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Gating Mechanism of the Cloned Inward Rectifier Potassium Channel from Mouse Heart

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Abstract. The complementary DNA encoding the inward rectifier potassium channel was cloned from the adult mouse heart by using the polymerase chain reaction. The clone had the nucleotide sequence identical to that of the IRK1 gene cloned from a mouse macrophage cell line. Northern blot analysis revealed that the transcript of this gene was mainly expressed in the ventricle, where the inward rectifier K^+ channel plays a predominant role in maintaining the high negative value of the resting membrane potential. The current expressed by injection of the complementary RNA of the cloned gene into *Xenopus* oocytes showed a marked inward rectification that depends on the driving force of K^+. A region of negative slope conductance was observed in the current-voltage relationship at potentials positive to the reversal potential. When the extracellular K^+ concentration was raised, the increase in outward current amplitude resulted in the "crossover" of outward current-voltage relations. The fast time-dependent increase in current amplitude was recorded upon membrane repolarization from a potential positive to the reversal potential. The kinetics of the time-dependent current was very similar to that of the intrinsic gating mechanism of the native cardiac inward rectifier K^+ channel. Our results suggest the existence of the intrinsic gating mechanism, accounting for the extent of rectification in the current-voltage relationship in the expressed channel.

Key words: Inward rectification — K current — Gating kinetics — *Xenopus* oocytes — IRK1

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Introduction

In the heart, the inward rectifier K^+ channel generates the background conductance in Purkinje fibers and ventricular muscles (Kameyama, Kiyosue & Soejima, 1983; Noma et al., 1984; Sakmann & Trube, 1984; Hume & Uehara, 1985). The current-voltage (*I-V*) relationship of this channel plays an important role not only in maintaining the well-polarized resting membrane potential but also in determining the shape of the action potential (Carmeliet & Vereecke, 1979; DiFrancesco & Noble, 1985; Hume & Uehara, 1985). The decrease in conductance at depolarized potentials helps maintain the long plateau phase, and the outward current that flows at membrane potentials positive to the reversal potential (E_{rev}) facilitates the final repolarization of the action potential.

The inward rectifier K^+ channels are present in a variety of cell types and have been studied extensively in skeletal muscle (Hestrin, 1981; Leech & Stanfield, 1981) and egg cells (Hagiwara & Takahashi, 1974; Hagiwara, Miyazaki & Rosenthal, 1976; Hagiwara & Yoshii, 1979). The rectification property of this current is unique in that it depends on the driving force of K^+ when the extracellular K^+ concentration ($[K^+]_o$) is altered, and in that the suppression of the outward current upon membrane depolarization is virtually instantaneous. In guinea-pig cardiac myocytes, both the blockade of the channel pore by intracellular Mg^{2+} and the intrinsic gating are considered to be the major mechanisms of the rectification property (Matsuda, Saigusa & Irisawa, 1987; Vandenberg, 1987; Matsuda, 1988; Ishihara et al., 1989; Matsuda & Cruz, 1993). The virtually instantaneous suppression of the outward current on membrane depolarization is due to the Mg^{2+} block of the channel. However, the blocked channels transit to the closed state during depolarization, and the extent of rectification is mainly determined by the gating kinetics in the steady-state (Ishihara et al., 1989).

Recently, a new family of genes was shown to encode K^+ channels which display the property of inward rectification (Ho et al., 1993; Kubo et al., 1993*a,b*). They are distinctive among known channels in that they have only two membrane-spanning domains, yet they contain the conserved region for providing the K^+ channel pore in the proposed topology. When expressed in *Xenopus* oocytes, one of these genes, IRK1, cloned from the mouse macrophage cell line (J774.1), generated the protein which showed very close electrophysiological properties to those of the inward rectifier K^+ channel in cardiac myocytes (Kubo et al., 1993*a*). To understand the molecular mechanism of the inward rectifier K^+ channel, it is of great interest to study the structure-function relationship using the gene encoding this channel obtained from cardiac tissues.

In the present study, the expression of the IRK1 gene in the heart was confirmed by using the polymerase chain reaction (PCR). The expression pattern of the gene in the heart was examined by Northern blot analysis. It showed a higher expression of the gene in the ventricle, supporting the idea that the protein encoded by IRK1 is the main unit of the inward rectifier K^+ channel in the heart. We have performed electrophysiological experiments using stage III and IV oocytes and recorded the fast activation phase of the expressed current to compare the biophysical properties of the expressed channel with those of the native inward rectifier K^+ channel.

Materials and Methods

Northern Blotting

Total RNA was isolated from a cultured J774.1 mouse macrophage cell line (a gift from the Japanese Cancer Research Resources Bank) or various tissues by the acid guanidine-thiocyanate-phenol-chloroform method (Chomczynski & Sacchi, 1987). The internal 571-bp region of the mouse macrophage IRK1 gene (sequence position at 165 to 735, Kubo et al., 1993*a*) was amplified by PCR from the first strand complementary DNA (cDNA) synthesized from J774.1 RNA, sequenced and used as the cDNA probe. Northern blot hybridization was performed by the standard method with minor modifications (Sambrook, Fritsch & Maniatis, 1989). Briefly, RNA (10 μg per lane) was size-fractionated by formaldehyde gel electrophoresis and transferred to a nylon membrane by capillary elution. To check the loading and transfer efficiency, each RNA sample contained 1 μl of ethidium bromide (400 μg/ml) routinely and the 18 and 28 S RNA bands that were of equivalent intensity in all lanes were visualized on the filter. Filter was baked at 80°C for 2 hr and prehybridized at 42°C for 4 hr in 50% formamide, 6× SSPE, 5× Denhardt's solution, 0.5% SDS and 0.1 mg/ml of salmon sperm DNA. Subsequently, the IRK1 cDNA probes, labeled with ^{32}P by random priming, were added to the fresh hybridization solution, and the filter was hybridized overnight at 42°C. It was then washed under a stringent condition (1× SSPE, 0.1% SDS at room temperature, then at 65°C). Expression of the IRK1 gene was detected by autoradiography.

