4.1 and HCN2 plasmids, both Kir2.1•E224G and HCN2 plasmids, both Kir4.1 and K2P1, or both Kir2.1•E224G and K2P1 plasmids were used. When two plasmids were used, the ratio was 1:1. Co-expression of GFP was used to identify the effectively transfected cells.

Culture of human iPSC-derived cardiomyocytes (CMC-100-110-001, Cell Dynamic International) was described previously in detail [(39)]. Briefly, human iPSC-derived cardiomyocytes were cultured in a 37°C , 5% CO_2 incubator according to the vendor's instructions, and transduced on days 8 to 20 of culture. Electrophysiological recordings were performed 48 h later after viral transduction.

Electrophysiology

Recordings of membrane potentials and whole-cell ramp currents were performed with the EPC-10 USB amplifier and a Dell 745 computer with PatchMaster software (HEKA Elektronik), and data were analyzed as described previously [(39)]. Briefly, recordings were performed at a sampled rate of 2 KHz. Patch pipettes with resistances of 2.0 to 3.5 $\text{M}\Omega$ were used. Series resistances are small than 10 $\text{M}\Omega$. The resistance was compensated at least 80% to minimize voltage errors. Whole-cell ramp currents were recorded with a standard 2.2-s voltage ramp from -140 mV to $+80\text{ mV}$ each 15 s with voltage-clamp techniques. Membrane potentials were continuously recorded with whole-cell current-clamp techniques during changes of bath solutions. Holding potential was -80 mV to measure whole-cell currents of CHO cells that heterologously expressed Kir2.1 alone or both Kir2.1 (Kir4.1 or Kir2.1•E224G) and HCN2 channels and of human iPSC-derived cardiomyocytes. Holding potential was -20 mV to measure whole-cell currents of CHO cells that heterologously expressed HCN2 or HCN4 channels alone. Electrophysiological data were analyzed with FithMaster (HEKA Elektronik), IGOR Pro (WaveMetrics), and Excel (Microsoft). All data are presented as means $\pm$ SEM. Two-tailed Student's t-tests were used to evaluate significance of differences between two groups of data.

For electrophysiological recordings in CHO cells, the pipette solution contained 140 mM KCl, 1 mM MgCl_2 , 10 mM EGTA, 1 mM $\text{K}_2\text{-ATP}$, and 5 mM HEPES (pH 7.4) and the bath

solution contained 135 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 15 mM glucose, 10 mM HEPES (pH 7.4). For electrophysiological recordings in human iPSC-derived cardiomyocytes, the pipette solution contained 20 mM KCl, 120 mM K-aspartate, 1 mM MgCl₂, 5 mM Na₂-ATP, 0.5 mM Na₂-GTP, 10 mM EGTA, and 5 mM HEPES (pH 7.4) and the bath solution contained 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES (pH 7.4). The total concentration of Na⁺ and K⁺ in bath solutions was constant, so Na⁺-based bath solutions with various low [K⁺]_e were obtained by replacing K⁺ and replacing it with equimolar Na⁺. The 30 mM stock solutions of ivabradine were prepared in dimethyl sulfoxide, aliquoted, and stored at -20 °C.

Results

Kir2.1 and HCN channels reconstitute two levels of resting membrane potential

We first studied how human Kir2.1, HCN2, or HCN4 channels maintain resting membrane potential when heterologously expressed in CHO cells in response to changes of [K⁺]_e from 5 mM to 2 mM, which occurs under severe hypokalemia [(26)]. CHO cells that expressed Kir2.1 channels alone hyperpolarized from -74.0 ± 0.7 mV to -97.2 ± 1.0 mV (n=9), because Kir2.1 channels maintained resting membrane potential as predicted by the Nernst equation for K⁺. Kir2.1 channels had reversal potentials of -75.8 ± 0.7 mV and -96.8 ± 1.3 mV (n=5) in 5 and 2 mM [K⁺]_e, respectively (Fig. 1A). In contrast, CHO cells expressing either HCN2 or HCN4 channels alone did not dramatically change resting membrane potential (-25.0 ± 1.6 mV vs. -26.0 ± 1.5 mV, n=5 for HCN2; -22.1 ± 1.7 mV vs. -23.8 ± 1.8 mV, n=5 for HCN4) when [K⁺]_e was decreased (Fig. 1B–1C). Thus, Kir2.1 and HCN channels maintain resting membrane potential comparable to their reversal potentials, respectively.

We next examined how Kir2.1 K⁺ currents counterbalance HCN2 cation currents and determine resting membrane potential in CHO cells that heterologously express both Kir2.1 and HCN2. These cells had resting membrane potentials in 5 mM [K⁺]_e that were dependent on relative expression levels of the two channels. We focused on the CHO cells in which Kir2.1 currents overcame opposing HCN2 cation currents resulting in a resting membrane potential of -74.2 ± 0.3 mV (n=91) in 5 mM [K⁺]_e, mimicking physiological conditions of human cardiomyocytes and cardiac Purkinje fibers. When [K⁺]_e was reduced to 2 mM, these CHO cells experienced rapid hyperpolarization from around -74 mV to -93.5 ± 0.7 mV (n=91) in phase 1 due to Kir2.1 channel function. Subsequently, Kir2.1 and HCN2 currents re-balanced at such a hyperpolarization to determine the resting membrane potential in phase 2, because both Kir2.1 and HCN2 channels were inhibited by reduction of [K⁺]_e from 5 to 2 mM and HCN2 channels were also activated by hyperpolarization induced by Kir2.1 channels in 2 mM [K⁺]_e (Fig. 1B). When Kir2.1 outward K⁺ currents dominated, the CHO cells remained on stable hyperpolarization of -97.2 ± 0.8 mV (n=33), consistent with reversal potentials (-95.9 ± 1.8 mV, n=12) of their whole-cell ramp currents. Blockade of Kir2.1 currents confirmed that this population of the cells had very small HCN2 currents (Fig. 2A).

