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ION CHANNELS, RECEPTORS AND TRANSPORTERS

Human Kir2.1 channel carries a transient outward potassium current with inward rectification

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Abstract We have previously reported a depolarization-activated 4-aminopyridine-resistant transient outward K^+ current with inward rectification ($I_{to.ir}$) in canine and guinea pig cardiac myocytes. However, molecular identity of this current is not clear. The present study was designed to investigate whether Kir2.1 channel carries this current in stably transfected human embryonic kidney (HEK) 293 cells using whole-cell patch-clamp technique. It was found that HEK 293 cells stably expressing human Kir2.1 gene had a transient outward current elicited by voltage steps positive to the membrane potential (around −70 mV). The current exhibited a current–voltage relationship with intermediate inward rectification and showed time-dependent inactivation and rapid recovery from inactivation. The half potential ($V_{0.5}$) of availability of the current was −49.4± 2.1 mV at 5 mM K^+ in bath solution. Action potential waveform clamp revealed two components of outward currents; one was immediately elicited and then rapidly inactivated during depolarization, and another was slowly activated during repolarization of action potential. These properties were similar to those of $I_{to.ir}$ observed previously in native cardiac myocytes. Interestingly, inactivation of the $I_{to.ir}$ was strongly slowed by increasing intracellular free Mg^{2+} ($Mg^{2+}{}_i$, from 0.03 to 1.0, 4.0, and 8.0 mM). The component elicited by action potential depolarization increased with the elevation of $Mg^{2+}{}_i$. Inclusion of spermine (100 μM) in the pipette solution remarkably inhibited both the $I_{to.ir}$ and steady-state current. These results demonstrate that the $Mg^{2+}{}_i$-dependent current carried by Kir2.1 likely is the molecular identity of $I_{to.ir}$ observed previously in cardiac myocytes.

Keywords Kir2.1 channel · Inward rectification · I_{K1} · Transient outward current with inward rectification

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Introduction

It is well recognized that depolarization-activated outward K^+ currents play an important role in myocardial repolarization of different species, and they have distinctive biophysical and pharmacological properties [1]. These outward currents include transient outward currents (I_{to}) [10, 13, 19, 44]; rapid and slow delayed rectifier K^+ currents (I_{Kr} and I_{Ks}) in guinea pig [36], dog [43], pig [21], and human [24] cardiac myocytes; and inward rectifier K^+ current (I_{K1}) in cardiac myocytes of all species. Ultra-rapid delayed rectifier K^+ current (I_{Kur}) is also expressed in rat [3], dog [43], pig [21], and human [24] atrial myocytes.

I_{to} has been identified using whole-cell voltage-clamp techniques in cardiac cells from a wide range of species since the 1960s, including rat [17], rabbit [13], elephant seal [31], ferret [4], dog [40, 44], pig [21], and humans [2, 23].

Kenyon and Gibbons [18] reported that 4-aminopyridine (4-AP) decreased I_{to} in sheep cardiac Purkinje fibers. Subsequently, 4-AP has been used as a selective inhibitor of transient outward K^+ current, and 4-AP-sensitive and 4-AP-resistant components of I_{to} have been reported in sheep Purkinje fibers [6] and in rabbit [47] and dog [40, 44] cardiac myocytes. The 4-AP-sensitive and 4-AP-resistant components often are termed I_{to1} and I_{to2}, respectively, by Tseng and Hoffman [40]; I_{to2} is a Ca^{2+}-activated transient outward Cl^- current ($I_{Cl.Ca}$) [21, 44, 47]. The third element of transient outward current with inward rectification ($I_{to.ir}$) was described in dog ventricular cells [25] and guinea pig cardiomyocytes [26]. $I_{to.ir}$ is another 4-AP-insensitive transient outward current carried by K^+ ions and sensitive to inhibition by Ba^{2+} and/or the omission of extracellular K^+ (K^+_o). Zhabyeyev and colleagues also reported I_{to} using whole-cell patch recording in guinea pig ventricular myocytes dialyzed with low-K^+ (10 mM) solution [46]. It has been suggested the I_{to} recorded using a low-K^+ pipette solution [46] or the $I_{to.ir}$ determined using a physiological-K^+ pipette solution [25, 26] is likely carried by the inward rectifier K^+ channel; however, this is challenged by the classical notion that the cardiac I_{K1} channel acts as a diode [30] which activates upon hyperpolarization of the membrane [34], and limited current passes through the channel in the outward direction under physiological conditions [30, 34].

During the study of human Kir2.1 channels stably expressed in human embryonic kidney (HEK) 293 cells, we observed a 4-AP-insensitive I_{to}, similar to the $I_{to.ir}$ observed in dog and guinea pig ventricular myocytes [25, 26]. The present study was designed to characterize this I_{to} in HEK 293 cells stably expressing human Kir2.1 gene and to investigate whether Kir2.1 channel contributes to the molecular identity of $I_{to.ir}$ previously observed in cardiac myocytes.

Materials and methods

Gene transfection and cell culture

HEK293 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and grown in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum in 5% CO_2 and 95% air at 37°C. Human Kir2.1 gene kindly provided by Dr. Carol A. Vandenberg (University of California at Santa Barbara, CA, USA) [35] was inserted into the pCDNA3 vector. The Kir2.1/pCDNA3 plasmid (4 μg) was then transfected into HEK 293 cells using Lipofectamine 2000™ and selected using 1,000 μg/ml G418 (GE Healthcare, Hong Kong). Colonies were picked with cloning cylinders (Sigma-Aldrich, St Louis, MO, USA) and examined for channel expression using whole-cell current recordings as described previously [9, 39]. The selected cell line stably expressing human Kir2.1 channel was maintained in DMEM medium containing 400 μg/ml G418 and 10% fetal bovine serum.

