Differential subunit composition of the G protein–activated inward-rectifier potassium channel during cardiac development

Bernd K. Fleischmann,1 Yaqi Duan,2 Yun Fan,3 Torsten Schoneberg,4 Andreas Ehlich,5 Nibedita Lenka,2 Serge Viatchenko-Karpinski,2 Lutz Pott,6 Juergen Hescheler,2 and Bernd Fakler7

1Institute of Physiology I, University of Bonn, Bonn, Germany. 2Institute of Neurophysiology, University of Cologne, Cologne, Germany. 3Department of Biomedical Engineering, Fourth Military Medical University, Xian, China. 4Department of Molecular Biochemistry, University of Leipzig, Leipzig, Germany. 5Axiogenesis AG, Cologne, Germany. 6Institute of Physiology, Ruhr-University Bochum, Bochum, Germany. 7Institute of Physiology II, University of Freiburg, Freiburg, Germany.

Parasympathetic slowing of the heart rate is predominantly mediated by acetylcholine-dependent activation of the G protein–gated potassium (K⁺) channel (IK,ACH). This channel is composed of 2 inward-rectifier K⁺ (Kir) channel subunits, Kir3.1 and Kir3.4, that display distinct functional properties. Here we show that subunit composition of IK,ACH changes during embryonic development. At early stages, IK,ACH is primarily formed by Kir3.1, while in late embryonic and adult cells, Kir3.4 is the predominant subunit. This change in subunit composition results in reduced rectification of IK,ACH allowing for marked K⁺ currents over the whole physiological voltage range. As a consequence, IK,ACH is able to generate the membrane hyperpolarization that underlies the strong negative chronotropic effect occurring in late- but not early-stage atrial cardiomyocytes upon application of muscarinic agonists. Both strong negative chronotropy and membrane hyperpolarization can be induced in early-stage cardiomyocytes by viral overexpression of the mildly rectifying Kir3.4 subunit. Thus, a switch in subunit composition is used to adopt IK,ACH to its functional role in adult cardiomyocytes.

Introduction

Potassium (K⁺) channels can be generally classified according to their outward- or inward-rectifying current-voltage (I-V) relation (1). Outward rectification is observed in voltage-gated K⁺ (Kv) channels, which are primarily involved in repolarization of the action potential (AP). In contrast, inward-rectifier K⁺ (Kir) channels stabilize the membrane potential near the equilibrium potential for K⁺ (E_K) in many types of excitable and nonexcitable cells (1). Inward-rectification results from a voltage-dependent block of these channels by the intracellular polyamines spermine and spermidine (2, 3) that occlude the channel pore when K⁺ flux is directed outward at potentials positive to E_K. As a consequence, Kir channels display high K⁺ conductance around E_K and at a limited voltage range positive to E_K, while at further depolarized potentials channels are blocked, and, therefore, no longer conductive. Accordingly, the voltage range where stabilization of the membrane potential occurs is defined by the strength of the polyamine-block that may be strong or weak and varies among Kir subunits (reviewed in ref. 4).

In cardiac myocytes, several types of Kir channels are expressed, the strong rectifier IK1 (5), the ATP-sensitive K⁺ channel IKATP (6) and the acetylcholine-activated (ACh-activated) K⁺ channel (IK,ACH) (7). Different from the others, IK,ACH is activated by the Gßδ dimer (8, 9) released from the pertussis toxin–sensitive G protein Gi (10) upon stimulation of the muscarinic type 2 (M2) receptor. IK,ACH is a heterotetramer assembled from Kir3.1 and Kir3.4 subunits (11) and is primarily expressed in sinusoidal and atrial cardiomyocytes. In these cells, release of ACh from parasympathetic nerves leads to negative chronotropy – a phenomenon that results from IK,ACH-mediated hyperpolarization of the membrane potential (7, 12). The negative chronotropic effect is further promoted by muscarinic inhibition of L-type calcium channels and hyperpolarization-activated nonselective cation channels (13) that decrease the threshold level and slow diastolic depolarization (14, 15).