Cloning of the Inward Rectifier K^+ Channel cDNA

To isolate IRK1 transcripts expressed in the heart, PCR was performed. First strand cDNA was synthesized from total RNA of the Balb/c mouse heart or the rat heart by using reverse transcriptase and used as templates for PCR. To amplify the entire coding region of the gene, oligonucleotide primers were synthesized based on the nucleic acid sequence of macrophage IRK1 (Kubo et al., 1993*a*); the 5′ oligonucleotide primer consisted of the sequence, −8 to +14, while the 3′ primer consisted of complementary nucleotides of the sequence, +1312 to +1332 within the 3′ noncoding region. To detect the closely related gene in the rat heart, degenerative primers were designed based on the sequences of IRK1 and ROMK1, another member of the gene family (Ho et al., 1993). They were AA(AG)GAIGGI(AC)GITG (TC)AA for the 3′ primer and (AG)TTIAT(AG)T(TC)I(AG)T(TC)TG (AG)TC for the 5′ primer. The PCR products were subcloned into pGEM-T (Promega, Madison, WI), and sequences of the clones were determined by using Kilo-Sequence Deletion Kit (Takara Shuzo, Japan) and Taq dye primer cycle sequencing kit (Applied Biosystems, Japan).

In Vitro Transcription and Oocyte Injection

The cDNA encoding the entire coding region of IRK1 obtained from the mouse heart was used for the expression study. The cDNA subcloned into pBluescript II KS (Strategene, San Diego, CA) was linearized at the 3′ end, and complementary RNA (cRNA) was synthesized with T7 RNA polymerase in the presence of m7GpppG cap analogue.

Adult female *Xenopus laevis* were purchased from Hamamatsu Seibutsu Kyozai (Japan). Following surgical removal from frogs, oocytes were treated with collagenase at 1 mg/ml (Boehringer Mannheim, Germany) in Ca^{2+}-free Barth's solution for 2 hr to remove overlying follicle cells. Stage V and VI oocytes (Dumont, 1972) were injected with 50 nl of the cRNA at a concentration of 5 ng/μl by using the previously described method (Yamane et al., 1993), except for the experiments in Figs. 3 and 4, in which cRNA of 100 ng/μl were injected. In recording the fast time-dependent change of the current on voltage change in the membrane, stage III and IV oocytes were selected, which have smaller capacitances compared to stage V and VI oocytes, and 20 nl of the cRNA solution (5 ng/μl) was injected using the same apparatus. Oocytes were incubated for 1–6 days in Barth's solution and tested for the functional expression of channels.

The composition of Barth's solution was (in mM): NaCl, 88; KCl, 1; $NaHCO_3$, 2.4; $MgSO_4$, 0.8; $CaCl_2$, 0.6; $Ca(NO_3)_2$, 0.3; and pH was adjusted to 7.6 with HCl. $CaCl_2$ and $Ca(NO_3)_2$ were omitted for Ca^{2+}-free Barth's solution.

Electrophysiology

Oocytes were impaled with two glass pipettes (Clark Electromedical Instruments, England), filled with 3 M KCl and voltage-clamped using a CA-1a amplifier (Dagan, Minneapolis, MN), which incorporates the bath-clamp circuit to prevent the voltage drop across the bath electrode when the current amplitude is substantially large. To facilitate the rise time of voltage steps, pipettes which have resistance lower than 0.2 MΩ were used. Voltage stimulation and data acquisition were performed using pCLAMP software (ver. 5.5.1, Axon Instruments, Foster City, CA) on an IBM computer (personal system/2, Model 30). The sampling rate for recording time-dependent currents in Figs. 6 and 7 was 100 kHz. Data were analyzed and plotted using pCLAMP and SigmaPlot (Jandel Scientific, San Rafael, CA) software. Neither ca-

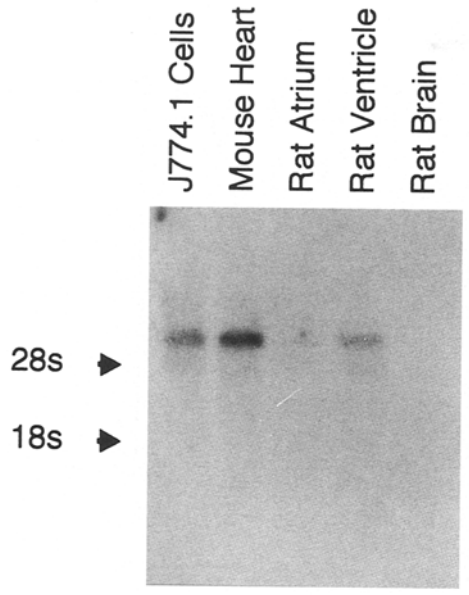

Fig. 1. Expression of IRK1 in cardiac tissues. Ten micrograms of total RNA derived from J774.1 mouse macrophage cells, mouse whole heart, rat atrium, rat ventricle and rat brain were blotted on a filter and probed with a random-labeled cDNA fragment of IRK1. The mobilities of 28 and 18 S ribosomal RNA are given on the left.

pacitive nor leakage currents were subtracted from current traces in this study. Experiments were performed at room temperature (20–22°C).

The advantage of using oocytes in stage III and IV was assessed by measuring the total membrane capacitance (C_m) and the time course of capacitive currents. Capacitive currents were recorded by applying small (10 or 20 mV) depolarizing voltage steps from a holding potential of −30 mV at 10 mM $[K^+]_o$. C_m was estimated by integrating the capacitive transient, and the time constant of capacitive current was obtained by fitting a single exponential curve. In stage V oocytes (around 1.0 mm in diameter, $n = 4$), C_m were 161.0 ± 11.4 nF (mean ± SD) and the time constants were 0.36 ± 0.04 msec. In stage III and IV oocytes (0.4–0.7 mm in diameter, $n = 10$), values of C_m ranged between 25 and 79 nF (56 ± 18.1 nF) and the time constants ranged from 0.05 to 0.22 msec (0.15 ± 0.05 msec).