Solutions and chemicals

Tyrode solution contained (mM) NaCl 140, KCl 5.0, $MgCl_2$ 1.0, $CaCl_2$ 1.8, NaH_2PO_4 0.33, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10.0, and glucose 10 (pH adjusted to 7.3 with NaOH). For whole-cell recordings, the pipette solution contained (mM) KCl 20, K-aspartate 110, $MgCl_2$ 1.0, HEPES 10, ethyleneglycoltetraacetic acid (EGTA) 5, guanosine triphosphate (GTP) 0.1, Na-phosphocreatine 5, and Mg-adenosine triphosphate (ATP) 5 (pH adjusted to 7.2 with KOH, ~10 mM). When accurate free Mg^{2+} concentrations in the pipette solution were administered, Mg-ATP was replaced by tris–ATP. The free Mg^{2+} in pipette solution was calculated using the Cabuf software created by Dr. G. Droogmans in the Department of Physiology, KU Leuven, Leuven, Belgium (http://www.kuleuven.be/fysiol/trp/cabuf). All reagents were obtained from Sigma-Aldrich. Stock solution of spermine was made with dimethylsulfoxide and was divided into aliquots and stored at −20°C.

Electrophysiology

Cells on a cover slip were transferred to an open cell chamber (0.5 ml) mounted on the stage of an inverted microscope and superfused with Tyrode solution at ~2 ml/min. The whole-cell patch-clamp technique was used as described previously [9, 12, 39]. The whole-cell membrane currents were measured using an EPC-10 amplifier and Pulse software (Heka Elektronik, Lambrecht, Germany). Borosilicate glass electrodes (1.2-mm OD) were pulled with a Brown–Flaming puller (model P-97, Sutter Instrument, Nato, CA, USA) and had tip resistances of 1–2 MΩ when filled with the pipette solution. A 3-M KCl agar bridge was used as the reference electrode. The tip potential was zeroed before the patch pipette touched the cell. After the gigaohm seal was obtained, the cell membrane was ruptured by applying gentle pressure to establish a whole-cell configuration. Series resistance (Rs) was 2–3 MΩ and was compensated by 50–70% to minimize voltage errors. The liquid junction potential (13.7 mV) calculated with the software of Clampex (http://www.Axon.com) was not corrected in the experiment and data analysis. Cell membrane capacitive transient was electrically compensated with the Pulse software. Current and voltage signals were low-pass filtered at 5 kHz and stored in the hard disk of an IBM

compatible computer. All experiments were conducted at room temperature (22–23°C).

Statistical analysis

Nonlinear curve-fitting was performed using Pulsefit (HEKA) and Sigmaplot (SPSS, Chicago, IL, USA). Paired and/or unpaired Student's *t* test was used as appropriate to evaluate the statistical significance of differences between two group means, and analysis of variance was used for multiple groups. Group data are expressed as mean±SEM. Values of $P<0.05$ were considered to be statistically significant.

Results

Initial discovery of transient outward current carried by Kir2.1 channel

When we used a conventional voltage step protocol (inset of Fig. 1a) to study Kir2.1 channel stably expressed in HEK 293 cells, a current with fast inactivation was recorded at −40 mV as indicated in control of Fig. 1b,c. The current disappeared when the inwardly rectifying component of Kir2.1 channel elicited by the hyperpolarization voltage steps was inhibited by application of 200 μM Ba^{2+} or removal of external K^+ ions (K^+_o) (Fig. 1b,c). No transient outward current was observed in HEK 293 cells without Kir2.1 gene transfection. We therefore assumed that this current was a depolarization-elicited transient outward current of Kir2.1 channel when the hyperpolarization steps (−120 to −50 mV) were returned to the holding potential (−40 mV).

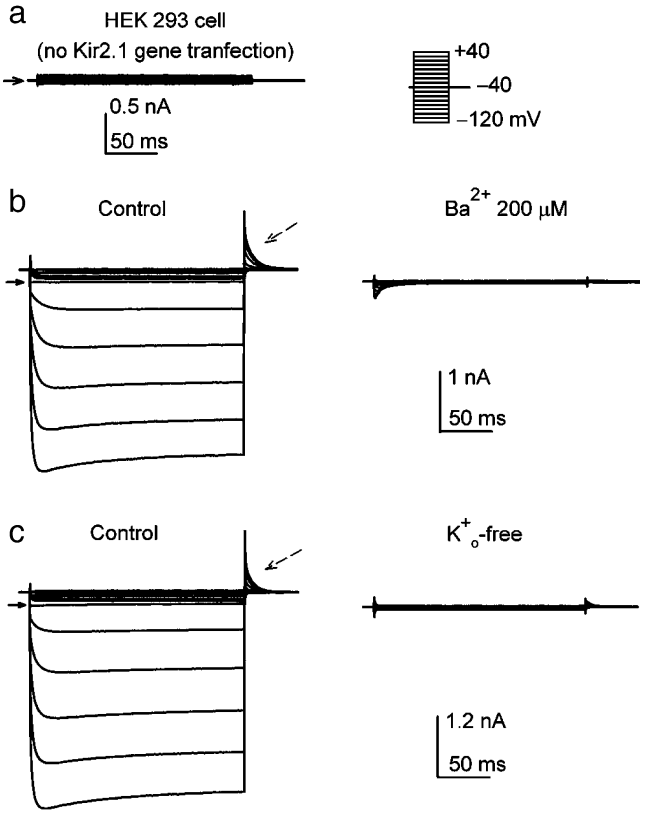

Fig. 1 Transient outward current recorded in HEK 293 cells stably expressing human Kir2.1 channels. **a** Only a small background current was recorded with the voltage steps to between −120 and +40 mV from a holding potential of −40 mV in HEK 293 cells without Kir2.1 gene transfection ($n=5$). Membrane capacitative transient was compensated electronically by the Pulse software. **b** Typical inward rectifier K^+ current (I_{K1}) was recorded in HEK 293 cells ($n=48$) using the protocol as shown in **a** followed by a transient outward current as hyperpolarization potentials returned to the holding potential (−40 mV, *broken arrow of left panel*). Both the inward rectifier and transient outward currents were almost fully inhibited by the application of 200 μM Ba^{2+} (5 min; $n=6$). **c** The inward rectifier and transient outward currents were abolished by omission of external K^+ (K^+_o, 5 min) in a representative cell expressing Kir2.1 gene ($n=5$)