In our previous work, the expression, modulation, and functional role of ion channels has been investigated in detail during early stages of development using ES cell–derived cardiomyocytes (reviewed in refs. 16, 17). When we investigated regulation of the spontaneous electrical activity in early-stage cardiomyocytes, we observed that, in contrast to adult cells, the muscarinic agonist carbacol (CCh) had a negative chronotropic effect without prominent hyperpolarization (14). As this result implied lack or low density of IK,ACH, we aimed to investigate the functional expression of IK,ACH and its possible involvement in the regulation of chronotropy during early and late embryonic cardiomyogenesis.

Here we provide evidence that IK,ACH is expressed in both early and late stages of embryonic development. The ACh-induced hyperpolarization observed in late-stage and adult cardiomyocytes is shown to be related to the subunit composition of IK,ACH.

Nonstandard abbreviations used: ACh, acetylcholine; AP, action potential; CCh, carbacol; EDSS, early developmental stage; E18.5, embryonic fixation potential for K⁺; IK,ACH, acetylcholine-activated K⁺ channel; IC50, 50% effective concentration; IC₆₀, 60% effective concentration; IC₇₀, 70% effective concentration; I-V, current-voltage; L-type, L-type calcium channel; M2, muscarinic type 2.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J Clin Invest. 114:994–1001 (2004). doi:10.1172/JCI200415925.
and $-56.7 \pm 2.3$ mV, $n = 9$ for late stage), while the duration of APs shortened during differentiation.

A striking difference was observed when CCh (1 μM) was applied to the two populations of cardiomyocytes. While a mild negative chronotropy was evoked in early-stage myocytes, decreasing the beating frequency by about 2-fold ($n = 8$; Figure 1, A–C), a pronounced negative chronotropic effect was observed in all late-stage myocytes, as CCh reduced the beating frequency by more than 10-fold ($n = 9$; Figure 1, A–C). This chronotropic effect was accompanied by a differential change in membrane potential. Thus, CCh application led to pronounced hyperpolarization in all late-stage myocytes tested (16.2 ± 1.4 mV, $n = 9$), while only 6 out of 10 early-stage myocytes responded to CCh with hyperpolarization, although with a smaller amplitude (7.9 ± 1.4 mV) (Figure 1, B–D).

Similar results for hyperpolarization of membrane potential were obtained with ES cell–derived cardiomyocytes (data not shown). In contrast to atrial myocytes, ventricular cardiomyocytes of either developmental stage did not exhibit membrane hyperpolarization in the presence of CCh ($n = 13$; Figure 1B).

The correlation between the CCh effect and activation of $I_{K,ACh}$ was further investigated with tertiapin (1 μM), a selective blocker of Kir3 channels (18). As shown in Figure 2A, tertiapin co-applied with CCh effectively reversed both CCh-mediated negative chronotropy (Figure 2B) and membrane hyperpolarization (Figure 2C) in late-stage atrial cardiomyocytes, while only minor effects were observed in early-stage atrial cardiomyocytes (Figure 2C). Sole application of tertiapin only minimally increased basal AP frequency in early- (9.2% ± 5%) and late-stage (12.4% ± 4%, data are mean ± SEM) atrial cells and did not affect at all the membrane potential ($n = 5$ and $n = 3$ for early- and late-stage atrial cardiomyocytes, respectively).

$I_{K,ACh}$ is expressed at similar densities at both stages of differentiation. Because of the distinct responses of early- and late-stage atrial cardiac myocytes to CCh, amplitude and activation of $I_{K,ACh}$ at each stage were further investigated. First, maximal $I_{K,ACh}$ elicited by CCh was recorded from early- and late-stage atrial cells at a holding potential of ~70 mV in 30 mM external K+. As shown in Figure 3A, the $I_{K,ACh}$ amplitude was almost identical in either type of cell (current amplitudes of 16.2 ± 1.8 pA/pF, $n = 12$, and 16.9 ± 3.4 mV, $n = 9$, for early- and late-stage atrial cardiomyocytes, respectively).