When Kir2.1 currents did not dominantly maintain a stable hyperpolarized resting membrane potential, the CHO cells exhibited two types of resting membrane potential

behaviors in phase 2. The first population of cells recapitulated the classical two stable levels of resting membrane potential observed in cardiac Purkinje fibers and human cardiomyocytes [(4, 23)] and permanently switched from hyperpolarization of -89.0 ± 1.5 mV to depolarization of -16.4 ± 1.5 mV ($n=30$) in an all-or-none way (Fig. 2B, top row). Application of 30 μ M ivabradine, a specific blocker for HCN channels, switched the depolarization back to hyperpolarization until washout of ivabradine (Fig. 2B, insert in top row). The second population spontaneously became depolarized and resting membrane potentials fluctuated between -94.4 ± 1.0 mV and -17.6 ± 1.8 mV ($n=31$, Fig. 2C, top row) with various patterns with two levels of resting membrane potential. The initial transition from hyperpolarization to depolarization was ignited either before or after maximal hyperpolarization was reached in phase 1.

These two groups of cells also recapitulated the N-shaped $I-V$ relationships that are observed in cardiac Purkinje fibers [(8)]. The first population mainly showed “a” type N-shaped whole-cell ramp currents that intersected with the voltage axis three times. The first and third zero-current potentials (-90.7 ± 2.5 mV and -16.1 ± 2.3 mV, $n=8$) determined the two levels of resting membrane potential. In fact, linear combinations of Kir2.1 and K2P1 ramp currents, which recorded individually in CHO cells in 2 mM $[K^+]_e$, generated N-shaped $I-V$ relationships that have three zero-current potentials (Fig. 2B, purple dash line in second row). When whole-cell currents were recorded each 15 s, these cells also simultaneously or separately displayed “b” type whole-cell ramp currents with a reversal potential of -16.9 ± 3.7 mV ($n=6$), comparable to the third zero-current potential of the N-shaped ramp currents. Sequential applications of Kir2.1 and HCN2 channel blockers confirmed that this population of the cells had very small HCN2 currents (Fig. 2B, second, third, and fourth rows). The second subgroup showed not only “a” type N-shaped ramp currents with the first and third zero-current potentials of -93.7 ± 1.7 mV and -19.3 ± 2.4 mV ($n=13$), respectively, and “b” type currents with a zero-current potential of -16.5 ± 2.0 mV ($n=5$) but also “c” type currents with a reversal potential (-99.0 ± 2.3 mV, $n=5$) that matches the first zero-current potentials of the N-shaped ramp currents (Fig. 2C, second, third, and fourth rows) during recordings of whole-cell currents each 15 s. The cells in this second population often dynamically displayed all three types of currents, consistent with their fluctuating resting membrane potential. These dynamic currents may reflect resting membrane potential that spontaneously fluctuated between the two levels or that remained depolarized or hyperpolarized. Since such dynamic changes of whole-cell currents were also observed in CHO cells that express Kir2.1 and K2P1 channels in sub-physiological $[K^+]_e$ [(39)] and K2P1 leak channels activates without time-dependence [(38)], slow activation kinetics of HCN2 channels should not be determinants of such dynamic changes of currents. Small HCN2 currents were isolated in these two populations of cells and the HCN2 current amplitudes were much larger than that found in the cells that did not show two levels of resting membrane potential (Fig. 2A–2C, third row). These results indicate that Kir2.1 currents dynamically counterbalance HCN2 cation currents, produce the N-shaped $I-V$ relationships with three zero-current potentials, and set two levels of resting membrane potential in sub-physiological $[K^+]_e$.

We also studied how Kir2.1 and HCN4 currents counterbalance and set resting membrane potential in 2 mM $[K^+]_e$. The CHO cells that express both channels exhibited three different

behaviors in resting membrane potential, similar to those observed in CHO cells that express both Kir2.1 and HCN2 channels. In response to changes of $[K^+]_e$ from 5 to 2 mM, the first population of the CHO cells simply shifted from -74.1 ± 0.5 mV to hyperpolarization of -95.4 ± 0.9 mV ($n=30$; Fig. 3A, top row), consistent with reversal potentials of -96.3 ± 0.9 mV ($n=19$) of the whole-cell ramp currents in 2 mM $[K^+]_e$. The second population recapitulated two stable levels of resting membrane potential and permanently depolarized to -19.0 ± 2.2 mV from hyperpolarization of -87.7 ± 2.0 mV ($n=13$; Fig. 3B, top row). The third group of the cells showed fluctuating membrane potential between -92.5 ± 1.4 mV and -24.3 ± 1.9 mV ($n=18$; Fig. 3C). The second subgroup exhibited “a” and “b” types of whole-cell ramp currents; the third dynamically exhibited “a”, “b”, and “c” types of whole-cell ramp currents. The first and third zero-current potentials of the “a” type N-shaped whole-cell ramp currents matched the two levels of resting membrane potential (Fig. 3A–3B, second and fourth rows). Small HCN2 currents were isolated in these three populations of cells and the HCN2 current amplitudes were much larger in cells that had two levels of resting membrane potential than in cells that did not showed the phenomenon (Fig. 3A–3C, third row). These results are consistent with those that were observed in CHO cells expressing either Kir2.1 and HCN2 channels or Kir2.1 and K2P1 channels [(39)] and indicate that Kir2.1 currents counterbalance these cation currents through the same mechanism even though K2P1 and HCN cation channels are gated by different mechanisms.