Depolarization-elicited transient outward current

To further confirm whether the transient outward current is elicited by depolarization voltage steps, a depolarization voltage protocol with 200 ms voltage steps to voltages between −70 and +40 mV from a holding potential of −80 mV (inset of Fig. 2a) was employed to characterize this current. Figure 2a shows the current tracings elicited by the depolarization voltage steps in a representative HEK 293 cell stably expressing Kir2.1 channel. A transient outward current that rapidly activated upon depolarization voltage steps positive to −70 mV (without correction of liquid junction potential) was insensitive to inhibition by the classical I_{to} blocker 4-AP (5 mM), and no 4-AP-sensitive current was observed. Similar results were obtained in four other cells. This transient outward current was fully inhibited by the application of 200 μM Ba^{2+} or omission of K^+_o from the bath medium, leaving a small background current. Ba^{2+}- or K^+_o-sensitive transient outward current was obtained by digital subtraction (Fig. 2b,c). Similar results were obtained in nine other cells in each experimental group. These results suggest that the I_{to} is most likely a K^+ current permeating through the Kir2.1 channel.

Voltage dependence of the transient outward current

The voltage dependence of the depolarization-activated I_{to} was determined using the same voltage-clamp protocol as in Fig. 2a. Ba^{2+}-sensitive current was used to determine the *I–V* relation of the current. Figure 3a shows the Ba^{2+}-sensitive current recorded in a representative cell at depolarization voltages between −70 and +40 mV from a holding potential of −80 mV. I_{to} was observed at −70 mV,

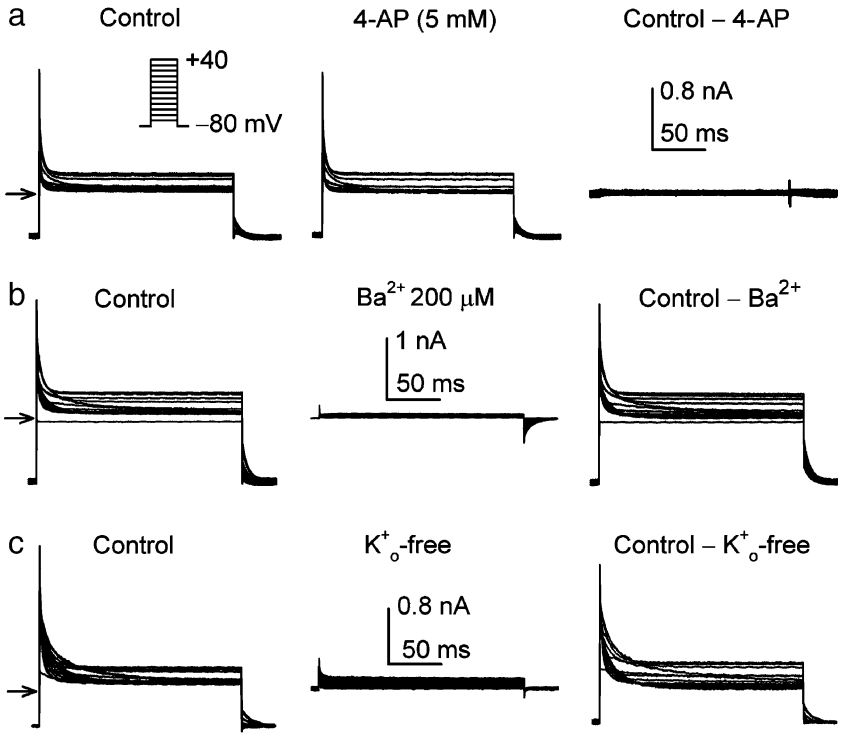

Fig. 2 Transient outward current elicited by depolarization voltages. **a** Representative currents elicited by 200-ms depolarization steps from −80 mV to test potentials between −70 and +40 mV (*inset*) in a representative cell during control and after application of 5 mM 4-AP for 8 min. No significant 4-AP-sensitive current was obtained by digital subtraction of the currents before and after 4-AP treatment (n=6). **b** Depolarization-elicited current was fully inhibited by the application of 200 μM Ba^{2+} in another cell. Ba^{2+}-senstive current was obtained by digital subtraction (n=10). **c** Depolarization-activated transient outward current was abolished by removing K^+_o in different cells (n=8). *Arrows* zero current level

just positive to the membrane potential of −72 mV, as indicated by the voltage step from the holding current, and augmented as the depolarization potential was made more positive from −70 to −40 mV (left panel). The current showed voltage-dependent inward rectification in the voltage range from −30 to 0 mV (middle panel) and reached a steady-state level at potentials between +10 and +40 mV (right panel).

Figure 3b schematically illustrates the method of current measurement in a recording with 200-ms step to −30 mV from a holding potential of −80 mV and indicates the measurements of the I_{to} (I_{peak}) and the steady-state current (I_{SS}). Figure 3c shows the mean values of *I–V* relationships of the I_{to} and I_{SS} in 11 cells. The amplitude of the I_{to} increased on depolarization, reached a steady-state level at potentials positive to −20 mV, and showed an intermediate