The standard external solution for recording the inward rectifier K^+ current contained (in mM): KCl, 10; *N*-methyl-D(−)-glucamine (NMG), 88; $MgCl_2$, 3; HEPES, 5; and pH was adjusted to 7.4 with HCl. When $[K^+]_o$ was increased, isomolar NMG was replaced with KCl. The endogenous Ca^{2+}-activated Cl^- current was not noticeable by using Ca^{2+}-free external solutions, especially when stage III and IV oocytes were used (Krafte & Lester, 1989).

Results

Expression of the Inward Rectifier K^+ Channel Gene in Cardiac Tissue

To determine the pattern of IRK1 expression in cardiac tissue, Northern blot analysis was performed (Fig. 1). Total RNA derived from the mouse whole heart, the rat atrium and rat ventricle were blotted on a filter, together with RNA from J774.1 mouse macrophage cells (positive control) and the rat brain. The region of IRK1, which encodes two proposed membrane-spanning segments, M1 and M2, and the pore forming region, H5, was used to probe the filter. After washing stringently, a single band of ~5.5 kb was clearly observed in the mouse whole heart lane, which was the same size as the band seen in the macrophage lane. A band with the same size was also observed in the rat ventricle, but not in the rat atrium lane. By PCR using degenerative primers, a cDNA clone of 571 bp was amplified from the rat ventricle, which had a 94.2% similarity at the nucleic acid level to the corresponding region of mouse IRK1 (*data not shown*). The deduced amino acid sequence of the cDNA clone, which contained M1, M2 and H5 regions, was identical to that of mouse IRK1, supporting that the 5.5 kb band in the rat ventricle lane represents the expression of the rat gene corresponding to IRK1. These results indicate that the IRK1 gene is expressed abundantly in the ventricle, but much less, if at all, in the atrium.

To ascertain that the transcripts expressed in the mouse heart do not possess any sequence differences from macrophage IRK1, which may be generated by the alternative splicing (Perez-Reyes et al., 1990), a cDNA containing the complete coding region of IRK1 was amplified by PCR using the first strand cDNA synthesized from mouse heart RNA as templates. When the PCR product was analyzed in the agarose gel electrophoresis, a single band was observed. The cloned cDNA fragment had the identical nucleotide sequence to that of macrophage IRK1 at nucleotide −8 to +1332 (Kubo et al., 1993*a*). The clone thus obtained from the mouse heart was used for the following expression study.

Marked Inward Rectification in the Current-Voltage Relationship of the Expressed Current

Oocytes were tested for the expression of inward rectifier K^+ channels 1–6 days after the injection of cRNA. The expression of the channels was easily noticed by measuring the resting membrane potential of the oocytes after inserting the electrode. When the channels were expressed, the potential was −52.5 ± 2.9 mV (mean ± SD, $n = 11$) with the standard external solution (Ca^{2+}-free, 10 mM $[K^+]_o$, whereas it ranged between −30 to −10 mV (−21.1 ± 7.7 mV, $n = 10$) in the same solution when the expression of exogenous channels was negligible. The resting membrane potential in the oocytes expressing the channels agreed well with the equilibrium potential for K^+ (E_K), calculated by assuming the internal K^+ concentration ($[K^+]_i$) in the oocytes was around 90 mM (Barish, 1983). When the membrane potential was stepped to various levels from a holding potential of −30 mV,

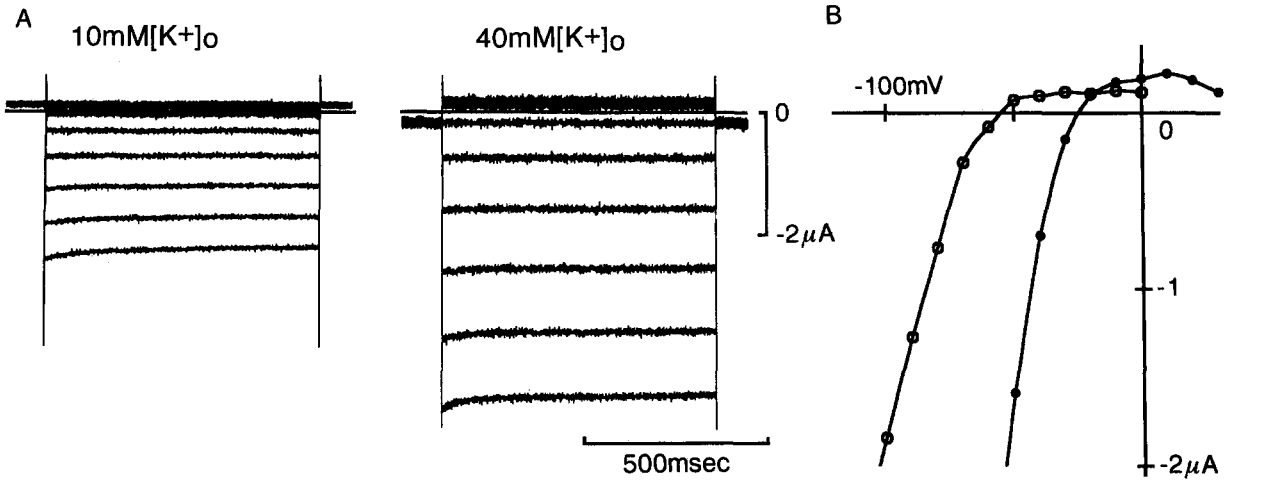

Fig. 2. Inward rectifier K^+ currents expressed by injection of the IRK1-cRNA into the oocyte. Fifty nanoliters of the cRNA at a concentration of 5 ng/μl were injected into the stage V oocyte and the expressed currents recorded one day after injection. (*A*) Families of currents elicited by 650 msec voltage steps from a holding potential of −30 mV at 10 mM (left) and 40 mM (right) $[K^+]_o$. Voltage steps were 0, −30, −50, −60, −70, −80, −90 and −100 mV from top to bottom (left), and 30, 10, −20, −30, −40, −50, −60, −70 and −80 mV (right). (*B*) The isochronal *I-V* relationship measured just after the capacitive transient at each voltage step in *A* (open circles, 10 mM; filled circles, 40 mM $[K^+]_o$). Note that there are "crossover" in the *I-V* curves and a slight negative slope at potentials positive to E_{rev}.

marked inward rectification was obvious in the current amplitudes. Figure 2*A* shows representative current traces recorded at 10 and 40 mM $[K^+]_o$ in the same oocyte. The isochronal *I-V* relationships obtained by measuring the current level after the capacitive transient in each voltage step were plotted in Fig. 2*B*. E_{rev} in each curve was close to the estimated E_K at each $[K^+]_o$. At membrane potentials positive to E_{rev}, the inward rectification was prominent and a slight negative slope was present in the *I-V* curve. Furthermore, the amplitude of the outward current increased by increasing $[K^+]_o$, resulting in the "crossover" of the two *I-V* curves. In the experiments in which different $[K^+]_o$ was tested, shifts in E_{rev} were equivalent to 24.7 ± 1.6 mV ($n = 8$) shift by an *e*-fold change in $[K^+]_o$, indicating that K^+ is the main charge carrier of these currents. The apparent "crossover" phenomenon was appreciated in three experiments.

