Kir2.2v: a possible negative regulator of the inwardly rectifying K^+ channel Kir2.2

Noriyuki Namba[a,b], Nobuya Inagaki[a], Tohru Gonoi[c], Yoshiki Seino[b], Susumu Seino[a,*]

[a]*Division of Molecular Medicine, Center for Biomedical Science, Chiba University, School of Medicine, 1-8-1, Inohana, Chuo-ku, Chiba 260, Japan*
[b]*Department of Pediatrics, Okayama University Medical School, Shikata-cho, Okayama 700, Japan*
[c]*Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, 1-8-1, Inohana, Chuo-ku, Chiba 260, Japan*

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Abstract We have cloned the human genes encoding the inwardly rectifying K^+ (Kir) channel subunits, Kir2.2 (hKir2.2) and its variant, termed hKir2.2v. When expressed in *Xenopus* oocytes, hKir2.2 produced strong inwardly rectifying K^+ currents, whereas the expression of hKir2.2v did not elicit significant currents. Coexpression of hKir2.2v with hKir2.2 showed an hKir2.2v inhibition of hKir2.2 K^+ currents, indicating that it acts as a negative regulator of hKir2.2 channel activity. Mutational analysis of hKir2.2v and studies of chimeras between hKir2.2 and hKir2.2v suggest that the intracellular C-terminal region of hKir2.2v participates in the negative regulation of the hKir2.2v channel activity.

Key words: Inwardly rectifying K^+ channel; Gene; Heteromultimer; *Xenopus* oocytes; Site-directed mutagenesis; Chimera

1. Introduction

Kir channels are characterized by a conductance which increases under hyperpolarization and decreases under depolarization and plays important roles in maintaining the resting membrane potential near the K^+ equilibrium potential (E_K) in excitable and nonexcitable cells [1]. Recently, expression cloning of Kir channel cDNAs has revealed a new family of K^+ channels having two transmembrane segments [2,3]. K^+ channels have thus far been divided into six subfamilies (Kir1.0–Kir6.0), based on their degree of identity [4-6]. It has been suggested that Kir channels function as multimers [7]. A recent study has shown that G-protein-gated atrial K^+ channels are a heteromultimer of GIRK1 (Kir3.1) and CIR (Kir3.4) [8]. In addition, we have shown that ATP-sensitive K^+ channels are a complex composed of at least two subunits, Kir6.2 and the sulfonylurea receptor [6]. These findings indicate that the assembly of Kir channel subunits is complex, and suggest that this complexity could account for functional diversity of Kir channels.

We have cloned the genes encoding the human Kir2.2 and its variant, termed hKir2.2v, and we show that hKir2.2v acts as a negative regulator of the hKir2.2 channel activity when it is coexpressed with hKir2.2. The domain of hKir2.2v which might be involved in the negative regulation also is discussed.

2. Materials and methods

2.1. General methods

Standard methods were carried out as described [9]. DNA sequencing was done by the dideoxynucleotide chain termination procedure using a sequencing kit (USB, Cleveland, OH) after subcloning appropriate DNA fragments into M13mp18, M13mp19 (Toyobo, Osaka, Japan), or pGEM-3Z (Promega, Madison, WI). Both strands were sequenced.

2.2. Screening of a human genomic library

Since several of the genes encoding Kir channels lack introns [6,10], a human genomic library was screened for novel clones. Seven hundred thousand plaques of a λFIXII human genomic library (Stratagene, La Jolla, CA) were screened under low-stringency hybridization conditions [11], using a ^{32}P-labeled hBIR DNA fragment as a probe [6]. The membranes were washed with 2×SSC/0.1% SDS at 42°C for 30 min and exposed to an X-ray film overnight at −80°C with an intensifying screen.