In addition, M2 receptor expression and levels of $G_i/G_o$ proteins were determined in radioligand binding assays and $[^{32}P]$ADP-ribosylation reactions. While M2 receptor expression differed between early- and late-stage murine-atria (Figure 3B), $G_i/G_o$ proteins linking receptor activity to the downstream signaling cascade were almost identical (Figure 3C). Together, these results suggested that the distinct CCh responses of early- and late-stage atrial cardiomyocytes are not due to different amounts of $I_{K,ACh}$.

Subunit composition of $I_{K,ACh}$ changes during development. We therefore turned to an investigation of the functional properties of $I_{K,ACh}$ activated to steady-state with CCh to exclude any influences of the M2 receptor $G$ protein cascade.

As shown in Figure 4, the time course of $I_{K,ACh}$-mediated currents recorded in response to a voltage step from 0 to ~100 mV (see Methods) was distinct in cardiomyocytes of either developmental stage as depicted by representative current traces. Thus, in late-stage atrial myocytes, the onset of current was largely instantaneous, while it was exponential in atrial cardiomyocytes of early stages (Figure 4A, upper panel). Analysis of currents from early-stage myocytes showed that the onset of current was monoexponential, with a time constant ($\tau_{on}$) of 13.9 ± 5.2 ms (mean ± SD of $n = 7$ cells). In late-stage myocytes, onset of current was either instantaneous (4 out of 8 cells) or exhibited a small exponential component (relative contribution of 0.16 ± 0.06; 4 out of 8 cells) with a $\tau_{on}$ of 16.9 ± 4.3 ms (mean ± SD) (Figure 4A, lower panel). The maximal $I_{K,ACh}$ amplitude was almost identical in atria-derived myocytes of both stages (mean ± SEM current density of 39.4 ± 9.4 pA/pF, $n = 11$ and 47.6 ± 6.3 pA/pF, $n = 11$, for early and late stages, respectively).

Very similar results were obtained in experiments with ES cell–derived cardiomyocytes (Figure 4B). Early-developmental-
stage (EDS) cells displayed exponential onset of current ($\tau_{on}$ of 15.9 ± 7.3 ms, mean ± SD of $n = 8$ cells), while the majority of late-developmental-stage (LDS) cardiomyocytes (6 out of 9 cells) exhibited instantaneous current onset. Some LDS cells (3 out of 9) displayed intermediate behavior with an $I_0/I_{max}$ ratio of about 0.5 (Figure 4B, lower panel). This was most likely due to the ES cell system, where complete synchronization of differentiation does not occur (16).

Exponential onset of currents mediated by heterologously expressed Kir3.1 and Kir3.4 channels in response to hyperpolarizing voltage steps was shown to reflect unbinding of polyamines from the channel. A negatively charged aspartate residue in the second transmembrane domain of the Kir3.1 subunit was identified as the major structural determinant (19). Neutralization of this residue, as in the Kir3.4 subunit, leads to a largely instantaneous onset of current. We therefore tested whether the distinct character of the onset of $I_{K,ACH}$ observed in early- and in late-stage atria– and ES cell–derived cardiomyocytes may be due to different integration of the Kir3.1 subunit into $I_{K,ACH}$. Various ratios of cRNAs coding for Kir3.1 and Kir3.4 were injected into Xenopus oocytes, and the onset of current was measured. As shown in Figure 5A, current onset was largely instantaneous at an excess expression of Kir3.4, while exponential activation became more and more prominent upon increasing the amount of Kir3.1. The relative contribution of the exponential current component decreased from 0.37 ± 0.04 (mean ± SD, $n = 6$) at a Kir3.1/Kir3.4 cRNA ratio of 10:1 to 0.03 ± 0.02 ($n = 4$) at a cRNA ratio of 1:10, respectively (Figure 5, B and C).

