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Cloning and functional expression of a cardiac inward rectifier K^+ channel

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Abstract

We have isolated a cDNA coding for an inward rectifier K^+ channel (RBHIK1) from rabbit heart. The cloned cDNA encodes a protein of 427 amino acids with two putative transmembrane segments. The primary structure of RBHIK1 is highly homologous to that of IRK1 which is an inward rectifier K^+ channel recently cloned from mouse macrophage by expression cloning. When expressed in *Xenopus* oocytes, RBHIK1 current showed strong inward rectification and was inhibited by extracellular Ba^{2+} and Cs^+. RNA blot analysis revealed the expression of RBHIK1 mRNA in various rabbit tissues, especially high level in the ventricular muscle.

Key words: K^+ channel; Inward rectifier; Heart; cDNA cloning; RNA blot analysis

1. Introduction

The inward rectifier K^+ channels, which were first discovered in skeletal muscle by Katz [1] more than 40 years ago, have subsequently been found in a variety of cell types, including cardiac myocytes [2–4]. They are thought to play important roles in determining the resting membrane potentials and in permitting long depolarizing responses. In the heart, electrophysiological studies have revealed significant differences of membrane properties in various anatomical regions. For example, the resting membrane potential of pacemaker cells is much less negative than that of non-pacemaker cells [5,6], and the sizes and shapes of action potential are markedly different depending on cell types. Different densities of inwardly rectifying K^+ channels are thought to contribute, to large extents, to such differnces. Recently, two kinds of inwardly rectifying K^+ channels were cloned by expression cloning using *Xenopus* oocytes. One channel (ROMK1) was cloned from rat kidney, which is regulated by ATP [7]. Another channel (IRK1) was cloned from mouse macrophage, whose functional properties closely resemble those of inward rectifier K^+ channels [8]. Both ROMK1 and IRK1 have only two transmembrane segments and correspond to the inner-core structure of the *Shaker* K^+ channel family [9]. Based on sequence similarity with ROMK1 and IRK1, the muscarinic K^+ channel (GIRK1) was cloned from rat heart [10]. GIRK1 mRNA is abundant in the atrium, which is consistent with electrophysiological findings on muscarinic K^+ channels. We here describe the cDNA cloning and functional expression of a cardiac inward rectifier K^+ channel. Messenger RNA specific for the K^+ channel was detected at the highest level in the ventricle.

2. Materials and Methods

2.1. cDNA cloning and sequencing

Polymerase chain reaction (PCR) was used to obtain a DNA probe for hybrid screening. Two degenerate oligonucleotide primers were synthesized according to the amino acid sequences conserved between IRK1 and ROMK1. The sense primer corresponded to amino acid residues 195 to 201 and the antisense primer corresponded to residues 290 to 296 of IRK1. The 5′ part of both primers was designed to contain *Eco*RI site to facilitate subcloning. PCR was carried out using rabbit heart cDNA as a template under the following conditions: 94°C, 1 min; 45°C, 2 min; 72°C 3 min for 30 cycles. The amplified products were digested with *Eco*RI and separated on 1% agarose gel. DNA bands of expected size (about 300 bp) were subcloned into pBluescript SK(+) vector (Stratagene) and sequenced by the dideoxy chain-termination method [11] using BcaBEST dideoxy sequencing kit (TAKARA). DNA fragment homologous to IRK1 was labeled with ^{32}P by the random priming method and used to screen a rabbit heart cDNA library in λZAPII. Approximately 6.3×10^5 plaques were screened under low stringency conditions and 29 positive clones were isolated. In vivo excision and rescue of pBluescript SK(−) from the positive λZAPII clones were performed according to the manufacturer's instructions (Stratagene). We selected one clone (RBHIK1) containing 2.3 kb insert for further characterization. DNA sequencing of RBHIK1 was performed on both strands using BcaBEST dideoxy sequencing kit.

2.2. Functional expression of RBHIK1

Electrophysiological measurements were carried out essentially the same as those reported previously [12]. The plasmid containing RBHIK1 was linearized by digestion with *Hin*dIII and capped run-off cRNA was synthesized in vitro with T3 RNA polymerase (Boeringer

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The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank Nucleotide sequence databases with Accession Number D21057.