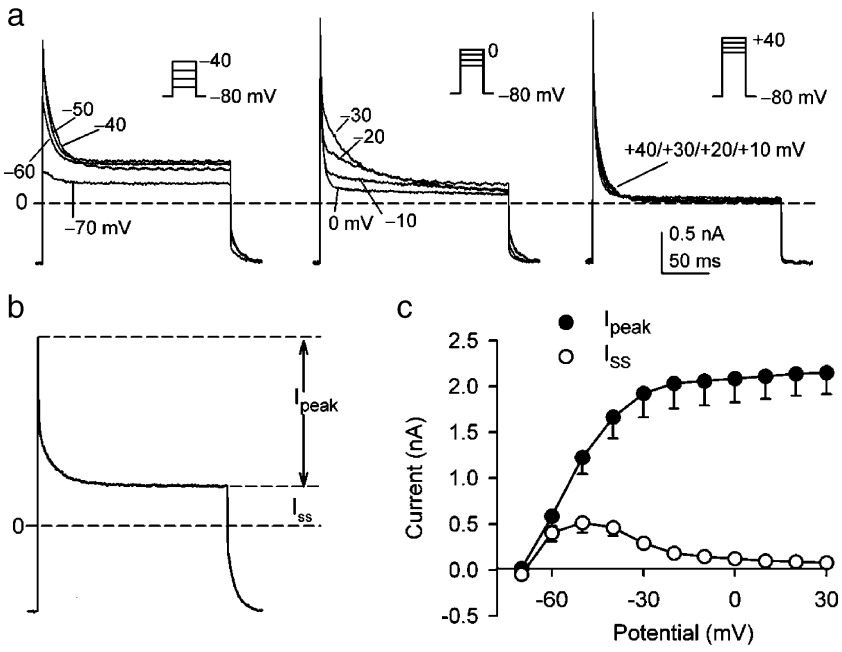

Fig. 3 Current–voltage (*I–V*) relations of depolarization-elicited currents. **a** Voltage dependence of depolarization-activated Ba^{2+}-sensitive current as shown in Fig. 2b. Current tracings are illustrated at potentials between −70 and −40 mV (*left*), between −30 and 0 mV (*middle*), and between +10 and +40 mV (*right*). **b** schematic showing current measurement in a Ba^{2+}-sensitive current tracing recorded with 300-ms step to −20 mV from a holding potential of −80 mV. I_{peak} time-dependent peak current, I_{SS} steady-state current. **c** *I–V* relationships of Ba^{2+}-sensitive currents (n=10) for I_{peak} and I_{SS}. The liquid junction potential was not corrected for the current recording and data analysis

inward rectification, whereas the *I–V* relationship for I_{SS} displayed a stronger inward rectification. These characteristics of I_{to} are similar to those of the $I_{to.ir}$ described previously in cardiac ventricular myocytes from dog [25] and guinea pig [26] hearts. These results suggest that Kir2.1 channel likely carries two components of outward currents upon depolarization. One is the steady-state current with strong inward rectification (i.e., classical I_{K1}) as widely observed in ventricular myocytes of mammalian hearts including those of humans [20]. Another is a transient outward current carried by Kir2.1 channel with intermediate inward rectification as $I_{to.ir}$ observed in ventricular myocytes of dog and guinea pig hearts [25, 26]. We therefore refer to this transient outward component as $I_{to.ir}$.

Time- and voltage-dependent kinetics of $I_{to.ir}$

Figure 4a shows the current trace recorded in a representative cell with a 200-ms voltage step from −80 to −20 mV. The transient outward component was best fitted to a bi-exponential function with time constants of 2.3 and 45.4 ms. The voltage dependence of the time constants τ_{fast} and τ_{slow} is shown in Fig. 4b. The fast inactivation τ_{fast} was voltage-dependent ($P<0.01$, $n=9$), and rate of rapid inactivation increased on depolarization to −20 mV. The voltage-dependence of slow inactivation τ_{slow} was not consistent, and rate of slow inactivation reduced only at −20, −10, and 0 mV ($P<0.05$ or $P<0.01$, $n=9$). The relative contributions of fast and slow components likely reflect the influence of Mg^{2+} and/or gating polyamines.

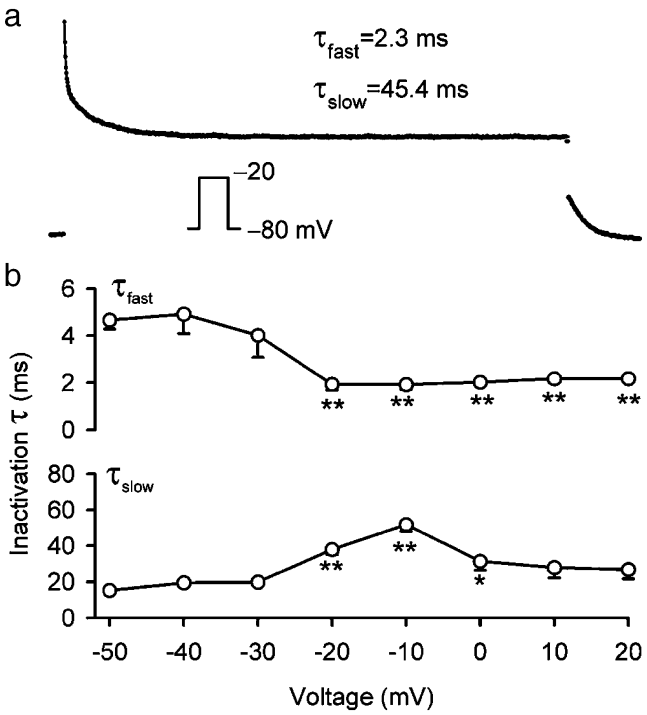

Fig. 4 Time-dependent inactivation of transient outward current. **a** Inactivation of the current on 200-ms depolarization from −80 to −20 mV was fitted to a bi-exponential function (*curve shown as solid line*, *points* are raw data) with time constants shown (τ_{fast} and τ_{slow}). **b** Voltage-dependence of time constants (τ_{fast} and τ_{slow}). ($n=9$, $^*P<0.05$, $^{**}P<0.01$ vs −50 mV)

To study the time-dependent recovery from inactivation and voltage-dependent availability (inactivation) of $I_{to.ir}$, we adopted the protocols used previously in dog and guinea pig ventricular myocytes [25, 26]. The recovery of $I_{to.ir}$ from inactivation was studied with a paired-pulse protocol (inset of Fig. 5a). The current during P2 (I_2) relative to the current during P1 (I_1) was determined as a function of the P1–P2 recovery interval. The recovery of $I_{to.ir}$ from inactivation was fitted to a monoexponential function with a time constant of 7.9±0.4 ms ($n=10$, Fig. 5b).

Figure 5c illustrates the voltage protocol used for determining the availability of $I_{to.ir}$ and current traces recorded. The variable of $I_{to.ir}$ availability was determined as the current at a given prepulse potential divided by the maximum $I_{to.ir}$ in the absence of a prepulse. Figure 5d shows the mean values of the channel availability fitted to Boltzmann distribution. The half potential ($V_{0.5}$) of $I_{to.ir}$ availability was −49.2±2.1 mV ($n=11$), and the slope factor was 15.2±0.6 mV. These results indicate that time- and voltage-dependent kinetics of $I_{to.ir}$ in HEK cells expressing Kir2.1 channel are similar to those of $I_{to.ir}$ in cardiac myocytes [25, 26].