HCN channels contribute to two levels of resting membrane potential in human iPSC-derived cardiomyocytes with enhanced Kir2.1 expression

We next studied the contribution of HCN channels to two levels of resting membrane potential in human iPSC-derived cardiomyocytes that are engineered to express Kir2.1 in sub-physiological $[K^+]_e$. We recently showed that low $[K^+]_e$ -induced K2P1 leak cation currents are the major inward cation currents in 75% of Kir2.1-expressing human iPSC-derived cardiomyocytes that showed the phenomenon of the two levels of resting membrane potential in 2 mM $[K^+]_e$ [(39)]. Since human iPSC-derived cardiomyocytes express HCN channels [(21)], we inhibited *K2P1* expression by transduction of cells with a previously validated *K2P1*-specific shRNA #1 [(22, 39)] and examined whether small HCN currents contribute to the inward cation currents in the remaining 25% of the Kir2.1-expressing iPSC-derived cardiomyocytes cells showing the phenomenon. 8.8% of Kir2.1-expressing human iPSC-derived cardiomyocytes that expressed *K2P1*-specific shRNA#1 had two levels of resting membrane potential in 2 mM $[K^+]_e$ [(39)]. These K2P1-deficient, Kir2.1-expressing iPSC-derived cardiomyocytes either spontaneously shifted from -87.7 ± 2.4 mV to -19.4 ± 2.5 mV ($n=11$) or fluctuated between -95.1 ± 1.8 mV to -21.0 ± 1.3 mV ($n=35$; Fig. 4A–4B). They exhibited “a” type N-shaped whole-cell ramp currents with three zero-current potentials. In addition, the first and third zero-current potentials (-93.4 ± 2.8 mV; -23.1 ± 1.5 mV, $n=20$) matched the two levels of resting membrane potential (Fig. 4C). As expected, these cells conducted very small K2P1-like inward leak Na^+ currents in 0 mM $[K^+]_e$ and the leak Na^+ current at -80 mV is -80 ± 19 pA ($n=8$), much smaller than that in the cardiomyocytes that were not treated with *K2P1*-targeted siRNA and had two levels of resting membrane potential (Fig. 4D).

Application of 30 μM ivabradine reversibly eliminated fluctuation of resting membrane potential between two levels in 2 mM $[\text{K}^+]_e$ in the K2P1-deficient, Kir2.1-expressing iPSC-derived cardiomyocytes ($n=5$; Fig. 4E). HCN currents were identified in this population of the cardiomyocytes (Fig. 4F). Interestingly, application of 30 μM ivabradine had no effect on the subpopulation of the K2P1-deficient, Kir2.1-expressing iPSC-derived cardiomyocytes that showed classical depolarization of two stable levels of resting membrane potential in 2 mM $[\text{K}^+]_e$. Three possibilities may explain this result: First, the small inward currents may suffice to balance Kir2 currents, setting two stable levels of resting membrane potential. Second, ivabradine might not completely block HCN currents or K2P1 inhibition might be not efficient enough to eliminate K2P1 currents. Third, inward cation currents other than K2P1 and HCN might account for this behavior. Previous reports actually suggest that inward Ca^{2+} flux may contribute to the two levels of resting membrane potential of cardiac Purkinje fibers in K^+ -free and Na^+ -free bath solutions [(35)]. These results indicate that HCN currents contribute to the two levels of resting membrane potential observed in Kir2.1-expressing human iPSC-derived cardiomyocytes that are deficient in K2P1.

Kir2.1 inward rectification is essential for two levels of resting membrane potential

Kir2.1 channels show strongly inward rectifying $I-V$ relationships, unlike the other members in the sub-family of Kir channels [(14)]. We hypothesize that Kir2.1 inward rectification is essential for maintenance of two levels of resting membrane potential, because Kir2.1 inward rectification contributes to the N-shaped $I-V$ relationships observed in cardiac Purkinje fibers [(8)] and CHO cells expressing Kir2.1 and HCN2, HCN4, or K2P1 [(39)]. We tested this hypothesis by analyses of two additional background K^+ channels, Kir4.1 channels and Kir2.1•E224G mutant channels. Kir4.1 channels exhibit intermediate inward rectification [(14)] and the Kir2.1•E224G mutation induces complete loss of inward rectification of Kir2.1 channels [(17, 36)]. We confirmed these channel properties in transfected CHO cells (Fig. 5A–5B).

In 5 mM $[\text{K}^+]_e$, CHO cells that expressed both Kir4.1 and HCN2 channels had one stable level of resting membrane potential between their reversal potentials, and the resting membrane potential was dependent on the relative expression levels of the two channels. To mimic the same conditions used for study of CHO cells expressing Kir2.1 and HCN2, we focused on the CHO cells that had resting membrane potential of -71.6 ± 1.0 mV ($n=9$) in 5 mM $[\text{K}^+]_e$ because of dominant Kir4.1 currents. The cells simply hyperpolarized to -92.3 ± 0.9 mV ($n=9$) in 2 mM $[\text{K}^+]_e$, and had whole-cell ramp currents with a reversal potential of -89.9 ± 0.9 mV ($n=11$; Fig. 5C). Sequential applications of Kir4.1 and HCN2 blockers confirmed that whole-cell ramp currents resulted from Kir4.1 and HCN2 ramp currents. As expected, small HCN2 ramp currents were isolated. The HCN2 current amplitude at -100 mV was -71.5 ± 19.8 pA ($n=4$).

We investigated directly that the impact of Kir2.1 inward rectification on the resting membrane potential by employing rectification-deficient Kir2.1•E224G mutant channels. CHO cells that expressed Kir2.1•E224G and HCN2 channels had a stable resting membrane potential between their reversal potentials in 5 mM $[\text{K}^+]_e$. We then focused on the subpopulation of CHO cells in which Kir2.1•E224G currents overcame HCN2 cation

currents and maintained a resting membrane potential of -70.2 ± 0.5 mV ($n=15$) in 5 mM $[K^+]_e$. These CHO cells hyperpolarized to -92.9 ± 0.8 mV ($n=15$) in 2 mM $[K^+]_e$ (Fig. 5D), matching reversal potential of whole-cell currents at -91.2 ± 0.6 mV ($n=10$). Small HCN2 ramp currents were isolated and the HCN2 current amplitude at -100 mV was -97.9 ± 25.4 pA ($n=5$). Three sets of data excluded the possibility that Kir4.1 or Kir2.1 Kir2.1•E224G currents were absolutely dominant so the cells expressing HCN2 and Kir4.1 or Kir2.1•E224G did not show two levels of resting membrane potential. These cells had resting membrane potentials of -71 mV in 5 mM $[K^+]_e$, more positive than the resting membrane potentials at -74 mV of the cells with Kir2.1 and HCN2 channels, which showed the phenomenon. Second, the HCN2 currents recorded in cells expressing HCN2 and Kir4.1 or Kir2.1•E224G were comparable to those found in CHO cells that expressed Kir2.1 and HCN2 and had two levels of resting membrane potential. Third, in 5 mM $[K^+]_e$ these cells had whole-cell currents at -120 mV of -1.7 ± 0.4 nA ($n=8$) and -2.5 ± 0.3 nA ($n=12$), respectively, compared to -3.5 ± 0.4 nA ($n=27$) in the cells with Kir2.1 and HCN2 channels.