2.3. Site-directed mutagenesis and construction of chimeras

Site-directed mutagenesis of hKir2.2v was performed by polymerase chain reaction overlap extension method [12]. The Kir2.2v mutants prepared (Kir2.2v mutants 1–7) are listed in Table 1. Two chimeras between hKir2.2 and hKir2.2v were also prepared: one, termed Kir2.2vC, was constructed by ligating a 529 bp *Sma*I-*Bsp*HI fragment of the hKir2.2 gene to a 842 bp *Bsp*HI-*Sma*I fragment of the hKir2.2v gene; the other, termed Kir2.2C, was constructed from a 529 bp *Sma*I-*Bsp*HI fragment of hKir2.2v mutant7, which encodes the same amino acid sequence in the P-region as does the hKir2.2 gene, and a 842 bp *Bsp*HI-*Sma*I fragment of the hKir2.2 gene. Various hKir2.2v mutants and the two chimeras were subcloned into pGEM-3Z, and their identities were confirmed by DNA sequencing.

2.4. Expression and electrophysiological analysis in Xenopus oocytes

10 μg each of pGEM-3Z carrying hKir2.2, hKir2.2v, various mutants of hKir2.2v, or the chimeras were transcribed in vitro by T_7 RNA polymerase after digestion with *Hin*dIII, according to the manufacturer's instructions. *Xenopus* oocytes were prepared as described previously [6]. The oocytes were injected with 60 nl of H_2O alone, hKir2.2 cRNA (0.25 ng), hKir2.2v cRNA (0.25–10 ng), hKir2.2vC cRNA (10 ng), or hKir2.2C (10 ng) cRNA. The oocytes were injected with the various hKir2.2v mutant cRNAs (10 ng each) (Table 1), and they were also coinjected with hKir2.2 cRNA and hKir2.2v cRNA at the concentrations indicated in Fig. 3. After 2–3 days, electrophysiological measurements were performed by two-electrode voltage clamp technique, as previously described [5]. The microelectrodes were filled with 3 M KCl; the resistance was 0.3–1.0 MΩ. The oocytes were bathed in a solution containing 90 mM KCl, 3 mM $MgCl_2$, and 5 mM HEPES (pH 7.4) with 300 μM niflumic acid to block endogenous chloride currents. The oocytes were voltage-clamped at 0 mV and voltage steps of 0.9 s duration were applied to the oocytes in 15 mV increments every 5 s. Recordings were made at 20–22°C.

3. Results

3.1. Cloning of genes encoding a human inwardly rectifying K^+ channel (hKir2.2) and its variant (hKir2.2v)

Among the 11 positive λ clones obtained from the λFIXII

*Corresponding author. Fax: (81) (43) 221-7803.
E-mail: seino@med.m.chiba-u.ac.jp

PII S0014-5793(96)00445-0

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hKir2.2v  MTAASRANPY SIVSSEEDGL HLVTMSGANG FGNGKVHTRR RCHNRFVKKN GQCNIEFANM DEKSQRYLAD IFTTCVDIRW   80
hKir2.2                                                 R                              M            80

               <----------M1---------->                             <-------P-------->        <-
hKir2.2v  RYMLLIFSLA FLASWLLFGV IFWVIAVAHG DLEPAEGRGR TPCVMQVHGF MAAFLFSIKT QNTISYGLRC VTEECPVAVF  160
hKir2.2                       I                                          E   T  G                  160

          ---------M2-------->
hKir2.2v  MVVAQSIVGC IINSFMIGAI MAKMVRPKKR AQTLLFSHNA VVALRDGKLC FMWRVGNLRK SHIVEAHVRA QLIKPRVTKE  240
hKir2.2                D            A                            L                             E   240

hKir2.2v  GEYIPLDQID IDVGFDKGLD HSFLVSPITI LHEIDEASPL FGISRQDLQM DDFEIVIILE GIVEATAMTT QARSSYLANE  320
hKir2.2                           RI                        -   ET       V     M                     319

hKir2.2v  ILWGHRFEPV LF-EKNQYKI DYLHFHKTYE VPSTPRCSAK DLVENKFLLP RANSFCYKNE LAFLSRDEED EADGDQDGRS  399
hKir2.2                E          S                              S      E                          399

hKir2.2v  REGLIPQARH DFDRLQAGGG VLEQRPYRRE SEI  432
hKir2.2   D   S                                 432
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Fig. 1. Comparison of the amino acid sequences of human Kir2.2 and Kir2.2v. Amino acids are indicated in single-letter code. The amino acid residues of hKir2.2 different from those of hKir2.2v are shown below hKir2.2v. Predicted transmembrane (M1 and M2) and pore (P) segments are indicated. Gaps introduced to generate alignment are indicated by dashes. The nucleotide sequence of hKir2.2v has been deposited in the Genbank with the accession number U53143.

