K⁺ Channel Regulator KCR1 Suppresses Heart Rhythm by Modulating the Pacemaker Current If

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Hyperpolarization-activated, cyclic nucleotide sensitive (HCN) channels underlie the pacemaker current If, which plays an essential role in spontaneous cardiac activity. HCN channel subunits (HCN1-4) are believed to be modulated by additional regulatory proteins, which still have to be identified. Using biochemistry, molecular biology and electrophysiology methods we demonstrate a protein-protein interaction between HCN2 and the K⁺ channel regulator protein 1, named KCR1. In coimmunoprecipitation experiments we show that KCR1 and HCN2 proteins are able to associate. Heterologously expressed HCN2 whole-cell current density was significantly decreased by KCR1. KCR1 profoundly suppressed IhCN2 single-channel activity, indicating a functional interaction between KCR1 and the HCN2 channel subunit. Endogenous KCR1 expression could be detected in adult and neonatal rat ventriculocytes. Adenoviral-mediated overexpression of KCR1 in rat cardiomyocytes (i) reduced If whole-cell currents, (ii) suppressed most single-channel gating parameters, (iii) altered the activation kinetics, (iv) suppressed spontaneous action potential activity, and (v) the beating rate. More importantly, siRNA-based knock-down of endogenous KCR1 increased the native If current size and single-channel activity and accelerated spontaneous beating rate, supporting an inhibitory action of endogenous KCR1 on native If. Our observations demonstrate for the first time that KCR1 modulates IHCN2/If channel gating and indicate that KCR1 serves as a regulator of cardiac automaticity.

INTRODUCTION

Hyperpolarization-activated cation channels are found in a variety of cardiac cells and neurons [1–3]. These channels activate in response to hyperpolarization to generate an inward current termed If ("funny") in cardiac cells, Ih ("hyperpolarization"-activated) in neurons, or Iq ("queer"). If has been proposed to give rise to a hyperpolarization-activated inward current with similar but not identical characteristics compared to native If [8,9,12]. These observations suggest that HCN channel function is likely to be modulated by regulatory proteins and β-subunits in myocardial tissue.

The K⁺ channel regulator 1 (KCR1), originally cloned from rat cerebellum, is a plasma membrane-associated protein with 12 putative transmembrane regions which is also expressed in rat cerebrum, and in rat and human heart [13,14]. The KCR1 protein can associate with rat ether-à-go-go (EAG) and human ether-à-go-go related (HERG) channel subunits [13–15]. Given the structural similarity and sequence analogy of HERG and HCN genes, we speculated that KCR1 might also interact with HCN channel subunits [8,9,12]. Therefore, we evaluated whether KCR1 and HCN2 proteins can associate. Secondly, we aimed to determine any possible functional modulation of IhCN2 and native If current characteristics by KCR1 in electrophysiological studies. Our results show that KCR1 and HCN2 proteins interact and demonstrate that KCR1 profoundly alters IhCN2 and If gating properties. Furthermore, KCR1 suppressed spontaneous rhythmicity in cardiocytes. Thus, our observations indicate that KCR1 serves as a regulatory protein of native If.

RESULTS

KCR1 and HCN2 associate in a protein complex

To determine whether HCN and KCR1 gene products can form a protein complex, we prepared protein extracts from CHO cells cotransfected with KCR1 cDNA incorporating triple FLAG tags at the 5' end (pCFLAG3-KCR1) and HCN2 cDNA. Control cells were transfected with pCFLAG3-KCR1 alone or cotransfected with HCN2 and the empty FLAG-epitope containing vector. Input lysates were assayed in Western blots using an anti-HCN2 antibody to show successful production and detection of the HCN2 protein (Figure 1A). In addition, cell lysates were immunoprecipitated with anti-FLAG-Sepharose and then blotted using the anti-HCN2 antibody. Indeed, a band with the expected molecular mass of HCN2 was detected by the anti-HCN2 antibody in cells cotransfected with HCN2 and pCFLAG3-KCR1.

Citation: Michels G, Er F, Khan IF, Endres-Becker J, Brandt MC, et al (2008) K⁺ Channel Regulator KCR1 Suppresses Heart Rhythm by Modulating the Pacemaker Current If. PLoS ONE 3(1): e1511. doi:10.1371/journal.pone.0001511