These results suggest that the ratio of Kir3.1 and Kir3.4 proteins present in the plasma membrane of atria and ES cell–derived cardiomyocytes may change during embryonic development. This hypothesis was further corroborated by quantitative RT-PCR performed on early- and late-stage atria. As illustrated in Figure 6A for 3 independent experiments, expression of Kir3.4 mRNA is low at early stages and increases markedly during development. In contrast, a marked decrease was observed with Kir3.1 mRNA. Consequently, the Kir3.4/Kir3.1 mRNA ratio increased during cardiac development by more than 600-fold (Figure 6B).

![Figure 2](image-url)

The CCh effect is blocked by the $I_{K,ACH}$-selective blocker tertiapin. (A) Hyperpolarization and negative chronotropy induced by CCh (1 $\mu$M) are reversed by tertiapin (1 $\mu$M) in a late-stage atrial cardiomyocyte. (B) CCh-induced deceleration of spontaneous APs is reversed by tertiapin in late-stage but not in early-stage atrial cardiomyocytes. Data are mean ± SEM of 6 early- and 7 late-stage cells ($P = 0.026$ for control and $P = 0.0003$ for CCh, unpaired Student’s $t$ test). (C) Tertiapin-induced depolarization of the membrane potential in CCh-treated early- and late-stage atrial cardiomyocytes ($P = 0.0007$, unpaired Student’s $t$ test). Recovery was estimated by determining the maximal diastolic potential prior to application of CCh and in presence of CCh and tertiapin. Asterisks denote statistically significant difference.

![Figure 3](image-url)

$I_{K,ACH}$ density, M2 receptor expression, and $G_i/G_o$-protein content in atrial cardiomyocytes during embryonic development. (A) Density of $I_{K,ACH}$ determined at a holding potential of −70 mV is identical at the two differentiation stages. Extracellular K+ was 30 mM, and data are mean ± SEM of 12 early- and 9 late-stage cardiomyocytes ($P = 0.9$, unpaired Student’s $t$ test). (B) M2 receptor expression in early- and late-stage atria determined from binding experiments with [3H]QNB. Data are mean ± SD of 3 early- and 2 late-stage cell preparations ($P = 0.002$, unpaired Student’s $t$ test). (C) $G_i/G_o$-protein content of atrial membranes determined by [32P]ADP-ribosylation analyzed densitometrically. One of 2 independent experiments with identical results is shown. Comparable protein loading was confirmed with Coomassie staining. Asterisk denotes statistically significant difference. Sta., standard.

![Figure 4](image-url)

![Figure 5](image-url)

![Figure 6](image-url)
Distinct rectification properties were also observed with heterologously expressed \( I_{K_{ACh}} \). Kir channels resulting from a Kir3.1/Kir3.4 cRNA ratio of 10:1 showed strong rectification with blocking of outward currents for potentials positive to \( E_K \) while channels forming upon excess injection of Kir3.4 cRNA (Kir3.1/Kir3.4 ratio of 1:10) conducted K+ currents over the whole voltage range tested (Figure 7C).

These results strongly suggest that the differences in rectification observed with \( I_{K_{ACh}} \) in early- and late-stage cardiomyocytes are due to changes in its subunit composition.

Overexpression of Kir3.4 in early-stage cardiomyocytes restores biophysical and functional features of late-stage cells. Kir3.4 and Kir3.1 subunits were overexpressed in early- and late-stage atrial cardiomyocytes in order to further explore whether the difference in rectification resulting from a developmental change in subunit composition of \( I_{K_{ACh}} \) is related to the distinct CCh-induced negative chronotropic and membrane hyperpolarization. As shown in Figure 8A, early-stage cells overexpressing Kir3.4 exhibited \( I_{K_{ACh}} \) with largely instantaneous onset and weak rectification (\( n = 8 \)) similar to that observed in nontransfected late-stage atrial cardiomyocytes. In contrast, when Kir3.1 was overexpressed in late-stage atrial cardiomyocytes, \( I_{K_{ACh}} \) displayed an exponential onset of current together with strong inward rectification (\( n = 5 \); Figure 8B).