human genomic library, four λ clones were further characterized and sequenced. Three clones contained the gene encoding human Kir2.2 [13,14], and the remaining clone contained a gene encoding a protein related to hKir2.2, designated hKir2.2v. The sequence of the λhKir2.2v DNA fragment revealed a single open reading frame encoding a 432 amino acid protein having 95% identity with hKir2.2 (Fig. 1). Comparison of the nucleotide sequences of the hKir2.2 gene, the hKir2.2v gene, and the previously reported Kir2.2 cDNA [13,14] confirms that both the hKir2.2 gene and the hKir2.2v gene are intronless in the protein-coding region. Comparison of the amino acid sequences of hKir2.2 and hKir2.2v shows that in the P-region, which is highly conserved among Kir channels [7,15,16], three amino acids differ between the two proteins: the amino acid residues 139, 142, and 145 of hKir2.2 in this region are Glu, Thr, and Gly, respectively, while they are Lys, Asn, and Ser in the corresponding positions in hKir2.2v. hKir2.2 has Asp (residue 173) in the second transmembrane segment that determines a strong rectifier [17,18], while hKir2.2v has Asn at the corresponding position.

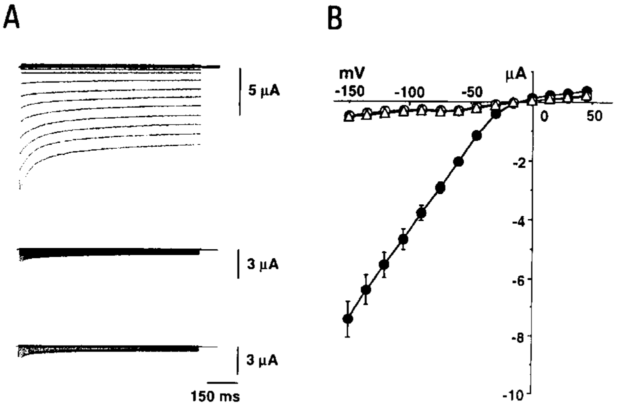

Fig. 2. Electrophysiological properties of hKir2.2 and hKir2.2v. (A) Representative traces of currents elicited by voltage steps from −150 to +45 mV in 15 mV increments in oocytes injected with hKir2.2 cRNA (top), hKir2.2v cRNA (middle), or water (bottom). The holding potential was set at 0 mV, according to the E_K value as predicted by the Nernst equation. (B) Current-voltage relations: hKir2.2 cRNA (*n*=20), closed circles; hKir2.2v cRNA (*n*=15), open circles; water (*n*=20), triangles. Values are means ± SEM.

3.2. *Electrophysiological analysis of hKir2.2 and hKir2.2v in Xenopus oocytes*

In the bath solution containing 90 mM K^+, the oocytes injected with hKir2.2 cRNA exhibited a rapidly activating and slowly inactivating large inward current at voltages below the reversal potential for K^+. A strong inward rectification was observed upon depolarization. External Ba^{2+} (500 μM) completely blocked the inward currents. By contrast, oocytes injected with hKir2.2v cRNA did not show any significant K^+ currents (Fig. 2).

3.3. *Coexpression of hKir2.2 and hKir2.2v and analysis of hKir2.2v mutants*

Since hKir2.2 and hKir2.2v are highly homologous, they might associate to form a heteromultimer. Therefore, we coexpressed hKir2.2 and hKir2.2v in *Xenopus* oocytes. As shown in Fig. 3, the inward currents seen in oocytes injected with hKir2.2 cRNA alone were decreased with coinjection with hKir2.2v cRNA in a dose dependent manner. The hKir2.2 currents were completely inhibited when hKir2.2 cRNA and hKir2.2v cRNA were coinjected at a ratio of one to five.