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The K⁺ channel regulator 1 (KCR1), originally cloned from rat cerebellum, is a plasma membrane-associated protein with 12 putative transmembrane regions which is also expressed in rat cerebrum, and in rat and human heart [13,14]. The KCR1 protein can associate with rat ether-à-go-go (EAG) and human ether-à-go-go related (HERG) channel subunits [13–15]. Given the structural similarity and sequence analogy of HERG and HCN genes, we speculated that KCR1 might also interact with HCN channel subunits [8,9,12]. Therefore, we evaluated whether KCR1 and HCN2 proteins can associate. Secondly, we aimed to determine any possible functional modulation of IhCN2 and native If current characteristics by KCR1 in electrophysiological studies. Our results show that KCR1 and HCN2 proteins interact and demonstrate that KCR1 profoundly alters IhCN2 and If gating properties. Furthermore, KCR1 suppressed spontaneous rhythmicity in cardiocytes. Thus, our observations indicate that KCR1 serves as a regulatory protein of native If.
Figure 1. Analysis of protein interaction between HCN2 and KCR1 and expression of KCR1 in various cell types. (A) KCR1 and HCN2 can associate in protein complexes. Coimmunoprecipitation of HCN2 and KCR1 from mammalian cell extracts. Protein extracts from cells transfected with pCFLAG3-KCR1 (lane 0), HCN2 plus pCFLAG3-KCR1 (lanes 1), HCN2 plus p3xFLAG-CMV (lanes 2) or non-transfected cells (lanes 3) assayed by Western blot using anti-HCN2 antibody. Input: input lysates were blotted to show that HCN2 protein was successfully produced and detected. Anti-FLAG-Sepharose: 700 μg of total lysate immunoprecipitated with anti-FLAG-Sepharose shows that HCN2 and KCR1 (pCFLAG3-KCR1) can be coimmunoprecipitated (lane 1). A-Sepharose: mock immunoprecipitation using protein A-Sepharose beads unlinked to FLAG-antibody to exclude any unspecific antibody binding. (B) KCR1 message (218 bp) can be detected in all cell types tested, except for non-transfected CHO cells (upper panel). Representative electrophoresis gel illustrating results obtained in single-cell RT-PCR experiments (as described in Methods) with three different cell types. CHO: Non-transfected Chinese hamster ovary cells; CHO+KCR1: CHO cells transfected with KCR1 cDNA; RatNN: Neonatal rat cardiomyocytes; RatA: Adult rat cardiomyocytes. GAPDH mRNA (318 bp) was used as a control and is found in all cell types tested, including non-transfected, KCR1-negative CHO cells (lower panel). Negative controls did not include RNA or reverse transcriptase and gave no amplicons (not shown). (C) Regional differences in expression of KCR1 in adult rat and pig heart. KCR1 mRNA levels determined by quantitative real-time PCR (qPCR; n = 3–4). RA = right atrium, RV = right ventricular free wall, LA = left atrium, LV = left ventricular free wall, Se = septum, SAN = sinoatrial node, AV = atrioventricular node. Values are mean ± SEM. *p < 0.05 vs. AV node, **p < 0.05 vs. SAN. Upper panel: Representative electrophoresis gel obtained from qPCR products of different cardiac regions (the AV-node region was divided in: AVS = superior part and AVI = inferior part), samples were loaded and normalized to GAPDH. doi:10.1371/journal.pone.0001511.g001
KCR1, whereas it could not be coimmunoprecipitated from extracts containing pCFLAG-3-KCR1 alone or HCN2 and the empty FLAG-epitope containing vector (Figure 1A). These results indicate that HCN2 and KCR1 associate in protein complexes in mammalian cells, while excluding any unspecific detection of KCR1 by the anti-HCN2 antibody and any unspecific coimmunoprecipitation of the FLAG-epitope and HCN2. In addition, unspecific coimmunoprecipitation by the anti-FLAG-Sepharose could be excluded by mock immunoprecipitation with normal A-Sepharose (Figure 1A).

**KCR1 reduces HCN2 current size and profoundly modulates HCN2 channel gating**

To evaluate a possible functional interaction of HCN2 and KCR1, the effect of KCR1 on I_{HCN2} was analysed. Whole-cell I_{HCN2} currents were recorded from CHO cells transfected with HCN2 (0.25 μg/well) alone or together with KCR1 (ratio 1:1, 1:2 or 1:3; total cDNA amount adjusted to 1 μg/well with the unrelated channel subunit Kv1.3AYA in all experiments). RT-PCR revealed no detectable KCR1 in non-transfected CHO cells (Figure 1B). Representative current recordings (Figure 2A) and mean current densities (Figure 2B) show that I_{HCN2} (79.6 ± 13.9 pA/pF at -130 mV, n = 9) was significantly decreased by KCR1 (15.8 ± 10.4 pA/pF, ratio 1:1, n = 14; 3.6 ± 2.1 pA/pF, ratio 1:2, n = 10; 2.5 ± 0.9 pA/pF, ratio 1:3, n = 17; p < 0.001). Moreover, KCR1 significantly shifted half-maximal activation of I_{HCN2} (−102.0 ± 2.1 mV) to more negative potentials (−109.8 ± 2.0 mV for HCN2 + KCR1, ratio 1:1; p = 0.046; n = 12).

To further confirm a functional modulation of HCN2 channel properties by KCR1 additional single-channel recordings were performed. Available data about HCN single-channel properties vary depending on cell-type and recording technique [16–20]. While we previously already showed characteristic stimulation of single HCN2 channels by forskolin [16], we now further proved that the channel openings in our experiments were produced by HCN channels demonstrating (i) a time- and voltage-dependent kinetic similar to macroscopic I_{HCN}/If current from HCN2 multi-channel recordings, which allows in contrast to “pure” one-channel recording cooperative HCN channel gating (Figure 3A), (ii) a HCN2 single-channel conductance of these multi-channel patches (24.0 ± 4.62 pS, n = 3) comparable to our cell-attached recordings (considering the symmetrical K+ solution in inside-out recordings) (Figure 3A), (iii) a typical increase of the open probability upon cAMP application in inside-out recordings (control: 24.7 ± 11.1% vs. Br-cAMP 1 mM: 51.0 ± 14.1%, n = 4, p < 0.05) (Figure 3B and 3C), and (iv) a reduction of HCN2 open probability by the specific I_{f} inhibitor ivabradine (50 μM) (25.8 ± 7.07% vs. 6.08 ± 3.45%, n = 4, p < 0.05) (Figure 3B and 3C).

Single-channel characteristics of cells cotransfected with HCN2 and KCR1 were distinct from cells expressing HCN2 alone. KCR1 profoundly reduced single-channel activity of I_{HCN2} (Figure 4A; Table 1). Moreover, KCR1 caused a hyperpolarization shift of the voltage of half-maximal I_{HCN2} activation (V_{0.5} for I_{HCN2} and

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**Figure 2. KCR1 suppresses recombinant I_{HCN2} current density.** (A) Representative HCN2 whole-cell currents in absence and presence of KCR1. (B) Mean current densities of heterologously expressed HCN2 alone or together with KCR1 demonstrate that KCR1 significantly (*, p < 0.001) reduced current density of recombinant I_{HCN2}. For data see text.