Moreover, when CCh (1 μM) was applied to Kir3.4-transfected early-stage atrial cardiomyocytes, a prominent negative chronotropic effect was observed in all cells tested, accompanied by a hyperpolarization of the membrane potential (3.3 ± 1.4 mV; mean ± SEM, \( n = 9 \); Figure 8, C and D). Both hyperpolarization and negative chronotropy were reversed by tertiapin (\( n = 9 \); Figure 8, C and D), as seen before in late-stage cells (Figure 2B and C). Interestingly, Kir3.4 overexpressing early-stage cells displayed significantly augmented beating frequencies (4.4 ± 0.3 Hz, \( n = 9 \); Figure 8D) compared with the untransfected controls (Figure 1C, \( P = 0.004 \); Figure 2B, \( P = 0.006 \), unpaired Student’s \( t \) test). Together, these results indicate that Kir3.4-dominant \( I_{K_{ACh}} \) is both necessary and sufficient for reconstituting a late-stage phenotype in early-stage atrial cardiomyocytes with respect to membrane hyperpolarization and negative chronotropy induced by muscarinic receptor stimulation.
A sequence, rectification of IK,
ACh is reduced at late stages of embryonic
development, which allows for a marked K+
conductance over the whole voltage range positive to EK.
So far, a change in subunit composition during development
has been reported only for the nicotinic ACh receptor. However, in
contrast to our findings on IK,ACh, nicotinic ACh receptors exchange
their embryonic γ subunit for the adult ε subunit (29–31). This subunit
exchange has been found to be accompanied by biophysical
modifications, in particular an increase in the mean open time and
prolongation of burst lengths (32).

The correlation between the distinct rectification properties of
the two Kir subunits and the distinct reaction of early- and late-
stage atrial cells on muscarinic agonists was most obvious in viral
transfection experiments. Thus, exogenous expression of Kir3.4
restored the negative chronotropic effect in early-stage cardiomyo-
cytes (Figure 8). The latter result further implied that early-stage
cardiomyocytes dispose of all the signaling elements required for
M2-induced activation of IK,ACh.

As structural correlates for the distinct rectification properties
observed among the various Kir channels, a number of sites have
been identified that govern the interaction between the Kir channel
pores and polyamines (4, 21). Among these, a residue in the second
transmembrane helix was found to be particularly effective and
discriminates between strong and weak rectifiers. Strong rectifi-
cers bear a negatively charged residue, while weak rectifiers present
with a neutral amino acid at this position (22–24). Kir3.1 exhib-
ts an aspartate residue at this site (D173), while an asparagine
residue is found in Kir3.4 (N179). Accordingly, the more Kir3.1
subunits are integrated into an IK,ACh channel, the more prominent
its rectification should be (25–27). This was indeed observed in
heterologous expression experiments in which the ratios of Kir3.1
and Kir3.4 cRNA were varied between 10:1 and 1:10 (see Figures 4
and 7). The close agreement between the properties of IK,ACh
and heterologously expressed Kir3.1 and Kir3.4 channels strongly sug-
gested that the ratio of Kir3.1/Kir3.4 subunits changes during
embryonic development. This conclusion is further supported by
quantitative PCR reported in Figure 6 as well as a report by Corey
and Clapham (28) demonstrating that Kir3.4 is the predominant
IK,ACh subunit in adult atria. Whether this reported change in
composition of IK,ACh is due to an altered subunit stoichiometry
of Kir3.1/Kir3.4 heteromultimers or due to an increase of Kir3.4
homomeric channels (28) presently remains unclear.