Activation of $I_{to.ir}$ during cardiac action potential

The $I_{to.ir}$ in cardiac myocytes was activated by action potential waveform [25, 26]. To study whether it is the case for $I_{to.ir}$ carried by Kir2.1 channel stably expressed in HEK 293 cells, the action potential recorded from human right ventricular myocytes [22] was used as the protocol to record membrane current (Fig. 6a). Two components of outward current currents were activated by the action potential waveform. One was a transient outward component evident immediately after depolarization, which is likely contributed by $I_{to.ir}$. Another component was activated during the phase 3 repolarization of the action potential. The two components of outward currents were abolished by the application 200 μM Ba^{2+} (Fig. 6b) or omission of K^+_o (data not shown). Very little Ba^{2+}-sensitive current was present during the plateau of the action potential (Fig. 6c). Similar results were obtained in a total of 11 cells. These results were similar to those observed in ventricular myocytes from dog and/or guinea pig hearts [25, 26], suggesting that the $I_{to.ir}$ observed in cardiac ventricular myocytes is most likely carried by Kir2.1 channel.

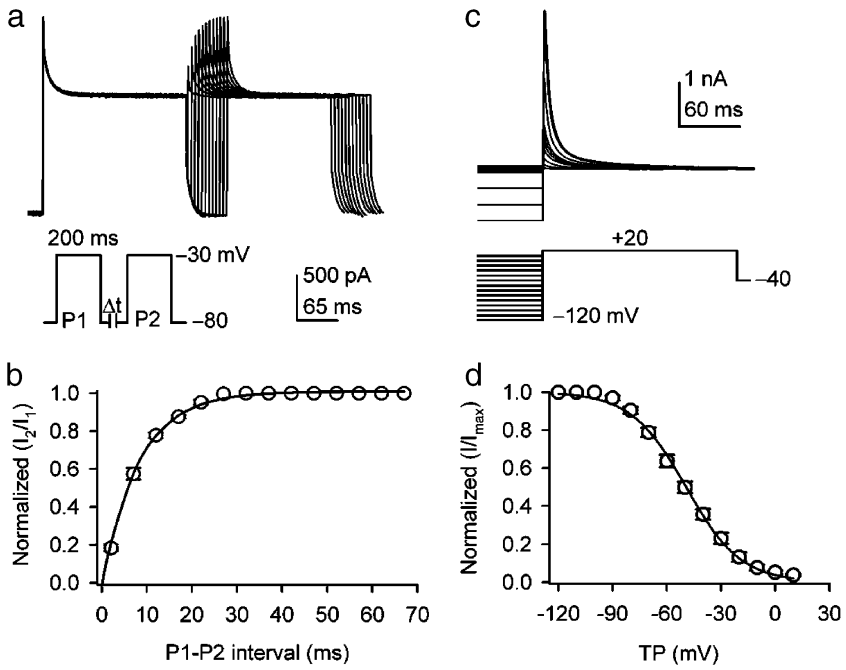

Fig. 5 Recovery from inactivation and voltage dependence of availability of $I_{to.ir}$. **a** Currents and protocol used to assess recovery of $I_{to.ir}$ from inactivation. Identical double pulses (200-ms, P1, and P2) from −80 to −30 mV (*inset*) were applied with a varying P1–P2 interval (Δt) delivered every 10 s. **b** Mean value of recovery curve (I_2/I_1) of $I_{to.ir}$ was fitted to a monoexponential function with a time constant of 7.9±0.4 ms (n=10). **c** Protocol and current traces used to assess the voltage dependence of steady-state inactivation (availability). Prepulses of 500-ms duration were applied to conditioning potentials between −120 and +10 mV, and currents were recorded during 200-ms test pulses to +20 mV. **d** Currents (I) recorded at +20 mV after 500-ms steps to the condition potential were normalized to the maximum current obtained (I_{max}). Data were fit to the Boltzmann relationship: $I/I_{max}=\{1+\exp[(V-V_{0.5})/S]\}^{-1}$, where V is the conditioning potential, $V_{0.5}$ is the potential for half-maximal inactivation (availability), and S is the slope factor. The $V_{0.5}$ of $I_{to.ir}$ availability was −49.2±2.1 mV (n=9)

Effects of intracellular free Mg^{2+} and spermine on $I_{to.ir}$

To examine whether $I_{to.ir}$ was affected by intracellular $Mg^{2+}{}_i$, we included different concentrations (0.03, 1, 4, or 8 mM) of free Mg^{2+} in the pipette solution. Figure 7a illustrates the currents recorded using the voltage steps as shown in the inset after 15 min dialysis for each concentration of free Mg^{2+} pipette solution. It is interesting to note that inactivation of $I_{to.ir}$ slowed with the increase of free $Mg^{2+}{}_i$. At −20 mV, an increase in inactivation τ_{fast} was observed at 8 mM free $Mg^{2+}{}_i$ (Fig. 7b, n=8, P<0.05 vs 0.03 mM), while increases in inactivation τ_{slow} were observed at 1, 4, and 8 mM free $Mg^{2+}{}_i$ (n=7–9, P<0.01 vs 0.03 mM free $Mg^{2+}{}_i$). Figure 7c displays the Ba^{2+}-sensitive current recorded in cells with action potential waveform using 0.03, 1, 4, or 8 mM free $Mg^{2+}{}_i$ pipette solutions. The action potential waveform protocol revealed that the duration of the component elicited by depolarization increased with the elevation of free $Mg^{2+}{}_i$ in the pipette solution; this is consistent with the slowing of $I_{to.ir}$ inactivation observed using the voltage step protocol (Fig. 7a,b). No significant change was observed for the second component activated by the repolarization during phase 3 of the action potential. Similar results were obtained in seven to nine cells for each free Mg^{2+} pipette solution concentration. These results indicate that $I_{to.ir}$ is greatly dependent on intracellular free Mg^{2+} concentration.