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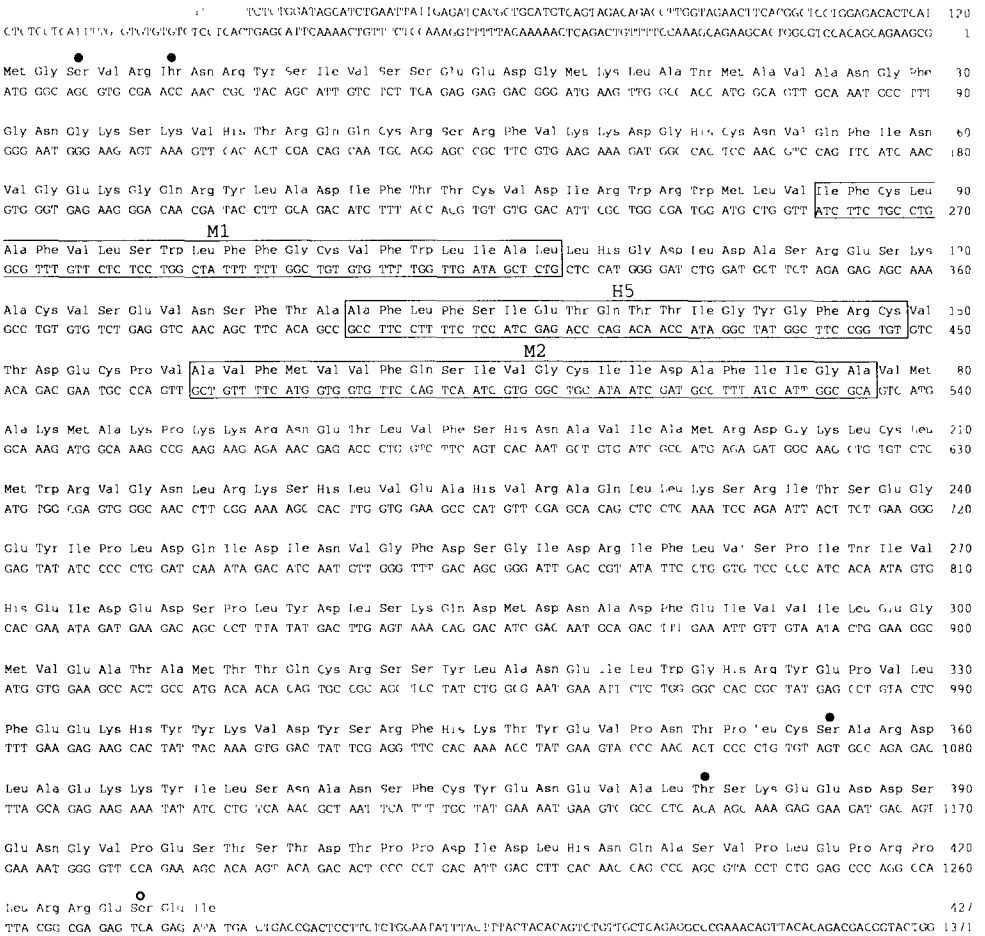

Fig. 1. Nucleotide and deduced amino acid sequences of RBHIK1. Nucleotides are numbered from the first residue of the ATG initiation triplet and amino acid residues are numbered from the initiating methionine. Putative transmembrane segments (M1 and M2) and pore forming region (H5) are boxed. Potential phosphorylation sites for protein kinase C (●) and for protein kinase A (○) are indicated.

Mannheim). Transcribed RNA was dissolved in water at a final concentration of 10 ng/μl for oocyte injection. *Xenopus laevis* oocytes (stage IV and V) were treated for 2 h with collagenase (2 mg/ml; WAKO) in modified Barth's medium and defolliculated manually with fine forceps. The oocytes were injected with the transcribed RNA (0.4–0.5 ng) and incubated at 19°C for 2–4 days in modified Barth's medium before electrophysiological measurements. Whole cell currents were recorded using a standard two-microelectrode voltage-clamp method with 3 M KCl filled electrodes. Bath solution contained 90 mM KCl, 3 mM $MgCl_2$, 5 mM HEPES (pH 7.4), and 0.3 mM niflumic acid to block the endogenous Cl^- current (the 90 mM K^+ solution). For the bath solution containing various concentration of K^+, Na^+ was replaced with K^+. Current records were low pass filtered at 3 kHz. All electrophysiological experiments were carried out at 21 ± 1°C.