So far, a change in subunit composition during development
has been reported only for the nicotinic ACh receptor. However, in
contrast to our findings on IK,ACh, nicotinic ACh receptors exchange
their embryonic γ subunit for the adult ε subunit (29–31). This sub-
unit exchange has been found to be accompanied by biophysical
modifications, in particular an increase in the mean open time and
prolongation of burst lengths (32).

Discussion
The results presented show that the G protein–activated K+ channel
IK,ACh changes its molecular composition in cardiomyocytes
during embryonic development: while the strongly rectifying Kir3.1 dominates at early stages, late-stage and adult IK,ACh is made
up mainly of the mildly rectifying Kir3.4 subunit. As a conse-
quence, rectification of IK,ACh is reduced at late stages of embryonic
development, which allows for a marked K+ conductance over the
whole voltage range positive to EK. Accordingly, IK,ACh activated by
ACh released from the vagal nerve leads to hyperpolarization of the
membrane potential, which in turn shunts excitation and largely
reduces electrical activity (AP frequency) (Figure 1). This negative
chronotropic effect does not occur in early-stage myocytes, since
Kir3.1-dominated IK,ACh is blocked by polyamines at the maximal
diastolic potential and, therefore, is unable to provide additional
K+ conductance upon vagal stimulation.

The Journal of Clinical Investigation

http://www.jci.org
Volume 114
Number 7
October 2004

Figure 6
Amount of Kir3.1 and Kir3.4 mRNA changes during embryonic devel-
Tigure 6
optment in atria. (A) Quantitative RT-PCR (Taqman) performed on early-
lymphatic atria, mRN levels in the different samples were normalized to 18S rRNA. Data are obtained
from 3 independent RNA preparations. (B) Ratio of Kir3.4/Kir3.1 mRNA
in early- and late-stage atria as determined from the data in A. Data are mean ± SD (P = 0.003, unpaired Student’s t test). Asterisk denotes statistically signiﬁcant difference.

Figure 7
Rectification of IK,ACh decreases during cardiac development. (A) Steady-state I-V relation of IK,ACh (voltage steps from –80 mV
to 40 mV in 20-mV steps) determined in early- (open squares)
and late-stage (ﬁlled squares) atria-derived cardiomyocytes. Rel.
current, relative current. (B) Original IK,ACh-mediated currents
induced by CCh and recorded at potentials of –80, –40, 0 mV
(gray traces) and 40 mV (black trace) in early- and late-stage
atria-derived cardiomyocytes; short lines indicate zero current.
Note the difference in outward current amplitude. (C) Steady-
state I-V relation determined in oocytes injected with Kir3.1
and Kir3.4 cRNA at the ratios indicated. (D) IK,ACh-mediated current
recorded in a late-stage ES cell–derived cardiomyocyte (recorded
with perforated patch) in response to a 500 ms voltage ramp
from –100 to 60 mV under symmetrical K+ conditions with 100
µM Cs+ present in the bath medium. Note complete block of
channels by Cs+ at –100 mV.
It is tempting to speculate that changes occurring in subunit composition of ion channels resulting in altered function may represent a more general motif of adaptation during development. Moreover, since pathologically altered cells can recapitulate their embryonic phenotype, similar changes may also occur in diseased cells.

**Methods**

**Harvest and isolation of embryonic cardiomyocytes.** For harvesting embryonic cardiomyocytes from murine atria, mice of the strain HIM:OF1 were bred using superovulation for precise staging (33). For isolation of early- and late-stage cardiomyocytes, E10.5–E12.5 and E16.5–E18.5 embryos were used, respectively. After embryos were harvested, hearts were dissected and atria separated from ventricles. For experiments with ES cells, the cell line D3 was used. ES cells were differentiated into cardiomyocytes, as described previously (34). EDS and LDS cells were obtained from embryoid bodies using enzymatic dissociation procedures as described previously (14). The isolated cells were plated on sterile, gelatin-coated glass coverslips and kept in the incubator for 24–48 hours. Spontaneously contracting cardiomyocytes could be observed within 12 hours after cell preparation. The experiments using mouse tissue were approved by the local ethical committee (University of Cologne).