doi:10.1371/journal.pone.0001511.g002
I_{HCN2+KCR1(1:2)} was $-58.5 \pm 4.8 \text{ mV}$, $n = 6$, vs. $-89.7 \pm 4.9 \text{ mV}$, $n = 5$, $p = 0.001$, with the slope factor $k$ being unchanged: $-15.8 \pm 6.3 \text{ mV}$ and $-12.4 \pm 2.7 \text{ mV}$, respectively; Figure 4B). We recently revealed an allosteric multi-state gating model comprising at least four open and five closed states of single HCN2 channel recordings [16,21]. In the present study KCR1 altered HCN2 channel open kinetics to only three open states (HCN2-$t_{open}$ values [ms]: 0.13 [47.5%], 0.86 [33.5%], 2.24 [18.5%], 5.93 [0.5%] ($n=8$) vs. HCN2+KCR1 (1:1)-$t_{open}$ values [ms]: 0.13 [54.7%], 0.87 [38.5%], 2.72 [6.8%] ($n=4$) and HCN2+KCR1 (1:2)-$t_{open}$ values [ms]: 0.13 [77%], 0.69 [21.9%], 2.36 [1.1%] ($n=5$)), while not significantly affecting the five closed states (HCN2-$t_{closed}$ values [ms]: 0.15 [55.7%], 0.90 [28.4%], 3.36 [5.5%], 5.52 [4.1%], 21.0 [6.3%] ($n=5$) vs. HCN2+KCR1 (1:1)-$t_{closed}$ values [ms]: 0.12 [64.9%], 0.55 [18.9%], 0.97 [12%], 2.96 [3.8%], 27.0 [0.4%] ($n=4$) and HCN2+KCR1 (1:2)-$t_{closed}$ values [ms]: 0.14 [76.3%], 0.83 [16.8%], 1.78 [3.1%], 7.80 [1.3%], 37.92 [2.5%] ($n=4$) (Figure 4C and 4D).

**KCR1 is highly expressed in the atrioventricular-node**

While functional interaction of HCN and KCR1 gene products in heterologous mammalian systems is interesting, it is even more important whether KCR1 is present in cardiac tissue and might also modulate native $I_f$. To clarify this situation we chose neonatal and adult rat ventriculocytes. To omit any interference of non-cardiac cells, we performed reverse transcriptase PCR analysis of single neonatal and adult rat ventricular myocytes. Indeed, in both cell types the expected band could be detected (Figure 1B), demonstrating endogenous expression of KCR1 in native cardiac cells.

To analyze regional expression of KCR1, we determined KCR1 mRNA levels of freshly dissected samples from different parts of adult rat and pig hearts by quantitative real-time PCR. In both species we consistently obtained highest KCR1 expression in the atrioventricular node (rat: 536 $\pm 48\%$, $n = 3$; pig: 637 $\pm 52\%$, $n = 3$; $p < 0.05$ vs. all other regions) and lowest levels of KCR1 in the sinus node (rat: 46.3 $\pm 8.10\%$, $n = 3$; pig: 52.7 $\pm 4.98\%$, $n = 3$; $p < 0.05$ vs. all other regions) (Figure 1C).

**KCR1 overexpression suppresses current density and single-channel activity of native $I_f$**

Considering our results in heterologous expression, overexpression of a construct carrying KCR1 should reduce $I_f$ current density in myocardium if HCN and KCR1 also interact in native tissue. In neonatal ventriculocytes $I_f$ density was indeed markedly reduced from 4.7 $\pm 0.3$ pA/pF ($n=14$) in control cells infected with...
adenoviral vectors expressing EGFP alone to 0.96 ± 0.1 pA/pF (n = 9) in KCR1-infected cells when measured at -130 mV (p<0.001) (Figure 5A, 5B and 5D). Similarly, I_f current size in adult rat ventricular myocytes was significantly suppressed from 3.36 ± 0.5 pA/pF (n = 11) in controls to 0.38 ± 0.08 pA/pF (at -130 mV; n = 10) in KCR1-infected myocytes (p<0.001). Direct modulation of I_f by KCR1 overexpression was further analysed in single-channel recordings. Notably, single-channel properties of native I_f in control cells more closely resembled those of heterologously expressed HCN2+KCR1 than of IHCN2 alone (Figure 6A, Table 2), indicating a possible modulation of HCN subunits by endogenous KCR1 in native cells. KCR1 further suppressed single-channel availability, open probability, and single-channel amplitude and conductance of native I_f (Figure 6A and 7A; Table 2), and shifted the voltage of half-maximal activation to more negative values (adult ventriculocytes: from -66.3 ± 4.3 mV, n = 5, to -95.8 ± 3.8 mV, n = 7; p<0.001, Figure 6B; neonatal ventriculocytes: from -56.4 ± 1.3 mV, n = 12, to -81.3 ± 3.2 mV, n = 10; p<0.0001, Figure 7B), without significantly affecting the slope factor k for adult (control: -11.9 ± 2.1 mV, KCR1: -17.8 ± 3.1 mV), and neonatal ventriculocytes (control: -12.1 ± 3.9 mV, KCR1: -16.7 ± 4.8 mV). These data indicate that KCR1 modulates single-channel behavior of I_f in cardiac tissue.

While KCR1 reduced the number of HCN2 open states from four to three in the heterologous expression system (CHO cells), in which no endogenous KCR1 was detectable, KCR1 had no effect on gating kinetics of native I_f in cardiomyocytes. In neonatal ventriculocytes we detected only three open and three closed states (I_f-tpopen values [ms]: 0.26 [58.9%], 0.87 [32.8%], 4.54 [8.3%] (n = 12) vs. I_f+KCR1-tpopen values [ms]: 0.25 [47.8%], 1.22 [43.1%], 5.56 [9.1%] (n = 10); I_f-tclosed values [ms]: 0.18 [57.5%], 1.57 [33.6%], 12.7 [8.9%] (n = 5) vs. I_f+KCR1-tclosed values [ms]: 0.15 [45.1%], 1.13 [37.3%], 16.5 [17.6%] (n = 6), Figure 7C and 7D), whereas in adult ventriculocytes we observed two more closed state (I_f-tpopen values [ms]: 0.23, 0.94, 4.64 (n = 7) vs. I_f+KCR1-tpopen values [ms]: 0.18, 0.99, 4.59 (n = 6); I_f-tclosed values [ms]: 0.16 [60.2%], 0.99 [4.7%], 12.1 [17.6%], 5.47