2.3. RNA blot analysis

Five microgram poly(A^+) RNA from the atrium, ventricle, cerebrum, cerebellum, kidney, liver, lung and skeletal muscle of rabbit were electrophoresed on 1% agarose gel containing formaldehyde and transferred by capillary action to GeneScreen Plus membrane (NEN). The filter was hybridized with ^{32}P labeled probe for 24 h in 50% formamide, 5 × SSPE, 0.1% SDS, 5 × Denhardt's solution and 200 μg/ml herring testis DNA at 42°C [13]. The DNA probe used was *Eco*RI–*Xba*I fragment (1.6 kb) from the 3′ terminal region of RBHIK1. The filter was briefly washed with 0.2 × SSC/0.1% SDS at room temperature, followed by two 15 min washes with 0.2 × SSC/0.1% SDS at 65°C. Autoradiography was performed on Kodak X-AR film for 40 h at −70°C with an intensifying screen.

3. Results and discussion

3.1. Primary structure of RBHIK1

A cDNA clone, RBHIK1, was isolated from a rabbit heart cDNA library. Nucleotide sequence analysis of RBHIK1 revealed one long open reading frame encoding a protein of 427 amino acid residues with a calculated M_r of 48,348 (Fig. 1). The nucleotide sequence of the open reading frame was 87% identical to that of IRK1 and the deduced amino acid sequence was 97% identical to that of IRK1. It contains only two putative transmembrane segments (M1 and M2), which is characteristic of the inwardly rectifying K^+ channels cloned so far [7,8,10]. RBHIK1 has four putative phosphorylation sites for protein kinase C (residues 3, 6, 357 and 383) and one putative phosphorylation site for protein kinase A (residue 425). There are some amino acid sequence differences observed between RBHIK1 and IRK1 in the segment between M1 and putative pore forming region H5, where the sequence of RBHIK1 is Ala-Ser-Arg-Glu and the sequence of IRK1 is Thr-Ser-Lys-Val. Since the

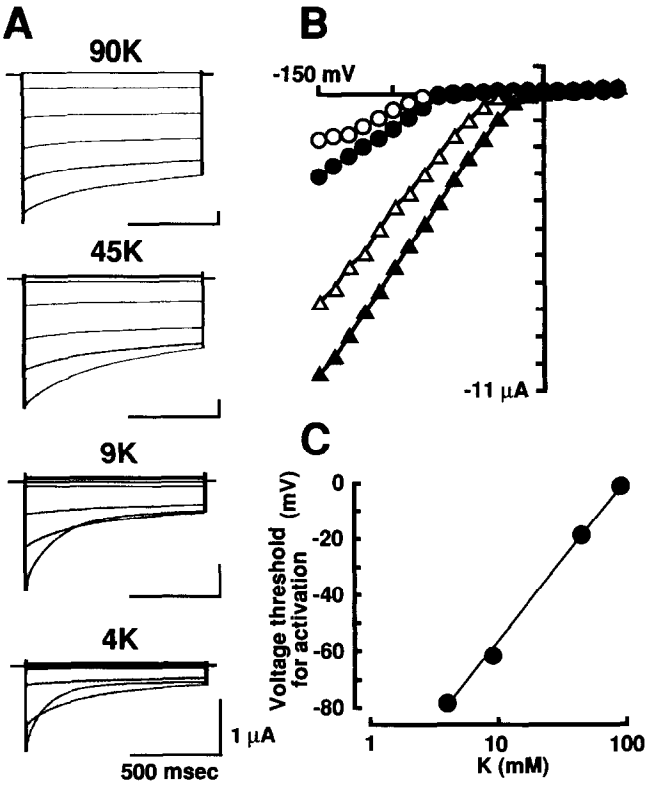

Fig. 2. Inwardly rectifying current through RBHIK1. A: Inwardly rectifying currents in an oocyte expressing RBHIK1. Current traces elicited by voltage steps to 50, 30, −30, −60, −90, −120, −150 mV for 1000 ms from a holding potential of 0 mV are shown. Extracellular K^+ was substituted with Na^+. (B) Current–voltage relationships obtained from the same oocyte of (A). Currents were elicited by voltage steps to test potentials ranging from 50 mV to −150 mV in −10 mV increment. Holding potential was 0 mV. Peak current amplitudes are plotted against membrane potential. Extracellular K^+ 4 mM (○), 9 mM (●), 45 mM (△), 90 mM (▲). (C) Semilogarithmic plot of extracellular K^+ concentration versus voltage threshold for activation (n = 6). The straight line shows E_K calculated from the Nernst equation.

regions surrounding H5 of *Shaker* the K^+ channel family are thought to form external mouth of the channel pore [14,15], these amino acid sequence changes between M1 and H5 may give rise to some functional differences between RBHIK1 and IRK1.