We also have examined *Xenopus* oocytes injected with various hKir2.2v mutant cRNAs (Table 1). However, none of the mutants prepared elicited any significant K^+ currents (data not shown). Interestingly, the hKir2.2v mutant (mutant7) which has the same amino acid sequence in the P-region as

Table 1
hKir2.2v mutants used in this study

Mutant	Channel activity
1. K139E	(−)
2. N142T	(−)
3. S145 G	(−)
4. K139E+N142T	(−)
5. K139E+S145G	(−)
6. N142T+S145G	(−)
7. K139E+N142T+S145G	(−)

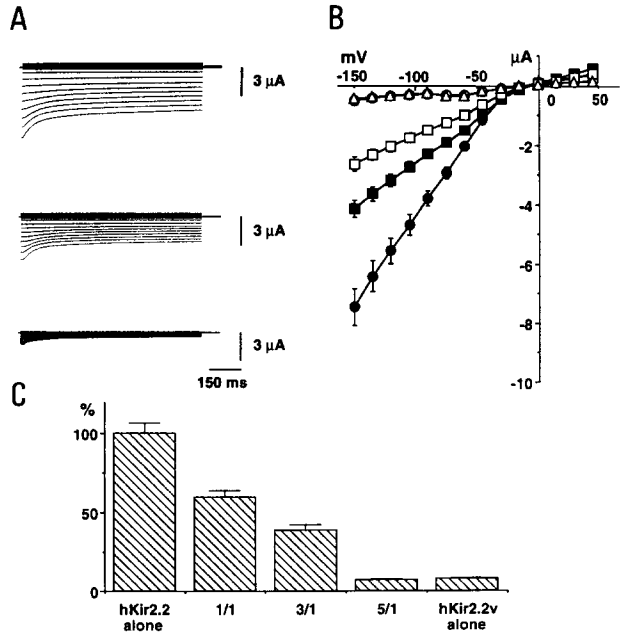

Fig. 3. Coexpression of hKir2.2 and hKir2.2v. (A) Representative traces of currents detected in oocytes injected with 0.25 ng of hKir2.2 cRNA plus 0.25 ng (top), 0.75 ng (middle), or 1.25 ng (bottom) of hKir2.2v cRNA. The holding potential was 0 mV. Voltage steps were applied as described in Fig. 2. (B) Current-voltage relations: 0.25 ng of hKir2.2 cRNA plus 0.25 ng (n=11, closed squares), 0.75 ng (n=18, open squares), or 1.25 ng (n=20, triangles) of hKir2.2v cRNA. Current-voltage relations in hKir2.2 alone (n=20, closed circles) or hKir2.2v alone (n=20, open circles) are shown for comparison. Values are means ± SEM. (C) hKir2.2v cRNA concentration dependent inhibition of Kir2.2 currents. The average K^+ current at −90 mV in oocytes injected with hKir2.2 cRNA alone is expressed as 100%. The ratios of hKir2.2v cRNA to hKir2.2 cRNA are indicated.

does hKir2.2 did not elicit significant K^+ currents. Coexpression of mutant7 with hKir2.2 also inhibited the hKir2.2 currents.

3.4. Analysis of chimeras

Expression of the chimera hKir2.2C having the intracellular C-terminal region of hKir2.2 in *Xenopus* oocytes elicited significant inwardly rectifying K^+ currents, whereas expression of the chimera hKir2.2vC having the intracellular C-terminal region of hKir2.2v did not generate significant currents (Fig. 4).

4. Discussion

It has been shown that Kir channels function as homomultimers or heteromultimers [8,19]. In addition, we have recently shown that Kir6.2 couples to the sulfonylurea receptor to form ATP-sensitive K^+ channel currents [6]. These studies indicate that the assembly of Kir channel subunits is complex, providing a basis for the functional diversity of Kir channels. In the present study we have identified a variant of hKir2.2, termed hKir2.2v. Although hKir2.2v has 95% amino acid identity with hKir2.2, the sequence of hKir2.2v in the ion-conducting P-region, which is highly conserved among Kir channel subunits, differs in three amino acids from hKir2.2. A notable difference among them is that hKir2.2v has Ser at residue 145 rather than Gly, which is at this position in hKir2.2. The Gly-Tyr-Gly motif is thought be important for K^+ permeation [7,15,16].