Figure 4. Effect of KCR1 on single recombinant HCN2 channel gating in one-channel patches. (A) Comparison of single recombinant HCN2 channels transfected in CHO cells alone (left) and with KCR1 (right). Middle, 20 consecutive single traces of each channel without and with KCR1. Single channels were hyperpolarized at continuous pulse mode for a total duration of 3 s (20 x 150 ms sweeps), with a holding potential of -35 mV and a test potential of -35 mV. Bottom, ensemble average current of one consecutive sweep of 3 s pulse duration. Scale bars, 50 ms, 6 pA (unitary current traces) for HCN2 alone (left) and 2 pA when co-transfected with KCR1 (right), or 1 s, 0.5 pA (ensemble average current) for HCN2 and HCN2+KCR1. (B) KCR1 significantly shifted IHCN2 activation to more negative potentials. Channel activation was measured by the parameter availability, plotted against the test potential and then determined by using the Boltzmann function. For data see text. (C) Open-time histograms: KCR1 reduced the number of HCN2 open states. Number of open events (square root) were plotted against the logistically binned open time durations for HCN2 alone and HCN2+KCR1 (pooled one- and multi-channel experiments). (D) Closed-time histograms: KCR1 did not affect the number of HCN2 closed states. Number of closed events (square root) were plotted against the logistically binned closed time durations for HCN2 alone and HCN2+KCR1 (pooled one-channel experiments only). doi:10.1371/journal.pone.0001511.g004

PLoS ONE | www.plosone.org 5 January 2008 | Issue 1 | e1511
Suppression of endogenous KCR1 increases $I_f$ current density, single-channel activity of native $I_f$ and spontaneous beating rate

While alteration of $I_f$ by KCR1 overexpression already indicated a direct interaction of HCN channels and KCR1 in native tissue, we further confirmed this finding by suppression of endogenous KCR1 using siRNA. Quantitative real-time PCR verified specific KCR1 knock-down by our siRNA in transfected CHO cells (normalized KCR1 mRNA: KCR1 100±0.09%; KCR1+pENTR/U6-KCR1siRNA 34.8±0.03%; KCR1+pENTR/U6-LacZsiRNA 91.1±0.08%) and neonatal cardiomyocytes (normalized KCR1 mRNA: control 100±0.07%; KCR1+pENTR/U6-KCR1siRNA 51.6±0.09%; KCR1+pENTR/U6-LacZsiRNA 96.5±0.1%). To evaluate the functional modulation of KCR1 knock-down we chose neonatal myocytes, because (i) these cells enable both analysis of KCR1-mediated effects on $I_f$ current and spontaneous beating activity, and (ii) adult cardiomyocytes exhibit a high rate of dedifferentiation during culture for >48 h [22].

Compared to control conditions suppression of endogenous KCR1 significantly increased whole-cell $I_f$ current density (6.09±0.6 pA/pF n = 11; p = 0.04 vs. control, Figure 5A, 5C and 5D). Moreover, KCR1 knock-down markedly increased the single-channel activity and altered the gating behavior of native $I_f$ (Figure 7, Table 2). In contrast to KCR1 overexpression, KCR1 knock-down shifted the voltage of the half-maximal activation to more positive values (−44.5±0.9 mV, n = 13; p < 0.05 vs. control; slope factor $k$ = −8.61±3.8 mV; p = n.s vs. control, Figure 7B). Consistent with our observations in experiments with enhanced and heterologous KCR1 expression, analyses of the multi-state gating revealed the

| Parameter          | HCN2 control | HCN2+KCR1 (1:1) | HCN2+KCR1 (1:2) |
|--------------------|--------------|----------------|-----------------|
| Open probability (%) | 32.6±8.79    | 23.4±13.14     | 0.99±0.32*      |
| Availability (%)   | 78.5±6.10    | 54.6±20.48     | 22.9±4.01*      |
| Mean open time (ms) | 1.15±0.15    | 0.76±0.16      | 0.44±0.06*      |
| Mean closed time (ms) | 2.94±0.45    | 1.23±0.44*     | 2.78±0.61       |
| Mean first latency (ms) | 37.2±6.27    | 38.0±17.14     | 42.7±5.94       |
| Amplitude (pA)     | −2.16±0.15   | −0.86±0.06*    | −0.68±0.01*     |
| Conductance (pS)   | 34.6±2.43    | 12.3±1.44*     | 8.39±0.63*      |
| $I_{peak}$ (pA)    | 809±170      | 398±204        | 43±14*          |
| number of experiments | 10 (6)       | 4 (4)          | 9 (4)           |

Single-channel parameters of HCN2 (control) and KCR1-cotransfected HCN2 channels in CHO-cells. Holding potential −35 mV, test potential −90 mV. $I_{peak}$ was measured from ensemble average currents. For closed time and latency analysis, only experiments containing just one detected open level were used for calculation. Numbers of experiments given in parentheses indicate number of experiments with only one channel in the patch. Pooled data are presented as mean±SEM. *p<0.05 vs. control.

1) In this case n = 12 experiments were taken for conductance calculation.

doi:10.1371/journal.pone.0001511.t001

| Figure 5. KCR1 reduces current size of native $I_f$ in neonatal rat cardiomyocytes. (A–C) Original whole-cell recordings of $I_f$ in neonatal rat cardiocytes: (A) control, (B) KCR1-infected and (C) KCR1siRNA-infected. (D) Mean current densities of $I_f$ in neonatal cells show that KCR1 overexpression reduced $I_f$ while suppression of endogenous KCR1 by KCR1siRNA significantly increased native $I_f$ (*, p<0.001). For data see text. doi:10.1371/journal.pone.0001511.g005 |
opposite effect by KCR1 knock-down on native If, i.e. an increase in the number of open states and a loss of one closed-state \( t_{\text{open}} \) values of If-KCR1siRNA [ms]: 0.26 [46.4%], 1.19 [10.0%], 1.26 [25.7%], 3.21 [17.9%] (n = 9); \( t_{\text{closed}} \) values of If-KCR1siRNA [ms]: 0.10 [79.9%], 8.38 [20.1%] (n = 5); Figure 7C and 7D).

To evaluate a possible functional modulation of KCR1 in spontaneously active tissue action potential recordings were performed in spontaneously beating neonatal cardiocytes. Control cultures beat spontaneously with a mean rate of 80.4 ± 6.5 bpm (n = 17). Cycle length tended to vary from beat to beat (Figure 8A). Maximal diastolic potential was 256.4 ± 3.5 mV. KCR1 overexpression entirely suppressed beating activity in neonatal cardiocytes (n = 20; maximal diastolic potential 258.6 ± 2.7 mV; p = n.s. vs. control). Figure 8B illustrates a representative recording of an action potential that was induced artificially by a short depolarizing current pulse in a KCR1-infected myocyte. Conversely, knock-down of endogenous KCR1 by siRNA resulted in an acceleration of the beating rate to 100.2 ± 4.6 bpm (n = 17; p<0.005 vs. control; maximal diastolic potential −55.8±3.3 mV; p = n.s. vs. controls, Figure 8C and 8D). This obvious inhibitory effect of KCR1 on action potential frequency further supports the critical contribution of If to spontaneous beating activity of neonatal cardiocytes [4] and the modulating effect of KCR1 on native If and automaticity.