**Electrophysiology on cardiomyocytes.** Isolated, spontaneously beating areas of embryoid bodies using enzymatic dissociation procedures as described previously (14). The isolated cells were plated on sterile, gelatin-coated glass coverslips and kept in the incubator for 24–48 hours. Spontaneously contracting cardiomyocytes could be observed within 12 hours after cell preparation. The experiments using mouse tissue were approved by the local ethical committee (University of Cologne).

Electrophysiology on cardiomyocytes. Isolated, spontaneously beating areas of embryoid bodies using enzymatic dissociation procedures as described previously (14). The isolated cells were plated on sterile, gelatin-coated glass coverslips and kept in the incubator for 24–48 hours. Spontaneously contracting cardiomyocytes could be observed within 12 hours after cell preparation. The experiments using mouse tissue were approved by the local ethical committee (University of Cologne).

All experiments were performed at 37°C. Solutions for current-clamp recordings had the following composition: 50 mM KCl, 80 mM Kasperlate, 1 mM MgCl₂, 3 mM MgATP, 10 mM EGTA, 10 mM HEPES, pH 7.4 (internal solution; pH adjusted with KOH); 140 mM NaCl, 5.4 mM KCl, 3.6 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 10 mM glucose, pH 7.4 (external solution; pH adjusted with NaOH). For voltage clamp experiments, solutions were: 40 mM KCl, 100 mM Kasperlate, 5 mM MgATP, 2 mM EGTA, 0.01 mM GTP, 10 mM HEPES, pH 7.4 (internal solution; pH adjusted with KOH); 140 mM NaCl, 2 mM MgCl₂, 5 mM NaCl, 1.8 mM CaCl₂, 5 mM glucose, 5 mM HEPES, pH 7.4 (external solution; pH adjusted with KOH). Steady-state recordings were performed in 30 mM extracellular K’. For perforated patch-clamp experiments in ES cell–derived cardiomyocytes the internal solution was: 55 mM KCl, 7 mM MgCl₂, 70 mM K₂SO₄, 10 mM HEPES, pH 7.4 (adjusted with KOH); the final concentration of amphotericin was 450 μg/ml. The external solution was: 140 mM KCl, 5 mM NaCl, 2 mM MgCl₂, 1.8 mM CaCl₂, 5 mM glucose, 5 mM HEPES, pH 7.4 (adjusted with KOH). Most of the voltage clamp recordings of

**Figure 8**

Overexpression of Kir3.4 in early-stage atrial cardiomyocytes reconstitutes the late-stage phenotype. (A) Left panel: I_K,ACH–mediated currents induced by CCh and recorded at potentials of –100 mV (gray trace) and 40 mV (black trace) in an early-stage cell overexpressing Kir3.4. Right panel: Steady-state I-V relation of the experiment in A (voltage steps from –100 mV to 40 mV in 20-mV steps). (B) Current traces and steady-state I-V relation as in A, but recorded from a late-stage cardiomyocyte overexpressing Kir3.1. (C) Hyperpolarization and negative chronotropy induced by CCh and reversed by tertiapin in an early-stage Kir3.4–overexpressing cardiomyocyte. (D) CCh–induced deceleration of spontaneous APs (left axis) and reversal by tertiapin (right axis) in early-stage cardiomyocytes overexpressing Kir3.4. Data are mean ± SEM of 9 cells.
**Research Article**

Is K<sub>AC3</sub> were performed in equimolar K<sup>+</sup> to increase current amplitude. All test substances were purchased from Sigma-Aldrich and were dissolved in extracellular or pipette (GTP-y-S) solution prior to use.