Blocking Effect of the Expressed Current by Ba^{2+} and Cs^+

We next examined the blocking effect of the expressed current by external Ba^{2+} and Cs^+ at 20 mM $[K^+]_o$. Figure 3*A* shows families of current traces recorded at various concentrations of Ba^{2+}. When 5 or 50 μM of Ba^{2+} was tested, the voltage-and time-dependent block of the currents was clearly observed, as has been previously reported for the IRK1-expressed current (Kubo et al., 1993*a*; Morishige et al., 1993). At 500 μM or 5 mM, the blocking effect of Ba^{2+} was so fast that the currents were hardly time-dependent on this time scale. At these concentrations, both the inward and outward currents were suppressed, leading to an almost linear relationship in the *I-V* curve (Fig. 3*B*). Similar results were obtained in nine experiments at 10 or 20 mM $[K^+]_o$. Figure 4 shows the blocking effect of the expressed current by external Cs^+. Cs^+ also blocked the inward currents in a voltage-dependent manner, but the outward currents were resistant to 5 mM of Cs^+ ($n = 10$). K^+-free external solution suppressed both inward and outward currents ($n = 4$, *data not shown*).

The findings shown above agree with the characteristics observed in the native cardiac inward rectifier K^+ channels (Hume & Uehara, 1985; Tourneur et al., 1987; Hirano & Hiraoka, 1988). We further analyzed the expressed current in detail to see if there were any differences in properties between the expressed channel and the native channel.

The Dependence of the Chord Conductance on E_K

One of the unique characteristics of the native inward rectifier K^+ current is that the conductance depends on the difference between the membrane potential and E_K, when $[K^+]_o$ is altered (Hagiwara & Takahashi, 1974; Hestrin, 1981; Harvey & Ten Eick, 1988). To test whether this is reproduced in the expressed current or not, the chord conductance defined by the following equation was calculated from *I-V* relationships.

$$gK = I/(V_m - E_{rev}), \quad (1)$$

where I is the current amplitude and V_m the membrane

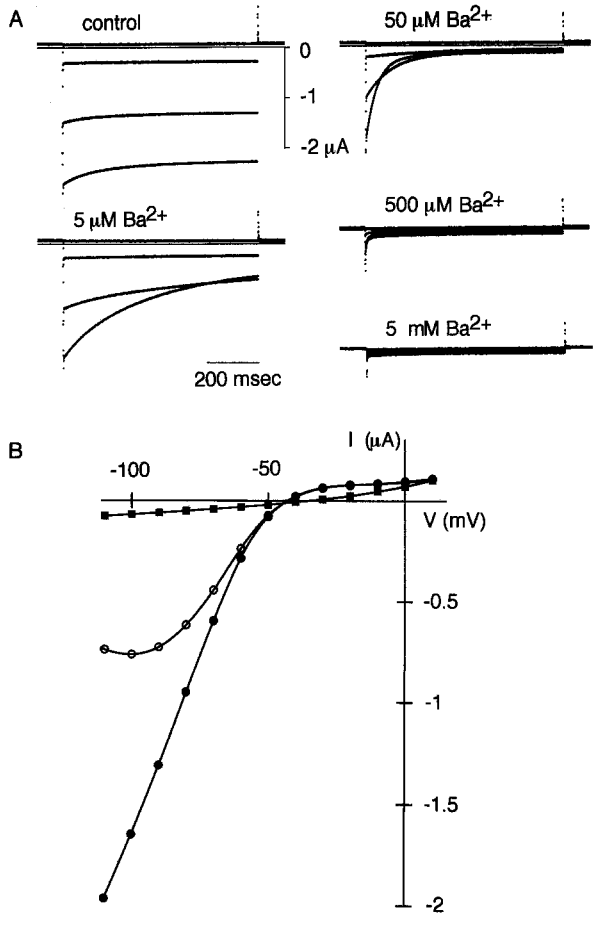

Fig. 3. Time- and voltage-dependent block of the expressed current by external Ba^{2+}. (*A*) Current traces recorded at different concentrations of Ba^{2+}. The concentration of Ba^{2+} applied to the external solution is indicated on the top of each family of traces. Currents were elicited by stepping the membrane to −120, −90, −60 and −30 mV from a holding potential of −20 mV. Horizontal lines indicate the zero current level. $[K^+]_o$ = 20 mM. (*B*) Isochronal *I-V* relationships obtained from the same experiment shown in *A*. Filled circles, control; open circles, 5 μM Ba^{2+}; squares, 5 mM Ba^{2+}. Current amplitudes were measured at the end of 780 msec step pulses. To show the suppression of outward currents by 5 mM of Ba^{2+} clearly, only relationships obtained at 5 μM and 5 mM Ba^{2+} are compared with the control curve.