DISCUSSION
In addition to the pore forming \( \alpha \)-subunit Na\(^+\), Ca\(^{2+}\) and K\(^+\) channels incorporate modulatory \( \beta \)-subunits, as well as scaffolding proteins, chaperones, cytoskeletal elements, and Ca\(^{2+}\)-sensing proteins in their higher order structures [23–25]. KCR1 is a membrane-associated protein with 12 putative transmembrane regions [13,14]. In the present study KCR1 expression was demonstrated in neonatal and adult ventriculocytes. By coimmunoprecipitation KCR1 was shown to form protein complexes with HCN2. KCR1 markedly altered If, and suppressed spontaneous action potential activity, indicating a functional interaction with HCN channels. Heterologously expressed HCN channels result in hyperpolarization-activated inward currents with similar, however, not identical properties compared to native If [8,9,12]. These observations suggest that HCN subunits are modulated by...
and Mint2 scaffold proteins [31] were reported to affect HCN containing Rab8b interacting protein [30], tamalin, S-SCAM distribution on the cell membrane [29]. Moreover, TPR-A modifies HCN1, but not HCN2 or HCN4 channel activity and cytops. Loss of KCR1 lead to (i) an increase of If current density, (ii) a channel behavior. More importantly, knock-down of endogenous Suppression of recombinant HCN2 single-channel activity by HCN2 proteins associate. It is well known that regulatory proteins additional proteins.

Using immunoprecipitation studies we now show that KCR1 and HCN2 proteins associate. It is well known that regulatory proteins can affect both expression and gating of pore forming β-subunits. Suppression of recombinant HCN2 single-channel activity by KCR1 clearly demonstrated a functional effect of KCR1 on HCN channel behavior. More importantly, knock-down of endogenous KCR1 also profoundly modulated native If function in ventricularcytes. Loss of KCR1 lead to (i) an increase of If current density, (ii) a higher activation of If on single-channel level, and (iii) a significant elevation of the spontaneous beating rate.

Currently, two distinct ranges of single channel conductances for native If and cloned HCN channels have been reported depending on the recording technique [low conductance currents of 1–1.5 pS using a corner frequency of 80–800 Hz [17,18,20] vs. an approximately 10-fold higher conductance using a filtering of 2000 Hz [16,19]], as has been discussed previously [32]. In the present study we further confirmed that current openings in our recordings indeed were produced by HCN channels as we demonstrated (i) a typical increase of channel activity by the application of cAMP to the inner side of inside-out patches, and (ii) a characteristic channel inhibition by the If/HCN specific blocker ivabradine. Moreover, we could clearly show time- and voltage-dependence of HCN single-channel recordings obtained from multi-channel patches which were comparable to macroscopic If/HCN currents. Given the evidence for cooperative gating between single HCN channels [20], expectedly, the sum of recordings from one-channel patches in the present study and in our previous report [16] did not result in average currents with identical kinetics compared to macroscopic If/HCN, emphasizing that for the evaluation of If/HCN averages it is essential to distinguish between recordings obtained from patches with one individual channel vs. multi-channel patches.

While differences of If/IHCN conductance in part might be caused by distinct recording techniques or by the expression of different HCN channel isoforms, it might also indicate HCN modulation by endogenous KCR1 within native channel complexes. Notably, in the present study single-channel parameters of native If more closely resembled those of HCN2+KCR1 than HCN2 alone (Table 1 and 2), like for instance single-channel conductance in ventricularcytes (7–9 pS). A similar single-channel conductance (7–10 pS) has been reported for recordings of If in neurons [19,33], which also express KCR1 [13], supporting the hypothesis of potential If modulation by KCR1 in native tissue. Beside single-channel conductance a possible interaction of endogenous KCR1 and HCN was especially evident for single-channel open kinetics. While four open states could be obtained for HCN2 alone in heterologous expression, KCR1 reduced HCN2 open kinetics to only three states, which was however similar to native If. Consistent with this notion, knock-down of endogenous KCR1 increased kinetics of native If to four open states and increased single-channel conductance. These observations further indicate that KCR1 functionally interacts with native If in cardiac tissue.

Interestingly, KCR1 overexpression shifted the voltage-dependence of If activation to more negative potentials, whereas KCR1 knock-down resulted in a shift of the half-maximal activation to more positive values. Low expression levels of KCR1 in sinus node cells might contribute to larger If current size and more positive voltages of mid-activation of If, whereas higher expression of KCR1 in the atrioventricular node and working myocardium could explain much smaller If currents and the more negative threshold potentials of If activation in these regions. However, it should always be acknowledged that (i) the regional distribution of HCN channel isoforms (e.g. HCN4 as the predominant isoform in sinoatrial node region), (ii) the variety of heteromultimerization, and (iii) the influence of other known/unknown modulating proteins together determine the biophysical behavior of native If. This might explain why single channel conductance of native If in sinoatrial node cells was not reported to be higher than in working myocardium or neurons [16,17,19,33].