We next transduced human iPSC-derived cardiomyocytes with Kir2.1•E224G mutant channel viral particles and analyzed their resting membrane potentials and background currents, because human iPSC-derived cardiomyocytes with enhanced Kir2.1 expression can show two levels of resting membrane potential and have N-shaped whole-cell ramp currents with three zero-current potentials in 2 mM $[K^+]_e$ (Fig. 6A). In response to lowering $[K^+]_e$ from 5.4 mM to 2 mM, the majority of human iPSC-derived cardiomyocytes with overexpression of Kir2.1•E224G mutant channels simply hyperpolarized from -73.4 ± 0.4 mV to -99.1 ± 0.6 mV ($n=46$), but a small fraction of the cells hyperpolarized to -94.3 ± 1.5 mV ($n=9$) in phase 1 and then slowly shifted to -86.9 ± 2.7 mV in phase 2 (Fig. 6B). Small and slow depolarization in this subset of human iPSC-derived cardiomyocytes might result from K2P1 channels that slowly change ion selectivity and conduct inward cation currents in 2 mM $[K^+]_e$ [(22)].

None of analyzed human iPSC-derived cardiomyocytes that express Kir2.1•E224G mutant channels exhibited two levels of resting membrane potential, compared to 35% of human iPSC-derived cardiomyocytes that express Kir2.1 (Fig. 6C). These results are consistent with results obtained in CHO cells that express both Kir2.1•E224G and HCN2 channels. These experiments showed that Kir2.1 channels set two levels of resting membrane potential of the cardiomyocytes with inward rectification.

Discussion

Kir2.1 channels set two levels of resting membrane potential through a common mechanism

In this report, we showed that Kir2.1 currents counterbalance hyperpolarization-activated HCN2 or HCN4 cation currents in sub-physiological $[K^+]_e$, generating the N-shaped $I-V$ relationships with three zero-current potentials and setting two levels of resting membrane potential in CHO cells that express these channels. Our previous studies indicated that Kir2.1 currents counterbalance low $[K^+]_e$ -induced K2P1 leak cation currents through a similar mechanism, also producing the phenomenon of the two levels of resting membrane potential in transfected CHO cells. Thus, Kir2.1 channels primarily set the two levels of

resting membrane potential when co-expressed with K2P1, HCN2, or HCN4 channels, although these channels conduct inward cation currents and are gated by different mechanisms. Second, our previous studies show that Kir2.1 channels are required for human iPSC-derived cardiomyocytes to display the phenomenon of the two levels of resting membrane potential in sub-physiological $[K^+]_e$. K2P1 channels were responsible for major inward cation currents that balance with Kir2.1 currents in 75% of Kir2.1-expressing human iPSC-derived cardiomyocytes showing the phenomenon; HCN channels contributed to inward cation currents in the remaining 25% of the cardiomyocytes that display two levels of resting membrane potential.

Previously, it was reported that when $[K^+]_e$ is reduced human adult cardiomyocytes depolarize to approximately -35 mV [(4, 23, 34)] rather than the approximately -20 mV, which we observed in Kir2.1-expressing human iPSC-derived cardiomyocytes and CHO cells that express both Kir2.1 and HCN channels. In addition to the difference in experimental conditions, voltage-gated K^+ channels such as I_{Ks} [(5)] and I_{Kr} in human adult cardiomyocytes may influence the third zero-current potential of the N-shaped currents because these channels are activated at potentials above -35 mV.

Finally, we found that CHO cells expressing both Kir2.1 and HCN2 (HCN4 or K2P1) channels, and Kir2.1-expressing human iPSC-derived cardiomyocytes display the two types of two levels of resting membrane potential. The first type of two levels of resting membrane potential recapitulates the classic two stable levels of resting membrane potential observed in cardiac Purkinje fibers and human adult cardiomyocytes [(8, 18, 30)]. In the second population, resting membrane potentials dynamically fluctuate between the two levels with various patterns, similar to the fluctuation of resting membrane potentials observed in macrophages and osteoclasts [(10, 11, 27, 32)] in physiological $[K^+]_e$. Although the I_{K1} background K^+ currents in these native cells are mediated by homomeric and/or heteromeric Kir2.1/Kir2.x channel complexes [(1, 20)], Kir2.1 and I_{K1} conductances share the same inward rectifying $I-V$ relationship. Therefore, we propose that Kir2.1 channels set two levels of resting membrane potential in cardiac Purkinje fibers, human adult cardiomyocytes, macrophages, and osteoclasts through a common mechanism. We hypothesize that Kir2.1 currents counterbalance inward cation currents through various cation channels that are gated by different mechanisms, regardless in physiological or sub-physiological $[K^+]_e$, and set two levels of resting membrane potential in various types of cells.

Ionic mechanisms underlying two levels of resting membrane potential

Previously proposed models of how Kir2.1 channels contribute to the two levels of resting membrane potential assume that Kir2.1 channels close at the depolarized membrane potential because of Kir2 strong inward rectification, so the membrane potential in the absence of Kir2.1 currents depolarizes due to the remaining active inward currents in the cell [(9, 33)], and there must be a net inward current to drive the membrane potential positive at depolarized membrane potential [(8)]. Our study offers insights into the mechanism of how Kir2 and HCN currents balance and set two levels of resting membrane potential. Together with results from our previous work on counterbalance between Kir2.1 and K2P1 currents [(39)], findings in this report supplement conventional assumptions. First, we directly