**RNA extraction and cDNA synthesis.** Total RNA was isolated from embryonic heart at different stages (E11.5, E18.5) using the RNA isolation kit from Qiagen. The extracted RNA was quantified with a spectrophotometer. One microgram of RNA was used for reverse transcription into cDNA using random hexamers (Roche Diagnostics) as primers and SuperscriptII Reverse Transcriptase (Invitrogen) following the manufacturer’s instructions. cDNA samples were stored at -20°C.

**Quantitative real-time PCR.** The mRNA levels of Kir3.1 and Kir3.4 as well as of IRS rRNA (as an endogenous control) were quantified by real-time PCR analysis (TaqMan chemistry; Applied Biosystems) on an ABI Prism 7700 sequence detection system (Applied Biosystems). All primers (see below) were designed with Primer Express 1.5 software (Applied Biosystems). Quantitative real-time PCR was performed in a total reaction volume of 25 µl containing 12.5 µl Taqman Universal Master Mix at a concentration of x2, 2.25 PM of each forward and reverse primer, and 0.75 PM dual-labeled fluorogenic internal probe in MicroAmp optical 96-well plates covered with MicroAmp optical caps (Applied Biosystems). 0.5 µl cDNA template was added to the mixture for amplification of Kir3.1 and Kir3.4, while 2 µl cDNA was used for IRS rRNA quantification. The PCR was carried out using the following cycling parameters: 50°C for 2 minutes and then 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

For quantification of Kir3.1 and Kir3.4 expression relative to that of IRS rRNA, the input cDNA levels were calculated from cycle number values (x10 the standard deviation of base-line emissions measured from cycles 3 to 10) provided by standard curves (linear plots of fluorescence versus cycle number) that were prepared from serial dilutions of early- (Kir3.1) and late-stage (Kir3.4) samples according to standard procedures (36).

Sequences of the primers or probes were the following: Kir3.1 forward primer (5′-CTGCGCAACAGCCACATG-3′); Kir3.1 reverse primer (5′-CCTCAGGTGTCTGCAGATG-3′); Kir3.1 probe (FAM-5′-CGCGCCAGATCCGCTGCA-3′-AMRA); Kir3.4 forward primer (5′-CTGGACACGACCTTTGGT-3′); Kir3.4 reverse primer (5′-CCATGCTCTTTAGTATAGCATTCC-3′); Kir3.4 probe (5′-CCATCTTAGCAGAGATGCGGTGTT-3′). The Kir3.4 probe used in the TaqMan reactions was designed to have nonfluorescent quenchers and minor groove-bending modifications.

**Electrophysiology on recombinant Is K<sub>AC3</sub>.** Oocyte handling and injection of cRNA specific for Kir3.1 and Kir3.4 was done as described previously (3). Electrophysiological recordings were performed 3–7 days after injection using a 2-microelectrode voltage clamp. For 2-microelectrode recordings, current and voltage electrodes were pulled from thick-walled borosilicate glass and had resistances between 0.1 and 0.5 MΩ when filled with 3 M KCl. Currents were recorded with a TurboTec 01C amplifier (npi electronic GmbH), digitized at 10 kHz (ITC-16, HEKA) and stored on hard drive. The bath solution was composed as follows: 117.5 mM KCl, 1.8 mM CaCl<sub>2</sub>, 10 mM HEPES (pH adjusted to 7.2 with KOH). All experiments were performed at room temperature (approximately 23°C). 1-V relations (Figure 5A) were obtained by plotting the steady-state current measured at voltages between –100 and 60 mV (increments of 20 mV) versus the command voltage; τ<sub>act</sub> and τ<sub>inact</sub> were determined as described above. Computational work was done on a Macintosh PowerPC using commercial software (IGOR; WaveMetrics) for fitting.

5. Sakmann, B. and Trube, G. 1984. Conductance properties of single inwardly rectifying potassium...