It is well established that spermine blocks the outward component of I_{K1} or Kir channel. To study whether spermine decreases $I_{to.ir}$, 100 μM spermine was included in a pipette solution containing 1 mM free $Mg^{2+}{}_i$. Spermine inclusion almost completely abolished $I_{to.ir}$ and reduced the steady-state component of Kir2.1 channel (Fig. 8a). Action-potential clamp voltage protocol displays that the initial component elicited by action potential depolarization was almost completely inhibited, and the second component activated by repolarization of the action potential was remarkably reduced by spermine inclusion; similar results were obtained in a total of eight HEK cells stably expressing human Kir2.1 channel. These results indicate that spermine blocks K^+ efflux through Kir2.1 channels, similar to those as described previously [11, 29, 42].

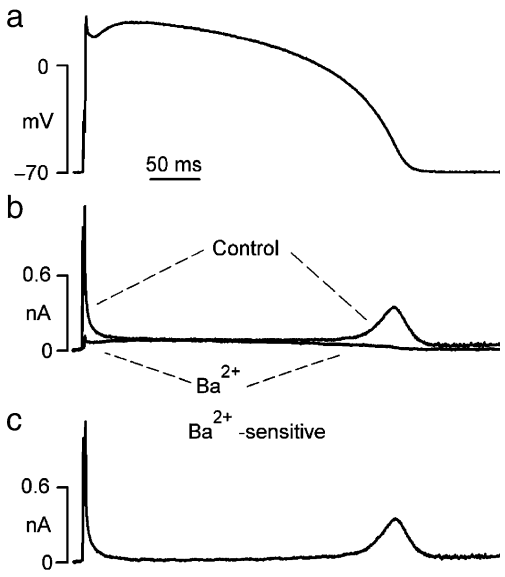

Fig. 6 Contribution of Kir2.1 channel currents to action potential. **a** Action potential recorded from a human ventricular myocyte was used as a voltage clamp protocol. **b** Currents activated by the action potential waveform before (*Control*) and after exposure to 200 μM Ba^{2+}. **c** Ba^{2+}-sensitive current during the action potential was obtained by digitally subtracting membrane currents before and after application of 200 μM Ba^{2+}. The early transient current is consistent with the properties of $I_{to.ir}$, as determined from square voltage steps. The transient current during phase 3 likely reflects the inwardly rectifying I_{K1}

Discussion

The present study demonstrates direct evidence that human Kir2.1 channel carries a transient outward potassium current with inward rectification. $I_{to.ir}$ is only present in HEK 293 cells stably expressing human Kir2.1 gene. Although a small 4-AP-sensitive I_{to} carried by A-type K^+ channels was reported in HEK 293 cells using a protocol with very negative holding potentials (−110 mV) [16], we did not observe the endogenous I_{to} with the protocol employed in the present study in HEK 293 cells that do not express human Kir2.1 channels (Fig. 1a). $I_{to.ir}$ was observed upon depolarization voltage steps only in cells stably expressing Kir2.1 gene (Fig. 1b,c) and was insensitive to the classical I_{to} blocker 4-AP (Fig. 2a); therefore, possible contamination of $I_{to.ir}$ by endogenous A-type K^+ channels should be very limited.

$I_{to.ir}$ was activated over a wide range of voltages positive to the membrane potential in HEK 293 cells stably expressing human Kir2.1 gene. The current was suppressed by the removal of K_o^+ or by application of Ba^{2+} (Figs. 1 and 2), indicating that the current is carried by human Kir2.1 channel. $I_{to.ir}$ inactivated rapidly after activation at more positive potentials (Figs. 3 and 4), recovered quickly from inactivation, and had an inactivation $V_{0.5}$ of −49 mV at normal K^+_o of 5 mM (Fig. 5). Action potential waveform protocol experiments revealed two components of outward currents. One component is immediately activated during

