

## Molecular cloning, functional expression and localization of a novel inward rectifier potassium channel in the rat brain

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### Abstract

We have cloned a novel inward rectifier potassium channel from a rat brain cDNA library and designated it RB-IRK2. The rat brain cDNA library was screened using a fragment of the mouse macrophage IRK1 cDNA as a probe. The amino acid sequence of RB-IRK2 shares 70%, 40% and 45% identity to mouse IRK1, rat ROMK1 and rat GIRK1, respectively. *Xenopus* oocytes injected with cRNA derived from RB-IRK2 expressed a potassium current which showed inward-rectifying channel characteristics similar to the IRK1 current, but distinct from the ROMK1 or the GIRK1 currents. However, the localization of RB-IRK2 mRNA in rat tissues, assessed by the Northern blot analysis, differed from that of mouse IRK1. These results indicate that the IRK family is composed of multiple genes, which express in different tissues and therefore may play heterogeneous functional roles in various organs, including rat central nervous system.

**Key words:** Inward rectifier potassium channel; cDNA library; Rat brain; Northern blot analysis; *Xenopus* oocyte

### 1. Introduction

Complementary DNAs of inward rectifier potassium channels have been cloned from the outer medulla of rat kidney (ROMK1) [1] and from a mouse macrophage cell line (IRK1) [2]. Both of these channels show only two transmembrane segments with one pore-forming region. More recently, a G protein-coupled muscarinic potassium channel (GIRK1) cloned from rat heart possesses a very similar molecular structure [3]. These K<sup>+</sup> channels can be electrophysiologically distinguished from each other at the whole cell and single channel current levels. Northern blot analysis shows ROMK1 mRNA to be expressed in kidney, spleen, lung, eye and forebrain [1], IRK1 in forebrain, cerebellum, heart, skeletal muscle

and a macrophage cell line [2], and GIRK1 in forebrain, cerebellum and heart [3]. Thus, K<sup>+</sup> channels with two transmembrane segments may form a family and play multiple functional roles in various organs. However, it is not known whether each member of this family is composed of multiple genes.

In the present study, to elucidate the possible diversity of the IRK channel family in the central nervous system, we have cloned from the rat brain cDNA library, electrophysiologically characterized and localized a novel member of the family.

### 2. Materials and methods

#### 2.1. Screening of rat brain cDNA library and DNA sequencing

A rat brain cDNA library was screened under a mild stringency condition using a *Bst*XI–*Not*I-digested IRK1 (~3.8 kb) as a probe. The rat brain cDNA library and mouse IRK1 cDNA were kindly provided by Dr. Shigetada Nakanishi (Kyoto University, Faculty of Medicine, Kyoto, Japan) [4] and Dr. Lily Y. Jan (UCSF, San Francisco, CA) [2], respectively. 4 × 10<sup>6</sup> Phage clones were screened with a <sup>32</sup>P-labelled probe. Hybridization was conducted in 5 × SSC, 30% formamide, 0.08% bovine serum albumin, 0.08% Ficoll, 0.08% polyvinylpyrrolidone, 0.1% sodium dodecyl sulfate (SDS) and 200 µg/ml denatured salmon sperm DNA, at 37°C for 17 h. Filters were washed once with 1 × SSC, 0.1% SDS at room temperature for 15 min and then twice with 0.1 × SSC, 0.5% SDS at room temperature for 20 min, and finally exposed to an X-ray film overnight at –70°C with an intensifying

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**Abbreviations:** IRK, inward rectifier potassium channel; cDNA, complementary deoxyribonucleic acid; cRNA, complementary ribonucleic acid; mRNA, messenger ribonucleic acid; SSC, saline-sodium citrate; SDS, sodium dodecyl sulfate; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase.

screen. DNA sequencing was performed on both strands using a sequencing kit (USB, Cleveland, OH) by specific oligonucleotide primers as previously described [5].

## 2.2. Functional expression of a rat brain inward rectifier potassium channel in *Xenopus* oocytes

The positive clone obtained was transcribed *in vitro* by T<sub>7</sub> RNA polymerase after digestion with *NotI* [5]. This transcript was dissolved in sterile water, and 50 nl of 1 µg/µl injected manually into defolliculated oocytes. After injection, oocytes were incubated in a modified Barth solution at 18°C, and electrophysiological studies were undertaken 48–96 h later.

Two-electrode voltage-clamp experiments were carried out with a commercially available amplifier (Turbo Clamp TEC 01C; Tamm, Germany) with microelectrodes which, when filled with 3 M KCl, had resistances of 0.5–1.5 MΩ. Oocytes were bathed in a solution which contained 90 mM KCl, 3 mM MgCl<sub>2</sub>, 5 mM HEPES (pH 7.4) and 150 µM niflumic acid to block endogenous chloride current. Voltage steps (1.5 s in duration) from the holding potential of 0 mV to potentials between +60 and –160 mV with 10 mV decrement were delivered at a rate of every 5 s. Experiments were performed at room temperature (20–22°C). Electrophysiological data were stored on video tapes using a PCM data recording system (NF Electronic Design, Tokyo, Japan), and subsequently replayed for computer analysis (EPAnalysis; Human Intelligence Inc., Rochester, MN).