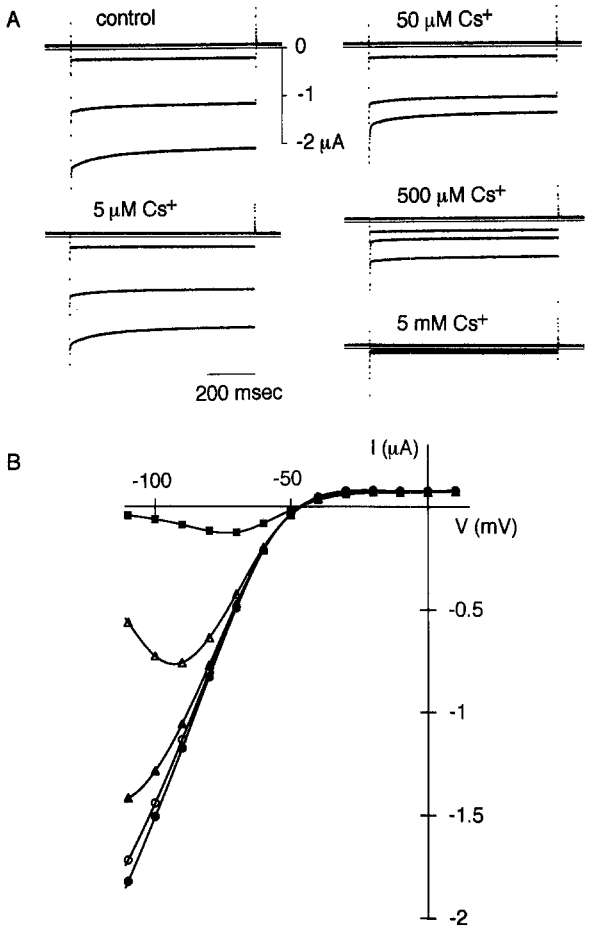

Fig. 4. Voltage-dependent block of the expressed current by external Cs^+. (*A*) Current traces recorded at different concentrations of Cs^+. The concentration of Cs^+ applied to the external solution is indicated on the top of each family of traces. The currents were elicited by stepping the membrane to −120, −90, −60, −30 and 0 mV from a holding potential of −20 mV. Horizontal lines indicate the zero current level. $[K^+]_o$ = 20 mM. (*B*) Isochronal *I-V* relationships obtained from the same experiment shown in *A*. Filled circles, control; open circles, 5 μM Cs^+; filled triangles, 50 μM Cs^+; open triangles, 500 μM Cs^+; squares, 5 mM Cs^+. Current amplitudes were measured at the end of 780 msec step pulses. Note that the outward currents were not blocked by external Cs^+.

potential. The experimental results showing little leakage current were selected, in which E_{rev} was close to the estimated E_K as in Fig. 2. The chord conductance obtained at 10 and 90 mM $[K^+]_o$ in the same cell was plotted against the membrane potentials in Fig. 5*A*. The relationship showed a sigmoidal voltage dependence, and the curve shifted to the right along with the shift in E_K by 54 mV when $[K^+]_o$ was raised from 10 to 90 mM. In Fig. 5*B*, the chord conductance obtained at various $[K^+]_o$ was expressed as a fraction of the maximum conductance in each experiment and plotted against the difference (ΔV) between the membrane potential and E_{rev} (n = 7). This clearly shows that the extent of rectification depends on ΔV, when E_K is altered by changing $[K^+]_o$.

The Time-dependent Activation of the Current on Hyperpolarization

In the macroscopic current, the suppression of the inward rectifier K^+ current upon membrane depolarization is virtually instantaneous, whereas the current change on membrane hyperpolarization shows a time-dependent activation following an instantaneous current jump (Hagiwara et al., 1976; Hestrin, 1981; Leech & Stanfield, 1981). We have shown that the time-dependent increase of the current upon membrane hyperpolarization reflects the kinetics of the intrinsic gating and that the relief of the channel by Mg^{2+} block is practically instantaneous in guinea-pig cardiac myocytes (Ishihara et al., 1989). To

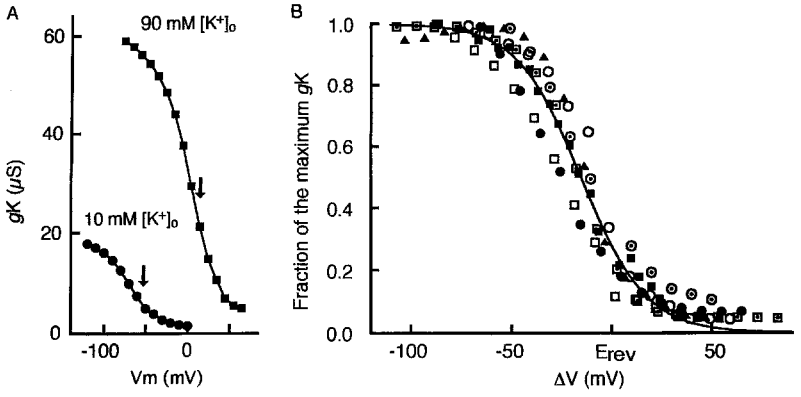

Fig. 5. (*A*) Relationship between the chord conductance, gK, and the membrane potential obtained at two different $[K^+]_o$ in the same cell (circles, 10 mM; triangles, 90 mM $[K^+]_o$). Arrows indicate E_{rev} in each experiment. (*B*) Relationship between gK, expressed as a fraction of the maximum gK in each experiment, and ΔV. Data were obtained at various $[K^+]_o$ (circles, 10 mM; triangles, 40 mM; squares, 90 mM $[K^+]_o$). Different symbols indicate different experiments (n = 7). Continuous curve was drawn using Eq. (3).

test whether or not the expressed channels in oocytes possess the intrinsic gating mechanism, current changes upon membrane hyperpolarization were recorded at a high sampling frequency in stage III and IV oocytes.

Figure 6*A* shows superimposed current traces recorded in the same oocyte by stepping the membrane potential to various levels from a holding potential of 10 and 40 mV, at 10 and 90 mM $[K^+]_o$, respectively. When the membrane was hyperpolarized to potentials negative to E_{rev}, a time-dependent increase was observed in the inward current. The time course of activation was approximated by a single exponential function at each membrane potential and the time constant decreased with hyperpolarization in the membrane. In Fig. 6*B*, time constants of the activation obtained at various $[K^+]_o$ were plotted against the membrane potential (left) and ΔV (right). It is obvious that the time constant depends on ΔV when E_K is altered by changing $[K^+]_o$. The relationship in the right graph (n = 7) was fitted by an equation,

$$\tau = K \exp(\Delta V/s), \tag{2}$$

where τ is the time constant in msec and s a slope factor. The values for K and s were 5.1 msec and 14.7 mV, respectively.