Electrophysiological studies have shown that expression of hKir2.2 in *Xenopus* oocytes produces inwardly rectifying K^+ currents, whereas expression of hKir2.2v does not. In addition, coexpression of hKir2.2 and hKir2.2v inhibited hKir2.2 currents, supporting the possibility that Kir channel subunits form a heteromultimer [8,20]. A recent report has shown that Kir3.3 has opposite effects on G-protein-evoked K^+ currents when coexpressed with Kir3.1 or Kir3.2: coexpression with Kir3.1 enhances the Kir3.1 channel activity, whereas coexpression with Kir3.2 suppresses the Kir3.2 channel activity [20]. Taken together, it is possible that some Kir channel subunits function as negative regulators when coassembled with other subunits.

We next explored the region of hKir2.2v that might be involved in the negative regulation of the channel activity. Since the P-region is thought to be important for ionic conductance [7,15,16], we first focused on this region and prepared various hKir2.2v mutants (Table 1). Surprisingly, none of these mutants, including mutant 7 which has the same amino acid sequence in the P-region as does hKir2.2, exhibited K^+ currents. Recent studies have suggested that the intracellular C-terminal region of Kir2.1 is involved in K^+ permeation as well as in rectification of the channel [21-23]. Therefore, we examined the functional properties of the two chimeras, hKir2.2C and hKir2.2vC, having the intracellular C-terminal region of hKir2.2 and hKir2.2v, respectively. hKir2.2C elicited inwardly rectifying K^+ currents similar to

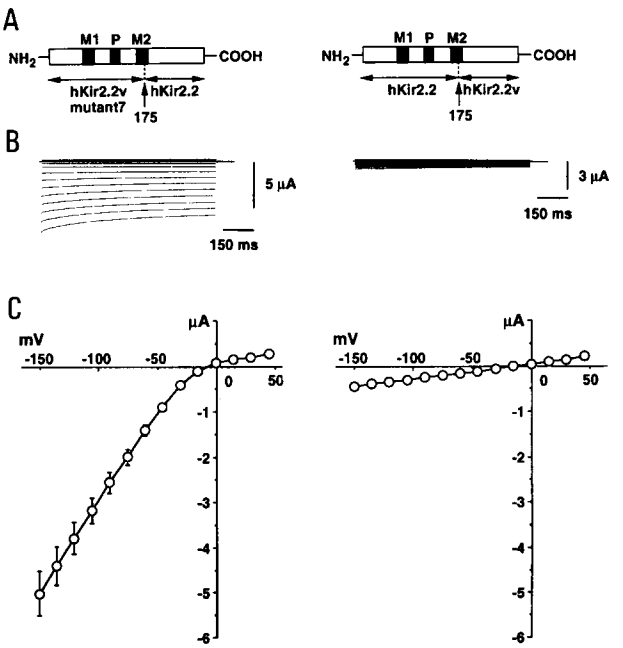

Fig. 4. Electrophysiological properties of chimeras. Left, hKir2.2C. Right, hKir2.2vC. (A) Schematic representation of the chimeric constructs, hKir2.2C and hKir2.2vC. hKir2.2C is derived from the N-terminal portion of Kir2.2v mutant 7 (see Table 1) and the C-terminal portion of Kir2.2. hKir2.2vC is derived from the N-terminal portion of hKir2.2 and the C-terminal portion of Kir2.2v. The number of the amino acid residue at the chimeric junction is indicated. (B) Representative traces of currents elicited by hKir2.2C or hKir2.2vC. The holding potential was 0 mV. Voltage steps were applied as described in Fig. 2. (C) Current-voltage relations (n=20, each). Values are means ± SEM.

those elicited by hKir2.2, whereas hKir2.2vC did not produce any significant currents, suggesting that the C-terminal region of hKir2.2v participates in the negative regulation of the hKir2.2 channel activity. Although the mechanism by which hKir2.2v suppresses the hKir2.2 channel activity is unknown at present, it is possible that the C-terminal region of hKir2.2v fails to form the normal channel pore structure when coassembled with hKir2.2, since it has been suggested that, in addition to the P region and part of the M2 region, the C-terminal region also comprises part of the pore [21-23]. An alternative possibility is that the C-terminal region of hKir2.2v interferes with formation of a functional multimeric structure. Mutational analysis of the C-terminal region of the Kir2.2v subunit should clarify which amino acid residues are involved in the negative regulation.

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