Table 2. Single-channel parameters of native If in adult and neonatal cardiomyocytes modulated by KCR1 and KCR1|αRNA

| Parameter | Adult myocyte If | Neonatal myocyte If |
|-----------|-----------------|---------------------|
|           | control         | KCR1-infected       |
| Open probability (%) | 4.10±0.74 | 0.94±0.24* |
| Availability (%) | 45.2±4.08 | 21.8±2.97* |
| Mean open time (ms) | 0.55±0.06 | 0.56±0.05 |
| Mean closed time (ms) | 1.85±0.28 | 2.22±0.10 |
| Mean first latency (ms) | 53.9±6.80 | 62.7±4.35 |
| Amplitude (pA) | −0.85±0.05 | −0.59±0.02* |
| Conductance (pS) | 8.81±0.25 | 6.10±0.26* |
| Ipeak (fA) | 70±9 | 27±2* |
| Availability (%) | 4.10±0.74 | 0.94±0.24* |
| Mean open time (ms) | 0.55±0.06 | 0.56±0.05 |
| Mean closed time (ms) | 1.85±0.28 | 2.22±0.10 |
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| Amplitude (pA) | −0.85±0.05 | −0.59±0.02* |
| Conductance (pS) | 8.81±0.25 | 6.10±0.26* |
| Ipeak (fA) | 70±9 | 27±2* |

*Significant difference vs. control; †Significant difference vs. KCR1-infected

DOI: 10.1371/journal.pone.0001511.t002
alteration of HCN channel subunits as underlying mechanism of KCR1-mediated modulation of If and cardiac automaticity. Further potential KCR1-induced regulatory mechanisms might involve alteration of HCN trafficking and cell-surface stability. Although such effects could not explain the changes of If single-channel gating, they might contribute to the observed KCR1-mediated actions and will be addressed in future studies.

KCR1 significantly suppressed spontaneous beating activity of neonatal cardiocytes, implying that KCR1 contributes to modulation of the spontaneous activity in cardiac cells. Interestingly, both KCNE2 and KCR1 originally were identified as potential regulatory subunits of HERG, the pore forming α-subunit of IKr [14,34]. These and our observations suggest, that both proteins may have a prominent role for the balance of inward (If) and outward (IKr) currents involved in cardiac pacemaker activity.

Given these obvious inhibitory effects of KCR1 on HCN channels and native If, alterations of KCR1 expression might cause changes in heart rate or ectopic spontaneous depolarizations in patients, though our in-vitro observations cannot readily be generalized to humans in-vivo. Interacting proteins like KCR1 inhibiting If and, thus, depressing spontaneous activity may provide a novel therapeutic target of cardiac arrhythmias.

MATERIALS AND METHODS

Plasmids, Small Interfering RNA and Adenovirus Preparation

The expression plasmids pAdCGI-HCN2 encoding the full-length sequence of mHCN2, pCGI-Kv1.3AYA and pAdCGI have been described [4,35]. The full-length coding sequence of rat KCR1 (kindly provided by Dr. H. Higashida, Kanazawa University, Japan) was cloned into the multiple cloning site of pAdCGI, to give pAdCGI-KCR1. The red fluorescent protein coding sequence (Red) of psDRed-Express-1 (Clontech, Palo Alto, CA) was cloned...
into pAdCGI-HCN2 in place of the EGFP sequence, to generate pAdCRI-HCN2. For immunoprecipitation studies, the rat KCR1 sequence was ligated in frame at the N-terminus to the three FLAG epitopes of vector p3xFLAG-CMV7.1 (Sigma GmbH, Munich), resulting in the plasmid pCFLAG3-KCR1.

The small interfering RNA (siRNA) nucleotide sequences for rat KCR1 (Accession no. GI 3513450) and control LacZ were as follows:

5′-GGAAACGTAGAGTTCAGTTCTC-3′
and 5′-CTACACAAATCAGCGATTT-3′ (Invitrogen), respectively. For transient transfection experiments the DNA oligonucleotides were cloned into pENTR/U6 (Invitrogen) to give pENTR/U6-KCR1 siRNA and pENTR/U6-LacZ siRNA, respectively, and for adenovirus generation into pAdHCRed to give pAdHCRed-KCR1 siRNA. Adenovirus vectors were generated by Cre-lox recombination of purified ψ5 viral DNA and shuttle vector DNA as previously described [4,35,36].

**Transient Transfections**

CHO-K1 cells (ATCC, Manassas, VA) were transfected with 1 μg/well plasmid DNA (as indicated) using Lipofectamine Plus (Life Technologies, Gaithersburg, MD) as directed by the manufacturer [37,38]. Three days posttransfection, electrophysiological recordings, RNA isolation and immunoprecipitations from whole cell protein extracts were performed.

**Rat Cardiomyocyte Isolation and Primary Culture**

Animal experiments were performed in accordance with institutional guidelines for animal use in research. Ventricular myocytes of adult rats (Sprague-Dawley, 200 to 300 g) were isolated using Langendorff apparatus as previously described [39,40]. Neonatal cardiomyocytes of Sprague-Dawley (1–2 days old) rats were enzymatically dissociated as previously described [4]. Action potential studies were conducted on 4- to 6-day-old monolayer cultures. For whole-cell patch-clamp experiments, 3- to 5-day-old monolayer cultures were dispersed by trypsin, and re-plated at a low density to study electrically isolated cells within 2 to 8 hours [4].

**Adenovirus Infection**

Infection of adult and neonatal myocytes was performed 2 h and 1–3 days after plating, respectively, at a multiplicity of infection (MOI) of 15 to 100 p.f.u. per cell.

**Immunoprecipitation**

Whole protein extracts were prepared by lysing the cells in washing buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 0.25% Triton X-100, and 5 mM NaF, supplemented with the mammalian protease inhibitors Aprotinin (Sigma). Extracts were then passed through a needle 15 times, followed by high-speed
centrifugation to sediment cellular debris. 700 μg of each extract were immunoprecipitated with protein A-Sepharose-linked anti-FLAG-M2 antibodies (Sigma). A set of identical control experiments was performed using beads not linked to antibody (normal A-Sepharose, Sigma). Immunoprecipitates were washed three times with lysis buffer followed by resuspension in SDS sample buffer (0.2% SDS, pH 6.4, 10% glycerol, 0.5% β-mercaptoethanol, 50 mM DTT), sonication, and electrophoresis on a 7.5% SDS-PAGE. The gel was transferred to Hybond ECL membrane (Amersham) for Western blotting with an anti-HCN2 antibody (Alomone Labs, Israel).