The Dependence of the Closed Channel Probability on E_K

In guinea-pig ventricular cells, the closed state probability of the inward rectifier K^+ channels at potentials positive to E_{rev} is determined by both the intrinsic gating kinetics and the voltage-dependent Mg^{2+} blockade. At potentials around E_{rev}, however, the steady-state closed channel probability is mainly determined by the gating mechanism (Ishihara et al., 1989). This property was also examined in the expressed channel. When the membrane was hyperpolarized from various test potentials, the amplitude of the time-dependent inward current varied depending on the probability of the channels to be in the closed state in the test potential. The current traces shown in Fig. 7*A* were recorded on hyperpolarizing voltage steps from three different conditioning potentials at 20 mM (left) and 90 mM (right) $[K^+]_o$. ΔV in the conditioning potentials were similar in the same row and the ΔV in the hyperpolarizing steps were E_{rev}-35 mV in the left and E_{rev}-38 mV in the right column. The time course of activation could be approximated by a single exponential function with the same time constant regardless of the conditioning potential at each $[K^+]_o$. The amplitude of the time-dependent component developed by increasing ΔV in the conditioning potential at both $[K^+]_o$, indicating the increase in the closed channel population.

To obtain the general relationship between the closed channel probability and the membrane potential, the amplitude of time-dependent currents recorded with various conditioning potentials was measured at different $[K^+]_o$. The amplitude was expressed as a fraction of the maximum value in each experiment and plotted against ΔV in the conditioning potential in Fig. 7*B* (n = 6). The sigmoidal relationships obtained at different $[K^+]_o$ were very similar to each other, indicating the dependence of the gating kinetics on ΔV.

The real closed channel probability, which should be obtained as a fraction of the steady-state current amplitude, was difficult to obtain in the present study (*see* Discussion). However, when we assume that the steady-state closed channel probability at potentials around E_{rev} is determined mainly by the gating kinetics as in the guinea-pig ventricular myocytes (Ishihara et al., 1989), the relationship in Fig. 7*B* represents the closed state probability at each membrane potential. The values for open state probability calculated according to this assumption were approximated by the following Boltzmann's relationship;

$$g\mathrm{K} = (1 + \exp((\Delta V - \Delta V_h)/v))^{-1}. \tag{3}$$

ΔV_h represents the ΔV where the activation becomes half, and v is the slope factor of the curve. ΔV_h and v were −9.5 and 8.9 mV, respectively, for the best fitting of the data in Fig. 7*B*.

The sigmoidal relationships between the chord conductance and ΔV in Fig. 5*B* should represent the open probability of the channel at different membrane potentials. When the relations in Fig. 5*B* were approximated

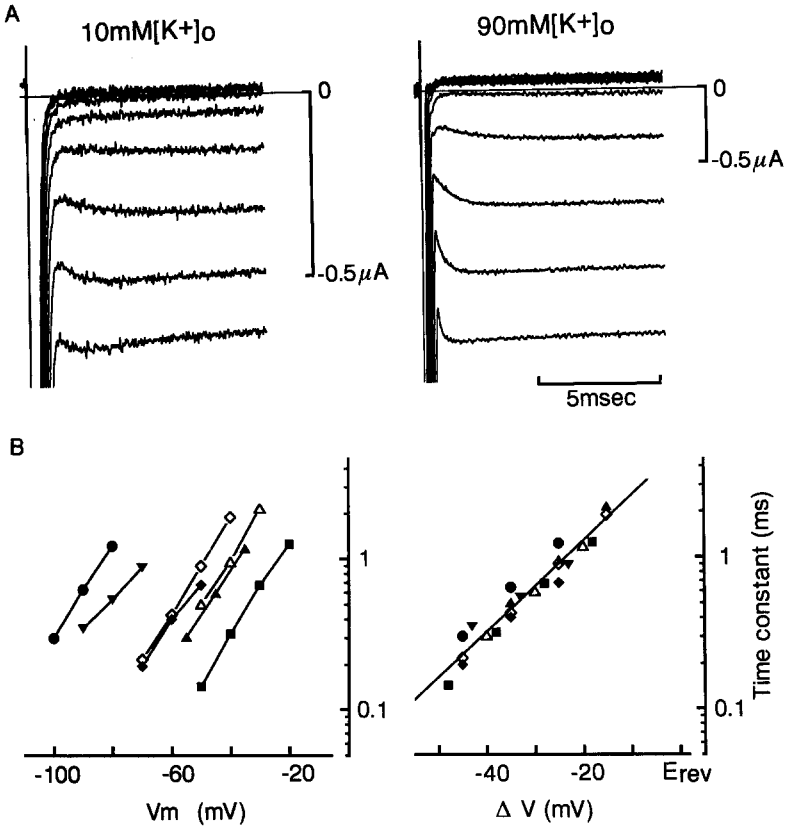

Fig. 6. (*A*) Voltage-dependent activation of the inward current upon membrane hyperpolarization at 10 mM (left) and 90 mM (right) $[K^+]_o$. Currents were recorded in the same oocyte by stepping the membrane potential to various levels from a holding potential of 10 and 40 mV, left and right panels, respectively. The potential range for the voltage step was −30 to −100 mV (left) and 30 to −50 mV (right) by 10 mV steps. Single exponential curves were superimposed to the time-dependent increase of the inward currents. The sampling frequency was 100 kHz. (*B*) Time constants of activation obtained at various $[K^+]_o$ were plotted against the membrane potential (left) and ΔV (right). Circles, 10 mM; inverted triangles, 20 mM; diamonds, 40 mM; triangles, 60 mM; squares, 90 mM $[K^+]_o$. Different symbols indicate different experiments (n = 7). The straight line in the right graph was drawn using Eq. (2).

to the Boltzmann's relationship, values for ΔV_h and v were −14.7 and 15.8 mV, respectively. The steeper slope factor obtained from the closed channel probabilities in Fig. 7*B* may contain some error caused by measuring the amplitude of the time-dependent current at around E_{rev}-40 mV, since values at potentials negative to this point are not available. However, it may be necessary to take into account the increase in open probability at depolarized potentials caused by the existence of the blocking kinetics by intracellular Mg^{2+} (Ishihara et al., 1989).