3.2. *Electrophysiological properties of RBHIK1*

The electrophysiological properties of RBHIK1 were examined using a *Xenopus* oocyte expression system. In the non-injected oocytes, small endogenous currents were observed which were little affected by extracellular K^+ or Ba^{2+}. In contrast, in the oocytes injected with RBHIK1 cRNA, large inwardly rectifying currents were observed which were sensitive to extracellular K^+ or Ba^{2+} as described below. The RBHIK1 current showed strong inward rectification (Fig. 2A,B). The voltage threshold for activation was about −78 mV in the solution containing 4 mM K^+; the peak current amplitude increased as the voltage became more negative and little outward current was observed as the voltage became more positive. The dependence of the amplitude and the voltage threshold for activation of RBHIK1 current on the extracellular K^+ concentration was studied (Fig. 2). The amplitude of RBHIK1 current increased as extracellular K^+ concentration increased. The threshold voltages for activation were −0.8 ± 0.8 mV (90 mM K^+), −18.3 ± 1.7 (45 mM K^+), −61.7 ± 1.7 (9 mM K^+) and −78.3 ± 1.7 (4 mM K^+)(n = 6). Assuming that intracellular concentration of K^+ is 90 mM [16], their values are close to the K^+ equilibrium potential (E_K) calculated from the Nernst equation in these extracellular solutions. Relationship of extracellular K^+ concentrations and the threshold voltage for activation is plotted in Fig. 2C. Cardiac inward rectifier K^+ channel exhibits substantial outward current above E_K and shows negative slope conductance when

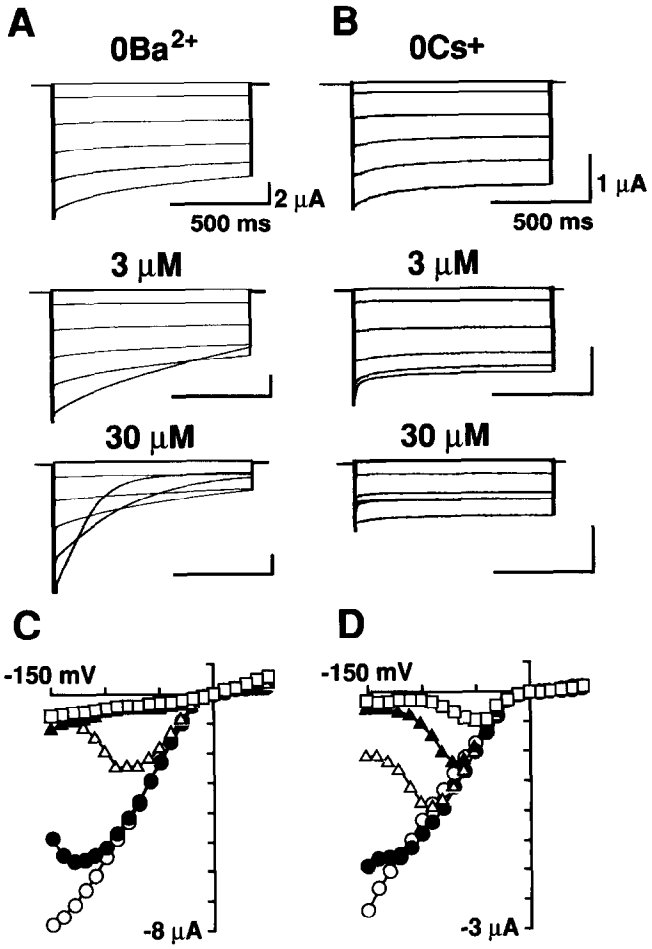

Fig. 3. (A,B) Block of RBHIK1 current by extracellular Ba^{2+}(A) and Cs^+ (B). Representative current traces elicited by voltage steps to 50, 30, −30, −60, −90, −120, −150 mV for 1000 ms from a holding potential of 0 mV are shown. Currents were elicited in the 90 mM K^+solution containing various concentration of Ba^{2+} or Cs^+. (C,D) Current–voltage relationships before and after the application of Ba^{2+} (C) in the same oocyte of A or Cs^+ (D) in the same oocyte of (B). Currents were elicited by voltage steps to test potentials ranging from 50 mV to −150 mV in −10 mV increment. Holding potential was 0 mV. Current amplitudes at the end of 1,000 ms pulses are plotted. (○) 0 μM Ba^{2+} or Cs^+(●) 3 μM Ba^{2+} or Cs^+(△) 30 μM Ba^{2+} or Cs^+(▲) 300 μM Ba^{2+} or Cs^+(□) 3 mM Ba^{2+} or Cs^+.