Single-cell RT-PCR analysis

cDNA synthesis and PCR reaction from two to three isolated single cells were performed using the OneStep RT-PCR Kit (QIAGEN, Hilden, Germany) as directed by the manufacturer with sense- and antisense primer specific for KCR1 (sense: 5'-GAG GAG ATC TTC CAC CTA CTA CGG-3'; antisense: 5'-TAG AAG TTGCCA ACA CATG AAG-3'; amplifying 218 base pairs and spanning one intron) and GAPDH (sense: 5'-GGT CGG TGT GAA CGG ATT TG-3'; antisense: 5'-GTT AGC CCC AGC CTT CTC CAT-3'; amplifying 318 base pairs). Amplification was performed in a Thermocycler gradient (Eppendorf, Hamburg, Germany), starting with 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s cycles (Denaturation: 95°C for 30 seconds; Annealing: 60°C for 30 seconds; Elongation: 72°C for 30 seconds) with lysis buffer followed by resuspension in SDS sample buffer (0.23 M Tris, pH 6.4, 10% glycerol, 0.33% SDS, 3.3 mM DTT), sonication, and electrophoresis on a 7.5% SDS-PAGE. The gel was transferred to Hybond ECL membrane (Amersham) for Western blotting with an anti-HCN2 antibody (Alomone Labs, Israel).

Preparation of sinus and atrioventricular node

Fresh hearts from adult rats (Sprague-Dawley, 200 to 300 g) and adult pigs (freshly received from slaughterhouse) were dissected. To locate the sinoatrial node we used three intersection lines: the sulcus terminalis, the lateral border of the superior vena cava, and the superior border of the superior vena cava or the right auricle. The atrioventricular node was prepared according to the anatomic landmarks of the triangle of Koch in the right atrium: the coronary sinus ostium, the membranous septum, and the septal/posterior comissure of the tricuspid valve. Finally the left and right atria, left and right ventricular free wall and ventricular septum were dissected. RNA was directly isolated from these fresh samples.

RNA isolation and Quantitative Real-time PCR

Total RNA was extracted from neonatal rat cardiomyocytes, and from cardiac tissue of adult rat and pig using the RNeasy kit (Qiagen), and first strand cDNA was reverse transcribed with Omniscript Reverse Transcription Kit (Qiagen) as described previously [41]. Quantitative real-time PCR was performed on a LightCyclerTM 2.0 instrument using FastStart DNA Master,Plus SYBR Green I (Roche Diagnostics). Gene-specific primers were the following: KCR1 for rat samples: forward-primer: 5'-TTC AGG AAG ATA CAG CCC AGA-3', backward-primer: 5'-GGG TTG GAA ATA CTA GGA GGG-3'; KCR1 for pig samples: forward-primer: 5'-GAC GAG ATC TTC CAC CTA CTA CGG-3', backward-primer: 5'-TAG AAG TTGCCA ACA CATG AAG-3'. GAPDH [glyceraldehyde-3-phosphate dehydrogenase] and SDHA [succinate dehydrogenase complex, subunit A, flavoprotein] expression of each sample was used as endogenous controls (GAPDH - primers: 5'-GGT CGG TGT GAA CGG ATT TG-3' and 5'-GTT AGC CCC AGC CTT CTC CAT-3'; GenBank accession No. NM_017008; SDHA-primers: 5'-TGG GAA GAA GAG GCC ATC TG-3' and 5'-CCA CCA CTG CAT AAATT CAT-3', GenBank accession No. NM_130428) [42]. Quantitative real-time PCR was carried out under the following cycling conditions: stage 1, 95°C for 10 minutes (rep 1); stage 2, 95°C for 10 s, 61°C for 3 s, and 72°C for 10 s (rep 30). Analysis of the PCR curves was performed with the second derivate maximum method of the LightCycler software [41,43,44]. All sample measurements were repeated at least three times and results are given as mean±SEM.

Electrophysiology and Data Analysis

Experiments were carried out using standard microelectrode whole-cell and cell-attached patch-clamp techniques with an Axopatch 200B amplifier and Digidata 1200 interface (Axon instruments, Foster City, CA, USA) at room temperature (21 to 23°C) [35,43,46]. Whole-cell and single-channel recordings were done as previously described, if not otherwise indicated [16,41,47]. For inside-out recordings both the pipette and bath solution were composed of (mM): KCl 160 mM, MgCl2 1 mM, HEPES 10 mM, EGTA 1 mM; pH 7.4 with KOH. In some experiments 8-bromo-adenosine 5',5'-cyclic monophosphate (8Br-cAMP, 1 mM) (Sigma) or ivabradine (50 μM; kindly provided by the Institut de Recherches Servier, Suresnes, France) were added to the bath solution, as indicated. In KCR1-infected myocytes action potentials were initiated by short depolarizing current pulses (2 ms, 500–800 pA).

Single-channel analysis were done using custom software as previously reported [16,43,47]. Linear leak and capacity currents were digitally subtracted using the average currents of non-active sweeps. For detailed gating analysis idealized currents were analyzed in 150 ms steps recorded at continuous pulse mode (total recording 20×150 ms). Closed-time and first-latency analyses were carried out only in one-channel patches. The open probability (defined as the relative occupancy of the open state during active sweeps), the availability (fraction of sweeps containing at least one channel opening), and Ipeak (the peak ensemble average current, obtained visually) were calculated from single-channel and multi-channel patches with a maximum of three active channels in one patch. Single-channel amplitudes were determined by direct measurements of fully resolved openings (conductance-calculation) or as the maximum of Gaussian fits on amplitude histograms, n, the number of the channels in the patch, was defined as the maximum current amplitude observed, divided by the unitary current. Peak current was corrected by division through n. The availability was corrected by the square root method: [1-availability (corrected)] is the nth root of [1-availability (uncorrected)]. The corrected open probability was calculated on the basis of the corrected number of active sweeps, i.e. total open time divided by (n×availability (corrected)×number of test pulses×pulse length). Time constants of open time (τopen) and closed time histograms (τclosed) were obtained by multi-exponential maximum likelihood estimates (MLE) on open and closed time distributions (shown here as logarithmic binned histograms from pooled data) is given by the “best fit” method [21]. Comparison of gating kinetics was performed as previously described [16].