Discussion

One of the aims of this study was to determine whether the IRK1 gene, originally cloned from a mouse macrophage cell line, is expressed in the heart, especially in the ventricle. The inward rectifier K^+ channels generate the conductance of the resting membrane in the ventricle (Kameyama et al., 1983; Noma et al., 1984; Sakmann & Trube, 1984), whereas the Ach-regulated K^+ channels supply the majority of conductance in the atrium (Soejima & Noma, 1984; Hume & Uehara, 1985). We have cloned the IRK1 gene from mouse and rat hearts. Northern blot analysis showed that the ventricle abundantly expressed genes highly homologous to the IRK1, including the IRK1 itself. This is compatible with the idea that IRK1 encodes at least one of the specific components of the inward rectifier K^+ channel in the heart.

Another aim of the present study was to test if the channel protein encoded by the single gene, IRK1, shows similar electrophysiological properties to those of the native inward rectifier K^+ channel in cardiac myocytes. This step was necessary to study the structure-function relationship of the channel. When uninjected or water-injected *Xenopus* oocytes were tested under the same experimental condition, no time-dependent current changes upon voltage steps were noticed, and *I-V* relations were almost linear or showed slight outward rectification at a potential range between −120 and +30 mV. Occasionally, the activation of the currents, which did not inactivate for many seconds, was observed with hyperpolarizing or depolarizing voltage steps beyond the above potential range. Therefore, our experiments were performed within the above potential range to avoid the influence of endogenous currents.

When IRK1 was expressed in *Xenopus* oocytes, the current showed strong inward rectification in the *I-V* relationship. One of our main concerns was to know whether the outward current flows at potentials positive to E_K, since the current at this potential range plays a significant role in facilitating the repolarization of the action potential in ventricular cells (Shimoni, Clark & Giles, 1992). Although the amplitude was small, the recorded outward current showed the following characteristics in the native cardiac inward rectifier K^+ current (Hume & Uehara, 1985). First, a slight, but apparently negative slope was present in the *I-V* relationship when

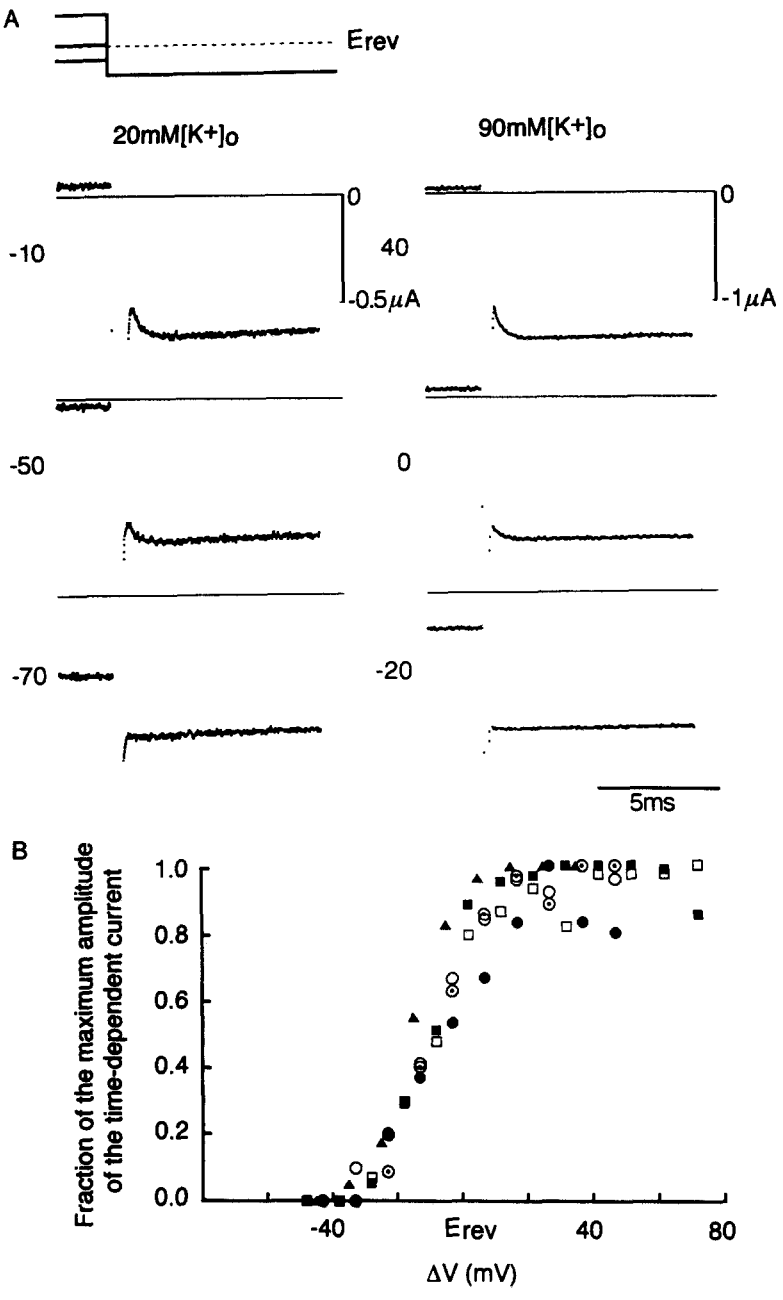

Fig. 7. (*A*) Dependence of the amplitude of the time-dependent activation on conditioning potential. Membrane was hyperpolarized to −80 mV (=E_{rev} − 35 mV) and −40 mV (=E_{rev} − 38 mV) from three different test potentials at 20 mM (left) and 90 mM (right) $[K^+]_o$, respectively. Numerals indicate the potential of the conditioning pulse. ΔV in the conditioning potentials were similar in the same row. The horizontal line superimposed on each current trace indicates the zero current level. Single exponential curves with time constants of 0.34 msec (left) and 0.32 msec (right) were superimposed on the time-dependent current components. Sampling frequency was 100 kHz. (*B*) Voltage dependence of closed state probabilities estimated from the amplitude of the time-dependent current component. Amplitude is expressed as a fraction of the maximum amplitude in each experiment and plotted against ΔV in the conditioning potential (circles, 20 mM; triangles, 40 mM; squares, 90 mM $[K^+]_o$). Different symbols indicate different experiments.

leakage or endogenous background currents were negligible (Figs. 2 and 4) or when the Ba^{2+}-sensitive component was examined (Fig. 3). Second, the "crossover" phenomenon was observed in the *I-V* relationships when $[K^+]_o$ was increased (Fig. 2).