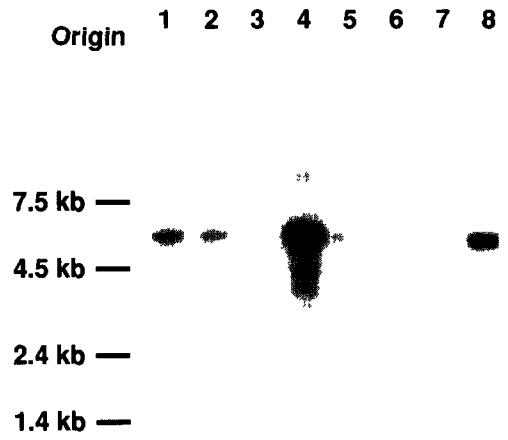

Fig. 4. RNA blot analysis of RBHIK1 mRNA. Poly(A$^+$) RNAs analysed were as follows: cerebrum (1), cerebellum (2), atrium (3), ventricle (4), kidney (5), liver (6), lung (7), skeletal muscle (8). The position of RNA size markers (BRL) are shown on the left. The integrity of each RNA was confirmed by re-probing the same filter with a β-actin cDNA probe (data not shown)

recorded in whole cell configuration [17–19]. Although RBHIK1 was cloned from rabbit heart, unlike native cardiac inward rectifier, it exhibited no substantial outward current and negative slope conductance when expressed in *Xenopus* oocytes. Inward rectifier K^+ channels show a linear current–voltage relationships if the internal surface of the membrane is exposed to a divalent ion-free solution. The mechanism responsible for inward rectification is thought to be the blockade of open channels by intracellular Mg^{2+} [20,21]. Therefore the discrepancy between the RBHIK1 current and the native inward rectifier K^+ current in cardiac cells may be attributed to the differences of intracellular ionic conditions between oocytes and cardiac cells. Inward rectifier K^+ currents are blocked by extracellular cations such as Ba^{2+} and Cs^+ [22–24]. So the effects of extracellular Ba^{2+} and Cs^+ on RBHIK1 current were examined. The RBHIK1 current was recorded in the 90 mM K^+ solution and $BaCl_2$ or CsCl was added to the solution without correcting the ionic strength. Fig. 3 shows typical results of Ba^{2+} (A,B) and Cs^+ (C,D) block of RBHIK1 current. Both cations blocked the RBHIK1 current in a voltage- and time-dependent manner, suggesting that they act as open channel blockers.

3.3. Distribution of RBHIK1 mRNA in rabbit tissues

The expression of RBHIK1 mRNA in various tissues of rabbit was examined by RNA blot analysis. An about 1.6 kb *Eco*RI–*Xba*I fragment from the 3′ terminal region of RBHIK1 (from nucleotide 706 to the 3′ end) was used as a probe, which detected a single band of mRNA with an approximate estimated size of 5.5 kb. The mRNA was expressed at the highest abundance in the ventricle, at high levels in the skeletal muscle, at moderate levels in the cerebrum and cerebellum, and at low levels in the kidney and lung (Fig. 4). There are some differences in distribution between RBHIK1 mRNA in rabbit tissues and IRK1 mRNA in mouse tissues. IRK1 mRNA was expressed at almost the same level in the skeletal muscle, heart and forebrain, which is much higher than in the cerebellum [8]. In contrast, RBHIK1 mRNA was expressed at almost the same level in the cerebrum and cerebellum, and much higher in the ventricle. There are remarkable differences of the expression of RBHIK1 mRNA between the atrium and ventricle. The highest expression of the 5.5 kb mRNA band was detected in the ventricle, but no expression was detected in the atrium under the experimental conditions we have employed. Our results are in good agreement with electrophysiological findings about the different sizes of inward rectifier K^+ current in the ventricle and atrium. The inward rectifier K^+ current is much larger in the ventricle than in the atrium [25]. The high expression of RBHIK1 mRNA in the ventricle supports that RBHIK1 accounts for a major inward rectifier current in rabbit ventricle. RBHIK1 will help to study the molecular mechanism of inward rectification in cardiac cells.

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