confirmed that Kir2.1 strong inward rectification is required for generation of two levels of resting membrane potential. Second, we are able to provide fair explanations how the depolarized membrane potential is achieved in order to make Kir2 channels close. When normal $[K^+]_e$ is reduced into sub-physiological levels, resting membrane potential of the cells hyperpolarizes and Kir2.1 outward currents decrease, then Kir2.1 currents re-balance small cation (e.g., HCN2, HCN4) currents in the hyperpolarized membrane potential (around -95 mV in this study) which Kir2.1 channels do not close, producing dynamic changes in $I-V$ relationships including N-shaped one with three zero-current potentials, and causing immediate transitions of resting membrane potential from the hyperpolarization at the first zero-current potential into the depolarization at the third zero-current potential which Kir2.1 channels are close because of complete inward rectification. In fact, at the depolarized membrane potential, cation channels such as HCN channels do not conduct active inward currents either. Second, our studies provide information on inward currents that counterbalance Kir2.1 currents to produce two levels of resting membrane potential. We showed that low $[K^+]_e$ -induced K2P1 leak cation currents or hyperpolarization-activated HCN2 or HCN4 cation currents counterbalanced Kir2.1 currents, reconstituting the phenomenon of the two levels of resting membrane potential. Interestingly, very small HCN2 or HCN4 currents suffice to play such a role, implying that Kir2.1 channels magnify the depolarizing effects of small inward currents on resting membrane potential.

Functional roles of Kir2.1 inward rectification in maintenance of resting membrane potential

Kir2.1 currents counterbalance K2P1 or HCN or other opposing cation currents, generating the N-shaped $I-V$ relationships with three zero-current potentials, and setting two levels of resting membrane potential. Two sets of experiments indicate that Kir2.1 inward rectification is essential for both the N-shaped $I-V$ relationships and the two levels of resting membrane potential. Weakly rectifying Kir4.1 or rectification-deficient Kir2.1•E224G K^+ currents counterbalance HCN2 cation currents in CHO cells that express these channels, producing stable whole-cell ramp currents with one zero-current potential and maintaining a stable resting membrane potential. Second, overexpression of Kir2.1•E224G mutant channels in human iPSC-derived cardiomyocytes did not result in N-shaped $I-V$ relationships with three zero-current potential and two levels of resting membrane potential; these cells had one stable hyperpolarized resting membrane potential. In contrast, human iPSC-derived cardiomyocytes engineered to express Kir2 exhibited the N-shaped $I-V$ relationships and two levels of resting membrane potential. Therefore, Kir2.1 channels use strong inward rectification to set two levels of resting membrane potential.

Physiological implications

Data from this study indicate that the balance between Kir2 and HCN2 or HCN4 currents results in two levels of resting membrane potential in sub-physiological $[K^+]_e$, which occurs under severe hypokalemia. Since HCN2 and HCN4 are highly expressed in cardiac Purkinje fibers, this mechanism likely accounts for the phenomenon of the two levels of resting potentials often observed in cardiac Purkinje fibers [(4, 7, 8, 18, 24, 29, 30)]. This work demonstrates how Kir2 and HCN channels affect resting membrane potential and impair

cardiac excitability during severe hypokalemia, and provides insights into pathological mechanisms of hypokalemia-induced cardiac arrhythmia.

Acknowledgments

We thank Steven A. Siegelbaum, Juliane Stieber, and Catherine Proenza for providing HCN plasmids and Stephen J. Tucker for providing rat Kir4.1 plasmids. This work was supported by the National Institute of General Medical Sciences, NIH, (R01GM102943), and the National Natural Science Foundation of China (81370296, 81370297, and 81570303).

Abbreviations

HCN	hyperpolarization-activated cyclic nucleotide-gated cation channel
iPSC	induced pluripotent stem cells
I-V	current-voltage
Kir2.1	inward rectifier K ⁺ channel subfamily 2 isoform 1
K2P1	two pore-domain K ⁺ channel isoform 1

References

1. Anumonwo JM, and Lopatin AN. Cardiac strong inward rectifier potassium channels. *J Mol Cell Cardiol* 48: 45–54, 2010. [PubMed: 19703462]
2. Biel M, Wahl-Schott C, Michalakakis S, and Zong X. Hyperpolarization-activated cation channels: from genes to function. *Physiol Rev* 89: 847–885, 2009. [PubMed: 19584315]
3. Cannon SC. Channelopathies of skeletal muscle excitability. *Comprehensive Physiology* 5: 761–790, 2015. [PubMed: 25880512]
4. Christe G. Effects of low [K⁺]_o on the electrical activity of human cardiac ventricular and Purkinje cells. *Cardiovasc Res* 17: 243–250, 1982.
5. Cui J. Voltage-Dependent Gating: Novel Insights from KCNQ1 Channels. *Biophys J* 110: 14–25, 2016. [PubMed: 26745405]
6. Doss MX, Di Diego JM, Goodrow RJ, Wu Y, Cordeiro JM, Nesterenko VV, Barajas-Martinez H, Hu D, Urrutia J, Desai M, Treat JA, Sachinidis A, and Antzelevitch C. Maximum diastolic potential of human induced pluripotent stem cell-derived cardiomyocytes depends critically on I(Kr). *PloS one* 7: e40288, 2012. [PubMed: 22815737]
7. Ellis D. The effects of external cations and ouabain on the intracellular sodium activity of sheep heart Purkinje fibres. *J Physiol* 273: 211–240, 1977. [PubMed: 599421]
8. Gadsby DC, and Cranefield PF. Two levels of resting potential in cardiac Purkinje fibers. *J Gen Physiol* 70: 725–746, 1977. [PubMed: 591921]
9. Gallaher J, Bier M, and van Heukelom JS. First order phase transition and hysteresis in a cell's maintenance of the membrane potential--An essential role for the inward potassium rectifiers. *Bio Systems* 101: 149–155, 2010.
10. Gallin EK. Voltage clamp studies in macrophages from mouse spleen cultures. *Science* 214: 458–460, 1981. [PubMed: 7291986]
11. Gallin EK, and Livengood DR. Inward rectification in mouse macrophages: evidence for a negative resistance region. *Am J Physiol* 241: C9–17, 1981. [PubMed: 7246764]
12. Geukes Foppen RJ, van Mil HG, and van Heukelom JS. Effects of chloride transport on bistable behaviour of the membrane potential in mouse skeletal muscle. *J Physiol* 542: 181–191, 2002. [PubMed: 12096060]
13. Herrmann S, Hofmann F, Stieber J, and Ludwig A. HCN channels in the heart: lessons from mouse mutants. *Br J Pharmacol* 166: 501–509, 2012. [PubMed: 22141457]