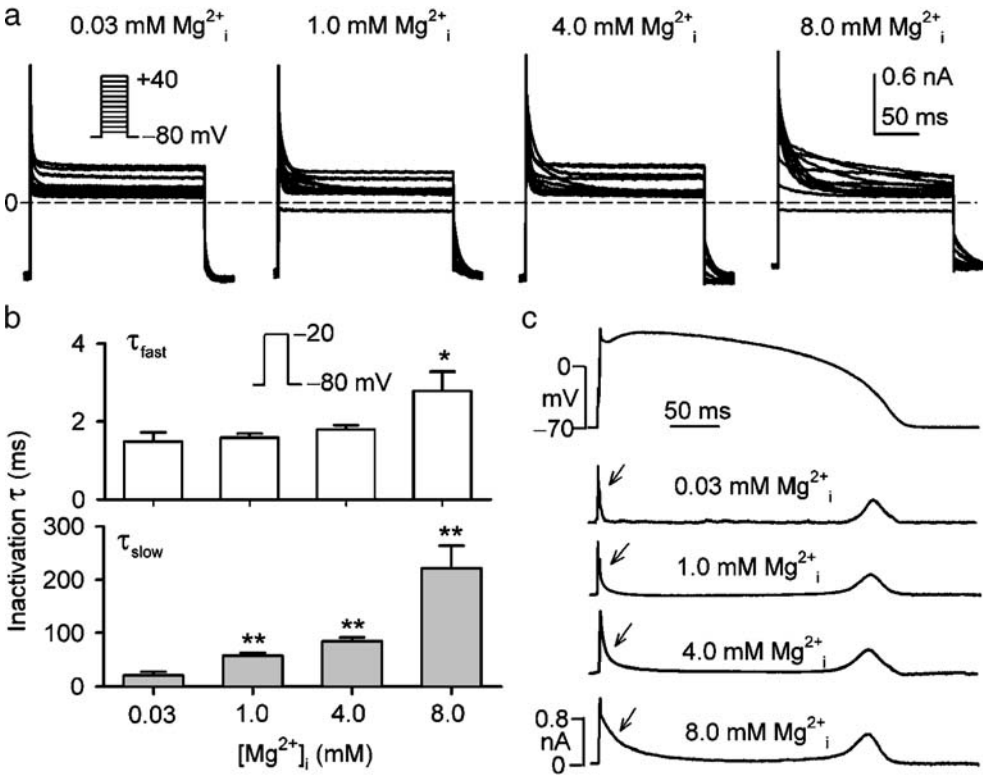

Fig. 7 Effect of intracellular free Mg^{2+} on $I_{to.ir}$. **a** Inactivation phase of $I_{to.ir}$ slowed with increasing free Mg^{2+}_i from 0.03, to 1.0, 4.0, and 8.0 mM. **b** Inactivation time constant (τ_{slow} and τ_{slow}, at −20 mV, n=7–10 for each group) increased (*P< 0.05; **P<0.01 vs 0.03 mM free Mg^{2+}_i). **c** Ba^{2+}-sensitive current elicited by action potential waveform, obtained by subtracting currents before and after application 200 μM Ba^{2+} with inclusion of different concentrations of free Mg^{2+}_i. Duration of the early transient current elicited by phase 1 of action potential increased with free Mg^{2+}_i elevation, consistent with the change of $I_{to.ir}$ inactivation determined from square voltage steps in **a**

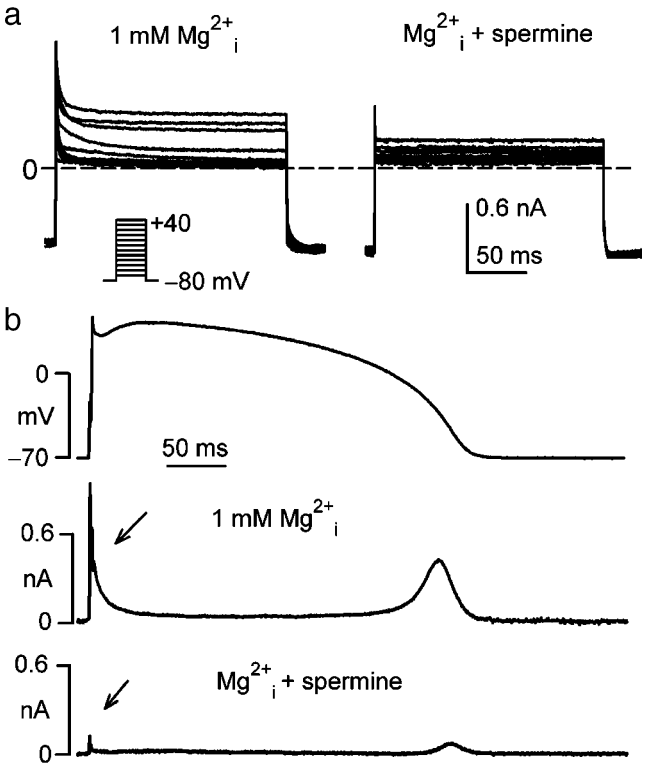

Fig. 8 Effect of spermine inclusion on $I_{to.ir}$. **a** Membrane current was recorded with the protocol shown in the *inset* with a pipette solution containing 1 mM free Mg^{2+} or 1 mM free Mg^{2+} plus 100 μM spermine. Spermine inhibited both $I_{to.ir}$ and I_{SS}. **b** Ba^{2+}-sensitive current elicited by the action potential waveform, obtained by subtracting currents before and after application 200 μM Ba^{2+} with a pipette solution containing 1 mM free $Mg^{2+}{}_i$ or 1 mM free $Mg^{2+}{}_i$ plus 100 μM spermine. No significant early transient current was observed during phase 1 of action potential, and only a small later transient current was recorded during phase 3

the depolarization phase and quickly inactivated at the plateau of the action potential. The other component is gradually activated during the repolarization (Fig. 6). These properties of $I_{to.ir}$ are similar to those of the $I_{to.ir}$ observed in dog [25] and guinea pig [26] ventricular myocytes. $I_{to.ir}$ was actually also observed in rabbit and human ventricular myocytes (the authors' unpublished observations).

Several properties of $I_{to.ir}$ recorded in the present study are similar to the $I_{to.ir}$ observed in native cardiac ventricular myocytes [25, 26]: (1) The current was sensitive to Ba^{2+} block or K^+_o removal; (2) the current displayed an intermediate inward rectification and a bi-exponential inactivation; (3) the current had similar values of availability potential ($V_{0.5}$) to $I_{to.ir}$ observed in native cardiac ventricular myocytes (−49.2 mV for $I_{to.ir}$ in this cell line, −44 mV and −51.6 mV for $I_{to.ir}$, respectively, in dog and guinea pig ventricular myocytes); (4) the current recovered quickly from inactivation (recovery τ: 7.9 ms for $I_{to.ir}$ in HEK cell line and 10.7 ms for $I_{to.ir}$ in cardiac myocytes); and (5) the current activated immediately upon depolarization of the cardiac action potential and inactivated quickly at plateau of the action potential (Fig. 6).

It is well recognized that I_{K1} in cardiac ventricular myocytes is dominantly contributed by Kir2.1 channels [8, 45], though possible Kir2.2 contribution is suggested [45]. $I_{to.ir}$ observed in native ventricular myocytes [25, 26] is most likely carried mainly by Kir2.1 channel. In addition, the transient outward current recorded using a low K^+ pipette solution in guinea pig ventricular myocytes [46] and the patch-duration-dependent 4-AP-insensitive I_{to} in cat ventricular myocytes [27] may also be carried by Kir2.1 channels. Therefore, molecular identity of the non-classical I_{to} observed under different conditions [25–27] is likely contributed mainly by Kir2.1 channel.