The availability or fractions of active sweeps were used for calculation of voltage-dependent activation. The voltage-dependence of activation was analyzed using the Boltzmann function: 

\[ Y = Y_{\text{max}} / \left(1 + e^{-N \cdot 0.5 \cdot (V - V_{0.5})}ight) \]

where \( V_{0.5} \) is the voltage of half-maximal activation and \( k \) is the slope factor [45]. Pooled data are presented as mean±SEM. An unpaired two-tailed t test was used for statistical examinations. Probability values of \( P < 0.05 \) were deemed significant. One-way ANOVA was calculated for multiple comparisons and considered significant for \( P < 0.05 \).
ACKNOWLEDGMENTS
We thank N. Henn and Iris Berg for skillful technical assistance, and Mr. Harbig for providing fresh hearts from adult pigs.

Author Contributions
Conceived and designed the experiments: UH GM. Performed the experiments: GM FE IK MB NG JE. Analyzed the data: GM. Contributed reagents/materials/analysis tools: DJ. Wrote the paper: UH GM.

REFERENCES
1. Pape HC (1996) Queer current and pacemaker: the hyperpolarization-activated cation current in neurons. Annu Rev Physiol 58: 299–327.
2. DiFrancesco D (1993) Pacemaker mechanisms in cardiac tissue. Annu Rev Physiol 55: 455–72.
3. Hoppe UC, Tanaka M, Takahashi H, Shahidullah M, Yokoyama S, Higashida H (1998) KCR1, a membrane protein that facilitates functional expression of non-cardiac hyperpolarization-activated mammalian cation channels. Nature 393: 387–91.
4. Santoro B, Liu DT, Yao H, Bartsch D, Kandel ER, et al. (1998) Identification of a gene encoding a hyperpolarization-activated pacemaker channel of brain. Cell 91: 217–29.
5. Ishii TM, Tanaka M, Xie LH, Nozaki A, Ohmori H (1999) Molecular characterization of the hyperpolarization-activated cation channel in rabbit heart sinoatrial node. J Biol Chem 274: 12035–9.
6. Seifert R, Scholten A, Gauss R, Mincheva A, Lichter P, et al. (1999) Molecular dissection of cardiac cation channel properties support a potential contribution of HCN channels and If to cardiac arrhythmias. Circulation 107: 485–9.
7. Santoro B, Liu DT, Yao H, Bartsch D, Kandel ER, et al. (1998) Identification of a gene encoding a hyperpolarization-activated pacemaker channel of brain. Cell 91: 217–29.
8. Kole MH, Hallermann S, Stuart GJ (2006) Single Ih channels in pyramidal neuron dendrites: properties, distribution, and impact on action potential output. J Neurosci 26: 1677–87.
9. Abbott GW, Sesti F, Splawski I, Buck ME, Lehmann MH, et al. (1999) MRP1 forms IKr potassium channels with HERG and is associated with cardiac arrhythmias. Cell 97: 175–87.
10. Hoppe UC, Marban E, Johns DC (2001) Distinct gene-specific mechanisms of arrhythmia revealed by cardiac gene transfer of two long QT disease genes, HERG and KCNQ1. Proc Natl Acad Sci U S A 98: 3353–40.
11. Hardy S, Kitamura M, Harris-Stanaul T, Dai Y, Phillips ML (1997) Construction of adenovirus vectors through Cre-lox recombination. J Virol 71: 1842–9.
12. Lam JS, Huang H, Levitz SM (2007) Effect of Differential N-linked and O-linked Mannosylation on Recognition of Fungal Antigens by Dendritic Cells. PLoS ONE 2: e1009.
13. Hoppe UC, Marban E, Johns DC (2000) Molecular dissection of cardiac channel pharmacology of mibebradil in human native T-type and recombinant Ca(v)3.2 calcium channels. Mol Pharmacol 61: 682–94.
14. Vetter K, Benke C, Birnau P, Krummenauer F, Seifert R, et al. (2005) Production of adenovirus vectors through Cre-lox recombination. J Virol 71: 1842–9.
15. Lam JS, Huang H, Levitz SM (2007) Effect of Differential N-linked and O-linked Mannosylation on Recognition of Fungal Antigens by Dendritic Cells. PLoS ONE 2: e1009.
16. DiFrancesco D (1986) Characterization of single pacemaker channels in cardiac sino-atrial node cells. Nature 324: 470–473.
17. DiFrancesco D, Mangoni M (1994) Modulation of single hyperpolarization-activated channels (If) by CaM in the rabbit sino-atrial node. J Physiol Lond 474: 473–482.
18. Smerone TA, Rho JM, Baram TZ (2005) Single channel properties of hyperpolarization-activated cation currents in acutely dissected rat hippocampal neurones. J Physiol 560: 371–80.
19. Dekker JP, Yellen G (2006) Cooperative gating between single HCN pacemaker channels. J Gen Physiol 128: 561–7.
20. Horn R (1987) Statistical methods for model discrimination. Applications to gating kinetics and permeation of the acetylcholine receptor channel. Biophys J 51: 253–65.
21. Bugajsky LB, Zak R (1989) Differentiation of adult rat cardiac myocytes in cell culture. Curr Res 64: 493–500.
22. An WF, Bowby MR, Betty M, Cao J, Ling HP, et al. (2006) Identification of A-type potassium channels by a family of calcium sensors. Nature 435: 553–6.
23. White BA, Yang Q, Kuryshye VA, Accili EA, Brown AM (1998) Cloning and expression of a novel K+ channel regulatory protein, KCaAP. J Biol Chem 273: 11745–51.
24. Wible BA, Yang Q, Janssen E, Sulik K, Beuckelmann DJ (1998) Hyperpolarization-activated inward current (Ig) in ventricular myocytes from normal and failing human hearts. Circulation 97: 53–63.
25. Kagan A, Melman YF, Krummerman A, McDonald TV (2002) 14-3-3 amplifies and prolongs adennergic stimulation of HERG K+ channel activity. Embo J 21: 1809–90.
26. Decher N, Bundis F, Vajna R, Steinmeyer A, McDonald TV (2002) 14-3-3 amplifies and prolongs adennergic stimulation of HERG K+ channel activity. Embo J 21: 1809–90.
27. Decher N, Bundis F, Vajna R, Steinmeyer A, McDonald TV (2002) 14-3-3 amplifies and prolongs adennergic stimulation of HERG K+ channel activity. Embo J 21: 1809–90.