14. Hibino H, Inanobe A, Furutani K, Murakami S, Findlay I, and Kurachi Y. Inwardly rectifying potassium channels: their structure, function, and physiological roles. *Physiol Rev* 90: 291–366, 2010. [PubMed: 20086079]
15. Hoekstra M, Mummery CL, Wilde AA, Bezzina CR, and Verkerk AO. Induced pluripotent stem cell derived cardiomyocytes as models for cardiac arrhythmias. *Frontiers in physiology* 3: 346, 2012. [PubMed: 23015789]
16. Jurkat-Rott K, Weber MA, Fauler M, Guo XH, Holzherr BD, Paczulla A, Nordsborg N, Joechle W, and Lehmann-Horn F. K⁺-dependent paradoxical membrane depolarization and Na⁺ overload, major and reversible contributors to weakness by ion channel leaks. *Proc Natl Acad Sci U S A* 106: 4036–4041, 2009. [PubMed: 19225109]
17. Kubo Y, and Murata Y. Control of rectification and permeation by two distinct sites after the second transmembrane region in Kir2.1 K⁺ channel. *J Physiol* 531: 645–660, 2001. [PubMed: 11251047]
18. Lee CO, and Fozzard HA. Membrane permeability during low potassium depolarization in sheep cardiac Purkinje fibers. *Am J Physiol* 237: C156–165, 1979. [PubMed: 474744]
19. Lieu DK, Fu JD, Chiamvimonvat N, Tung KC, McNerney GP, Huser T, Keller G, Kong CW, and Li RA. Mechanism-based facilitated maturation of human pluripotent stem cell-derived cardiomyocytes. *Circ Arrhythm Electrophysiol* 6: 191–201, 2013. [PubMed: 23392582]
20. Lopatin AN, and Nichols CG. Inward rectifiers in the heart: an update on I(K1). *J Mol Cell Cardiol* 33: 625–638, 2001. [PubMed: 11273717]
21. Ma J, Guo L, Fiene SJ, Anson BD, Thomson JA, Kamp TJ, Kolaja KL, Swanson BJ, and January CT. High purity human-induced pluripotent stem cell-derived cardiomyocytes: electrophysiological properties of action potentials and ionic currents. *Am J Physiol Heart Circ Physiol* 301: H2006–2017, 2011. [PubMed: 21890694]
22. Ma L, Zhang X, and Chen H. TWIK-1 two-pore domain potassium channels change ion selectivity and conduct inward leak sodium currents in hypokalemia. *Science signaling* 4: ra37, 2011. [PubMed: 21653227]
23. McCullough JR, Chua WT, Rasmussen HH, Ten Eick RE, and Singer DH. Two stable levels of diastolic potential at physiological K⁺ concentrations in human ventricular myocardial cells. *Circ Res* 66: 191–201, 1990. [PubMed: 2295138]
24. Miura DS, Hoffman BF, and Rosen MR. The effect of extracellular potassium on the intracellular potassium ion activity and transmembrane potentials of beating canine cardiac Purkinje fibers. *J Gen Physiol* 69: 463–474, 1977. [PubMed: 853287]
25. Proenza C, and Yellen G. Distinct populations of HCN pacemaker channels produce voltage-dependent and voltage-independent currents. *J Gen Physiol* 127: 183–190, 2006. [PubMed: 16446506]
26. Rastegar A, and Soleimani M. Hypokalaemia and hyperkalaemia. *Postgrad Med J* 77: 759–764, 2001. [PubMed: 11723313]
27. Ravesloot JH, Ypey DL, Vrijheid-Lammers T, and Nijweide PJ. Voltage-activated K⁺ conductances in freshly isolated embryonic chicken osteoclasts. *Proc Natl Acad Sci U S A* 86: 6821–6825, 1989. [PubMed: 2549551]
28. Roubille F, and Tardif JC. New therapeutic targets in cardiology: heart failure and arrhythmia: HCN channels. *Circulation* 127: 1986–1996, 2013. [PubMed: 23671179]
29. Shah AK, Cohen IS, and Datyner NB. Background K⁺ current in isolated canine cardiac Purkinje myocytes. *Biophys J* 52: 519–525, 1987. [PubMed: 2445390]
30. Sheu SS, Korth M, Lathrop DA, and Fozzard HA. Intra- and extracellular K⁺ and Na⁺ activities and resting membrane potential in sheep cardiac purkinje strands. *Circ Res* 47: 692–700, 1980. [PubMed: 7418129]
31. Siegenbeek van Heukelom J. Role of the anomalous rectifier in determining membrane potentials of mouse muscle fibres at low extracellular K⁺. *J Physiol* 434: 549–560, 1991. [PubMed: 2023129]
32. Sims SM, and Dixon SJ. Inwardly rectifying K⁺ current in osteoclasts. *Am J Physiol* 256: C1277–1282, 1989. [PubMed: 2735399]

33. Struyk AF, and Cannon SC. Paradoxical depolarization of BA2+- treated muscle exposed to low extracellular K+: insights into resting potential abnormalities in hypokalemic paralysis. *Muscle Nerve* 37: 326–337, 2008. [PubMed: 18041053]
34. Ten Eick RE, and Singer DH. Electrophysiological properties of diseased human atrium. I. Low diastolic potential and altered cellular response to potassium. *Circ Res* 44: 545–557, 1979. [PubMed: 428050]
35. Wiggins JR, and Cranefield PF. Two levels of resting potential in canine cardiac Purkinje fibers exposed to sodium-free solutions. *Circ Res* 39: 466–474, 1976. [PubMed: 183909]
36. Yang J, Jan YN, and Jan LY. Control of rectification and permeation by residues in two distinct domains in an inward rectifier K+ channel. *Neuron* 14: 1047–1054, 1995. [PubMed: 7748552]
37. Zaza A. Serum potassium and arrhythmias. *Europace* 11: 421–422, 2009. [PubMed: 19182234]
38. Zhou M, Xu G, Xie M, Zhang X, Schools GP, Ma L, Kimelberg HK, and Chen H. TWIK-1 and TREK-1 are potassium channels contributing significantly to astrocyte passive conductance in rat hippocampal slices. *J Neurosci* 29: 8551–8564, 2009. [PubMed: 19571146]
39. Zuo D, Chen K, Zhou M, Liu Z, and Chen H. Kir2.1 and K2P1 channels reconstitute two levels of resting membrane potential in cardiomyocytes. *J Physiol* 595: 5129–5142, 2017. [PubMed: 28543529]