The previous arguments that $I_{to.ir}$ in native cardiac myocytes might be a novel current [25, 26] were based on the observation that the current is neither the classical I_{to1} or I_{to2}, and the classical concept that (1) the cardiac ventricular I_{K1} channel is considered to act as a diode [30] that activates only on hyperpolarization of the membrane [34]; (2) there is very little current passing through the channel in the outward direction under physiological conditions [30, 34]; and (3) I_{K1} was inactivated during the upstroke and plateau phases of the action potential and is consequently available for repolarization only during phase 3 [37]. However, studies by others [27, 46] and ours [25, 26] on native ventricular myocytes of different species, in addition to the present study of HEK 293 cells stably expressing human Kir2.1 channels (Figs. 2 and 5), indicate that cardiac I_{K1} can be rapidly activated during depolarization, rapidly inactivated at plateau of the action potential, and re-activated gradually at phase 3 of the action potential (Figs. 6 and 7). Therefore, the contribution of cardiac I_{K1} during the depolarization of cardiac action potential should be revisited.

We found that the amplitudes of both $I_{to.ir}$ and I_{SS} were significantly inhibited by application of spermine in the pipette solution (Fig. 8); this supports the notion that spermine blocks cardiac I_{K1} or Kir2.1 [11, 29, 42]. However, inactivation of $I_{to.ir}$ was remarkably regulated by different concentrations of free Mg^{2+} in the pipette solution (Fig. 7), although our previous studies did not find any effect of 5 μM spermine inclusion or Mg^{2+} omission in the pipette on $I_{to.ir}$ in native cardiac myocytes with 10 min dialysis [25, 26].

The reports for the effect of free $Mg^{2+}{}_i$ on outward current of classical cardiac I_{K1} or Kir2.1 channel were controversial and dependent on various experimental conditions. It seems that the $Mg^{2+}{}_i$ block of outward component in I_{K1} or Kir2.1 channels is usually observed under conditions where the channel conductance is increased when high K^+_o or symmetrical K^+ condition is applied to record the current [15, 30] but not under conditions where physiological K^+_o is employed in cardiac

myocytes [28, 38]. The conductance increase of I_{K1} or Kir2.1 channels by a high K^+_o or symmetrical K^+ is likely related to the alteration of channel conformation, which may be highly sensitive to internal block by Mg^{2+}_i.

In the present study, a physiological concentration of K^+_o was used to record Kir2.1 channel current; no evidence of Mg^{2+} blocking effect was observed for this current. Instead, inactivation of $I_{to.ir}$ carried by Kir2.1 channel greatly slowed with the increase of free Mg^{2+}_i (Fig. 7a,b); the component elicited by phase 0 depolarization of the action potential significantly increased as the elevation of free Mg^{2+}_i from 0.03 to 1.0, 4.0, and 8.0 mM without affecting the component activated by phase 3 repolarization of the action potential; and the increase of free Mg^{2+}_i makes the current last long enough to contribute to phase 1 repolarization (Fig. 7c). Presumably, this is likely a complex interaction between Mg^{2+} and polyamines, with Mg^{2+} moving in and out of the channel with sufficient rapidity that current can flow but occupying enough to prevent polyamines like spermine from blocking.

It has been reported that cardiac physiological free Mg^{2+}_i is in the range of 0.6–1.1 mM [5, 14, 32]. Thus, duration of $I_{to.ir}$ is significant at physiological free Mg^{2+}_i (Figs. 6 and 7c). Therefore, $I_{to.ir}$ should contribute to action potential during the depolarization under physiological conditions. Due to its rapid activation and inactivation during the depolarization of the channel demonstrated in the previous reports [25, 26] and in the present study, the transient outward component of cardiac I_{K1} should make a significant contribution to K^+ efflux during phase 1 of action potential. The rapid recovery of the transient outward of I_{K1} from inactivation suggests that its contribution to the action potential should be independent of the heart rate.

However, the potential significance of the Mg^{2+}_i-dependent depolarization-activated component ($I_{to.ir}$) of I_{K1} had been ignored for a long time because non-physiological K^+_o and/or non-physiological Mg^{2+}_i were used for most previous studies. For instance, if 1 mM $MgCl_2$ is included in a pipette solution containing 5 mM K-ATP, 5 mM EGTA, and 0.1 mM GTP, the calculated free Mg^{2+} is only 0.014 mM using the Cabuf software (http://www.kuleuven.be/fysiol/trp/cabuf). At <0.03 mM free Mg^{2+} in pipette solution, $I_{to.ir}$ is quickly inactivated and is not easily differentiated from capacitative transient (Fig. 7a). Based on the Cabuf software, most Mg^{2+} molecules bind to ATP. In the present and our previous studies, Mg-ATP and 1 mM $MgCl_2$ were applied in pipette solution, and the Mg-ATP may not require Mg^{2+} molecules to bind, in which significant $I_{to.ir}$ was demonstrated [25, 26].

Cell excitability has generally been associated with the ability of inward currents to generate an action potential upstroke. The classical component of I_{K1} plays a role in the excitability of cardiac cells by stabilizing the resting potential [7, 33]. Because significant outward current carried by $I_{to.ir}$ can be elicited by depolarization at very negative potentials over a time course comparable to I_{Na}, $I_{to.ir}$ may play a role in maintaining cardiac excitability, especially under conditions where I_{Na} is reduced, such as myocardial ischemia. Earlier work by Murphy et al. demonstrated that cytosolic free Mg^{2+}_i level increased to >2.0 mM during cardiac ischemia [32]. Another study suggested that voltage-dependent changes in V_{max} (maximal velocity) of action potential with increased $[K^+]_o$ are poorly explained by changes in I_{Na} [41]. This discrepancy may be due to a participation of $I_{to.ir}$ in determining the V_{max}, which becomes particularly important when I_{Na} is reduced.

In summary, human Kir2.1 channel carries a transient outward current ($I_{to.ir}$) in HEK 293 cells stably expressing the Kir2.1 gene. Properties of the current are similar to those of $I_{to.ir}$ observed in dog and guinea pig ventricular myocytes. Therefore, the present study provides the direct confirmatory information that $I_{to.ir}$ observed in the present study and $I_{to.ir}$ previously observed in native cardiac myocytes shares the same molecular identity. Inactivation of $I_{to.ir}$ is strongly dependent on free Mg^{2+}_i. In native cardiac myocytes, the depolarization-activated $I_{to.ir}$ contributes significantly to phase 1 repolarization of action potential [25, 26] and may play an important role in maintaining cardiac excitability.

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