The outward current flowing at potentials positive to E_K is much more prominent in the native inward rectifier K^+ channels of guinea-pig or rabbit ventricular myocytes than that in the expressed channels shown in the present study (Hume & Uehara, 1985; Shimoni, et al., 1992). We think that this discrepancy is probably due to the species difference of the inward rectifier K^+ channel (Trautwein & McDonald, 1978). The amplitude of the outward current is small, and the negative slope in the outward *I-V* relationship is not steep in the native channels of mouse ventricular myocytes (Benndorf & Nilius, 1988) and the mouse macrophage cell line J774.1 (Gallin & Sheehy, 1985). The shape of the action potential in rat and mouse ventricular cells is also different from that in other species. It is characterized by a fast repolarization phase due to the early outward current activated on depolarization, followed by a short plateau phase, which should be necessary for extremely rapid heart rates (Josephson, Sanchez-Chapula & Brown, 1984; Benndorf & Nilius, 1988).

In the present study, we showed the existence of

time-dependent changes in the IRK1-expressed current. Although we have not performed any experiments to examine the Mg^{2+} block of the expressed current, our conclusion that the time-dependent kinetics reflects the intrinsic gating mechanism should be supported by the following findings. First, the time course of current activation on membrane hyperpolarization was approximated by a first-order kinetics, and the time constant decreased with hyperpolarization, *e*-fold for a 14.7 mV change in ΔV. The similar voltage dependence of the kinetics was reported for the inward rectifier K^+ channel in the starfish egg (14–17 mV, Hagiwara et al., 1976) and in the skeletal muscle (18 mV, Leech & Stanfield, 1981), but the weaker voltage dependence was found for that in cardiac myocytes of the guinea pig (25–31 mV, Tourneur et al., 1987; 28.8 mV, Ishihara et al., 1989) and of the cat (28 mV, Harvey & Ten Eick, 1988). The time constants in the expressed channel were in the milliseconds range as has been reported for the native cardiac channel. The time constant at E_K (K values in Eq. 2), which can be estimated from the linear relationship between the logarithm of the time constant and ΔV, was similar to that in guinea-pig cardiac myocytes (Ishihara et al., 1989), but was approximately 1/100 or 1/10 of the value in the starfish egg (Hagiwara et al., 1976) or in the skeletal muscle, (Leech & Stanfield, 1981), respectively.

Second, the amplitude of the time-dependent current observed upon membrane hyperpolarization depended on the conditioning membrane potential, also in an E_K-dependent manner. In the present study, the accurate measurement of the time-dependent current amplitude was hampered by the overlapping capacitive currents. We did not subtract the capacitive currents since they varied during experiments, especially when the composition of the external solution was altered. The time course of the capacitive current was not always well approximated by a single exponential curve. The presence of significant micro/macrovilli in the oocyte membrane, which makes the specific membrane capacitance significantly high (4–7 $\mu F/cm^2$, Dascal, 1987), and the polarized nature of oocytes may be the source of an uneven series resistance in oocytes. However, when the activation parameters was estimated from the relative amplitude of the time-dependent current (Fig. 7*B*), the slope factor of the curve was very close to that in the guinea-pig cardiac channel obtained by similar (Tourneur et al., 1987) and different experimental procedures (Ishihara et al., 1989).

We attempted to calculate the density of the expressed channels in the oocyte membrane to ask if the distinct channel density may have affected the result in this study. The unit conductance of the single inward rectifier K^+ channel was adopted from that in the guinea pig ventricular cell ($\gamma = 1.3\ pS \times [K^+]_o^{0.63}$, Sakmann & Trube, 1984), since a similar single channel conductance was reported for IRK1 expressed in oocytes (Kubo et al., 1993*a*). The membrane area was calculated from the total membrane capacitance, assuming a specific capacitance of 1 $\mu F/cm^2$. The channel density calculated by assuming the open probability at the maximum conductance to be 0.9 was $5.2 \pm 0.6 \times 10^7$ channels/cm^2 (mean $\pm$ SD, $n = 4$). This is in the same range as the channel density reported in the rabbit ventricle ($7.4–29.5 \times 10^7$ channels/cm^2, Noma et al., 1984) and in J774.1 cells (0.47 channels/μm^2, McKinney & Gallin, 1988).

As stated above, the overall electrophysiological properties of the native inward rectifier K^+ channel were well reproduced in the channel expressed by injecting the IRK1-cRNA into oocytes. This is in contrast to the *Shaker*-type K^+ channels, which do not exactly show the properties of the native voltage-gated K^+ channels, when a single type of gene is expressed in oocytes. The protein encoded by IRK1 may form a channel in multimer, like *Shaker*-type K^+ channels (Kubo et al., 1993*a*). That the main properties of the inward rectifier K^+ channel were reconstructed by expressing the single gene, IRK1, may suggest that the main unit of this channel is formed by a homomultimer. The inward rectifier K^+ channel is unique in that the voltage dependence of the gating mechanism depends on the driving force of K^+ when E_K is altered by changing $[K^+]_o$, but not on the absolute membrane potential. This strongly implies that the structure responsible for the gating exists within the pore region. The existence of the gating kinetics in the expressed channels, which lack the voltage sensor characteristic of voltage-dependent channels (Na^+, Ca^{2+}, *Shaker*-type K^+), may support this idea. The present study shows that the IRK1 gene is suitable for studying the mechanism of the E_K-dependent gating kinetics at the molecular level.

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