Figure 1. Kir2.1, HCN2, or HCN4 channels maintain resting membrane potential comparable to their reversal potentials in 5 and 2 mM $[K^+]_e$ when heterologously expressed in CHO cells.

(A–C) Resting membrane potentials (RMP, top) and whole-cell ramp currents (middle) of CHO cells that express human (A) Kir2.1, (B) HCN2, or (C) HCN4 channels are shown before and after Na^+ -based bath solutions were changed from 5 (bold black lines) to 2 (bold pink lines) mM K^+ . Top row: Membrane potentials monitored continuously during changes in $[K^+]_e$; time scale, 100 s. Middle row: Whole-cell ramp currents recorded in 5 (black lines) and 2 mM (pink lines) $[K^+]_e$; HCN2 and HCN4 channels had reversal potentials of -26.4 ± 1.4 mV ($n=5$) and -21.5 ± 0.7 mV ($n=9$) in 5 mM $[K^+]_e$, respectively, and -29.5 ± 1.5 mV ($n=5$) and -25.6 ± 0.4 mV ($n=9$) in 2 mM $[K^+]_e$, respectively. Bottom row: Resting membrane potentials (filled bars) of CHO cells that express Kir2.1 or HCN2 or HCN4 channels and reversal potentials (open bars) of these channels in 5 mM and 2 mM $[K^+]_e$ ($n=5-9$).

Figure 2. Kir2.1 and HCN2 currents counterbalance, generating N-shaped I - V relationships and two levels of resting membrane potential in transfected CHO cells in 2 mM $[K^+]_e$.

(A-C) Resting membrane potentials and whole-cell ramp currents of three populations of CHO cells that express both human Kir2.1 and HCN2 channels before and after $[K^+]_e$ was changed from 5 mM to 2 mM: (A) 35% of the cells remain on hyperpolarization in 2 mM $[K^+]_e$, (B) 32% of the cells spontaneously jump into permanent depolarization, and (C) 33% of the cells fluctuated between the two levels.

Top row: Resting membrane potentials. Blue numbers indicate phases 1 and 2 of changes in resting membrane potential. Time scale, 100 s. *Insert* in B, blockade of HCN2 channels with 30 μ M ivabradine eliminated the classical two levels of resting membrane potential; Resting membrane potentials were recorded when bath solutions were first changed from 5 to 2 mM $[K^+]_e$, then reversibly applied for 30 μ M ivabradine (teal bar), and eventually returned to 5 mM $[K^+]_e$.

Second row: (A) Whole-cell ramp currents in 5 (black line) and 2 mM (pink line) $[K^+]_e$; (B-C) Pink lines indicate 'a' type N-shaped ramp currents with three zero-current potentials in 2 mM $[K^+]_e$, blue lines indicate 'b' type ramp currents with a reversal potential matching the third zero-current potential of 'a' type N-shaped ramp currents, and green line indicates 'c' type ramp currents with a reversal potential comparable to the first zero-current potential. Often "a", "b", and/or "c" types of currents were recorded in the same cells during recordings of whole-cell ramp currents each 15 s. Dash purple line indicates a linear combination of Kir2.1 and K2P1 whole-cell ramp currents that were individually recorded in 2 mM $[K^+]_e$.

Third row: Pink and green lines indicate whole-cell currents before application of channel blockers; purple and orange lines indicate the currents after sequential application of 0.5 mM Ba²⁺ or both 0.5 mM Ba²⁺ and 2 mM Cs⁺ in 2 mM [K⁺]_e, respectively. Ba²⁺ completely blocks Kir2.1 currents, and Cs⁺ inhibits HCN2 currents. The HCN2 currents at -100 mV were -29.2 ± 19.9 pA (n=5) for **A**, -111.0 ± 42.1 pA (n=3) for **B**, and -95.1 ± 15.9 pA (n=6) for **C**.

Bottom row: (**A**) Resting membrane potentials (filled black and pink bars) of the cells and reversal potentials (open black and pink bars) of whole-cell ramp currents in 5 mM and 2 mM [K⁺]_e; (**B-C**) Two levels of resting membrane potential (patterned black bars) of the cells, the first and third zero-current potentials (patterned pink bars) of 'a' type N-shaped ramp currents, and reversal potentials of 'b' (open blue bars) and 'c' (open green bar) types of ramp currents in 2 mM [K⁺]_e (n=5-65).

Figure 3. Kir2.1 and HCN4 channels reconstitute two levels of resting membrane potential in CHO cells that express both channels in 2 mM $[K^+]_e$.

(A-C) Resting membrane potentials and whole-cell ramp currents of three populations of CHO cells that expressed both human Kir2.1 and HCN4 channels before and after reversible changes of $[K^+]_e$ from 5 mM to 2 mM.

Top row: (A) 50% of the cells remained on hyperpolarization in 2 mM $[K^+]_e$, (B) 21% of the cells spontaneously jumped into permanent depolarization, and (C) 30% of the cells fluctuated between two levels. Blue numbers indicate phases 1 and 2 of changes in resting membrane potential. Time scale, 100 s.

Second row: (A) Whole-cell ramp currents in 5 mM (black) and 2 mM (pink) $[K^+]_e$; (B-C) Pink, blue, and green lines indicate “a”, “b”, and “c” types of whole-cell ramp currents that were described in Fig. 2. The first and third zero-current potentials of “a” type currents and reversal potential of “b” type currents were -82.9 ± 3.8 mV ($n=5$), -22.2 ± 4.6 mV ($n=5$), and -19.3 ± 3.1 mV ($n=7$) for B and -88.9 ± 1.4 mV ($n=15$), -21.9 ± 1.9 mV ($n=15$), and -24.3 ± 2.3 mV ($n=4$) for C. Reversal potential of “c” type currents was -93.5 ± 1.3 mV ($n=10$).

Third row: Purple and orange lines indicate the currents after sequential application of 0.5 mM Ba^{2+} and both 0.5 mM Ba^{2+} and 2 mM Cs^+ in 2 mM $[K^+]_e$, respectively. The HCN2 currents at -100 mV were -20.2 ± 13.4 pA ($n=5$) for A, -35.6 ± 18.5 pA ($n=3$) for B, and -42.1 ± 7.8 pA ($n=9$) for C.

Bottom row: (A) Resting membrane potentials (filled black and pink bars) of the cells and reversal potentials (open black and pink bars) of whole-cell ramp currents in 5 mM and 2 mM $[K^+]_e$; (B-C) Two levels of resting membrane potential (patterned black bars), the first

and third zero-current potentials (patterned pink bars) of 'a' type N-shaped ramp currents, and reversal potentials of 'b' (open blue bars) and 'c' (open green bar) types of ramp currents in 2 mM $[K^+]_e$ (n=4–30).

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Figure 4. HCN channels contribute to two levels of resting membrane potential in K2P1-deficient, Kir2.1-expressing human iPSC-derived cardiomyocytes in 2 mM [K⁺]_e.

(A-B) Resting membrane potentials of two populations of K2P1-deficient, Kir2.1-expressing human iPSC-derived cardiomyocytes before and after [K⁺]_e was reversibly changed from 5.4 mM to 2 mM.

(C) Three types of whole-cell ramp currents recorded in 2 mM [K⁺]_e in the human iPSC-derived cardiomyocytes in A and/or B.

(D) Percentage of Kir2.1-expressing human iPSC-derived cardiomyocytes treated with control shRNA (filled black bar) or K2P1-targeted shRNA#1 (filled pink bar) that had two levels of resting membrane potential in 2 mM [K⁺]_e; K2P1-like whole-cell inward leak Na⁺ currents recorded in 0 mM [K⁺]_e in Kir2.1-expressing human iPSC-derived cardiomyocytes without (open black bar) and with (open pink bar) treatment with K2P1-targeted shRNA#1, which exhibited the phenomenon (* P = 0.0001, relative to control). (E) Resting membrane potential of K2P1-deficient, Kir2.1-expressing human iPSC-derived cardiomyocytes

recorded when Na^+ -based bath solutions were changed from 5.4 mM to 2 mM K^+ , 30 μM ivabradine was reversibly applied (bold teal line), and solution was returned to 5.4 mM K^+ . (F) Whole-cell ramp currents of K2P1-deficient, Kir2.1-expressing human iPSC-derived cardiomyocytes that show fluctuating resting membrane potentials in 2 mM $[\text{K}^+]_o$ before (pink line, “b” type current) and after sequential application of 0.5 mM Ba^{2+} (purple line) and both 0.5 mM Ba^{2+} and 30 μM ivabradine (teal line) (n=8–35).

Figure 5. Kir4.1 or Kir2.1•E224G channels maintain one stable level of resting membrane potential when co-expressed with HCN2 channels in CHO cells. (A–B) Resting membrane potentials (top) and whole-cell ramp currents (bottom) of CHO cells that express (A) rat Kir4.1 or (B) human Kir2.1•E224G mutant channels before and after $[K^+]_e$ was changed from 5 mM to 2 mM. Kir4.1 reversal potentials shifted from -74.2 ± 1.0 mV to -97.4 ± 0.7 mV ($n=5$), whereas reversal potentials of Kir2.1•E224G channels shifted from -72.7 ± 1.0 mV to -95.8 ± 0.7 mV ($n=6$). (C–D) Resting membrane potentials (top) and whole-cell ramp currents (middle) of CHO cells that express (C) both Kir4.1 and HCN2 channels or (D) both Kir2.1•E224G and HCN2 channels before and after $[K^+]_e$ was decreased from 5 mM to 2 mM. Time scale, 100 s. Middle row: Black and pink lines indicate whole-cell ramp currents in 5 and 2 mM $[K^+]_e$, respectively; purple and orange lines indicate the currents after sequential application of 0.5 mM Ba^{2+} and both 0.5 mM Ba^{2+} and 2 mM Cs^+ in 2 mM $[K^+]_e$, respectively; *inserts*, Magnifications of ramp currents between -150 pA to 100 pA, illustrating clearly small HCN currents. Bottom row: Resting membrane potentials (filled bars) of the cells and reversal potentials (open bars) of whole-cell currents recorded in 5 and 2 mM $[K^+]_e$ ($n=4-17$).

Figure 6. Kir2.1•E224G channels maintain one stable level of resting membrane potential in human iPSC-derived cardiomyocytes. (A-B) Resting membrane potentials (top and/or middle) and whole-cell ramp currents (bottom) of human iPSC-derived cardiomyocytes that over-express (A) Kir2.1 or (B) inward rectification-deficient Kir2.1•E224G mutant channels before and after [K⁺]_e was reversibly changed from 5.4 mM to 2 mM. Blue numbers indicate phases 1 and 2 of changes in resting membrane potential. Time scale, 100 s. Black line indicates whole-cell ramp currents recorded in 5.4 mM [K⁺]_e, and pink lines indicate currents recorded in 2 mM [K⁺]_e. Blue and green lines indicate “b” and “c” types of currents, respectively, whereas pink line indicates “a” type N-shaped current with three zero-current potentials. (C) The percentages of human iPSC-derived cardiomyocytes that express Kir2.1 (black bar) or Kir2.1•E224G (purple bar), which show two levels of resting membrane potential in 2 mM [K⁺]_e (* P < 0.001, n = 53 to 160 cells).