## 2.3. Northern blot analysis

Total RNAs from various organs were extracted by the guanidine thiocyanate method [6], and poly(A)<sup>+</sup> RNAs were isolated using Oligotex-dT mRNA kit (QIAGEN, Chatsworth, CA). Equal amounts (3 µg) of poly(A)<sup>+</sup> RNAs, as determined by absorbance at 260 nm, were separated in a 1% agarose-formaldehyde gel and transferred to a Hybond-N nylon membrane (Amersham, Arlington Heights, IL). Hy-

bridization using random primer-labeled probe was performed in 50% formamide hybridization buffer with an *Apal*-digested fragment (2.0 kb) of pRB-IRK2 at 42°C. A glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA probe was used as a control to ascertain that equivalent amounts of mRNA had been transferred. Blots were washed at moderate stringency (55°C, 0.5 × SSC, 0.1% SDS) and exposed to Kodak XAR-5 film with an intensifying screen at –70°C.

## 3. Results

### 3.1. Molecular cloning of inward rectifier potassium channel

After screening 4 × 10<sup>6</sup> rat brain cDNA clones, we obtained a 4.5 kb cDNA (RB18-15) insert that contains an open reading frame. This phage insert was subcloned into plasmids by rescue excision to generate pRB18-15. We used pRB18-15 as a template for preparing cRNA and DNA sequencing. We designated this clone RB-IRK2 based on its sequence (Fig. 1) and the electrophysiological properties of the expressed current (Fig. 2).

The nucleotide sequencing revealed that an open reading frame of 1281 bp (Fig. 1A) encodes a protein of 427 amino acids (Fig. 1B). The deduced amino acid sequence of RB-IRK2 showed 70% identity with mouse IRK1. The Kyte–Doolittle hydropathy plot [7] indicates two

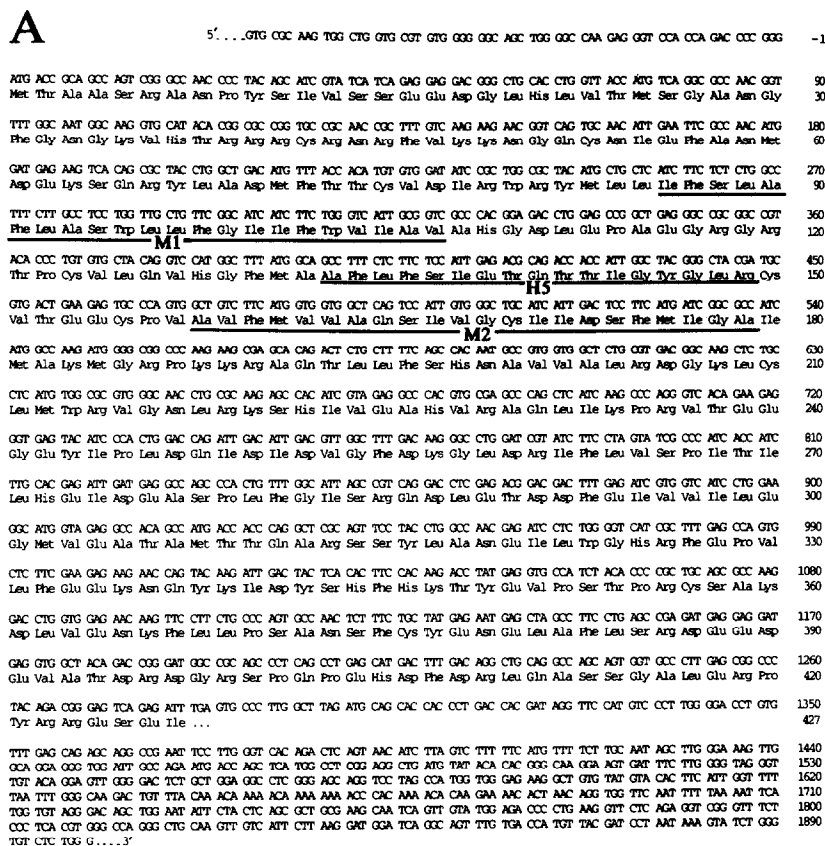


Fig. 1. (A) The nucleotide sequence and deduced amino acid sequence of the RB-IRK2 cDNA. The amino acids are numbered as groups of ten starting at the Met of the predicted amino-terminus. The nucleotides are numbered at the side of the sequence. Positive numbers are assigned to nucleotides 3' to the A of the initiation codon, which is assigned as +1. Nucleotides 5' to this codon are assigned negative numbers. Possible membrane spanning regions (M1 and M2) and potential pore region (H5) are indicated by lines below the sequence.

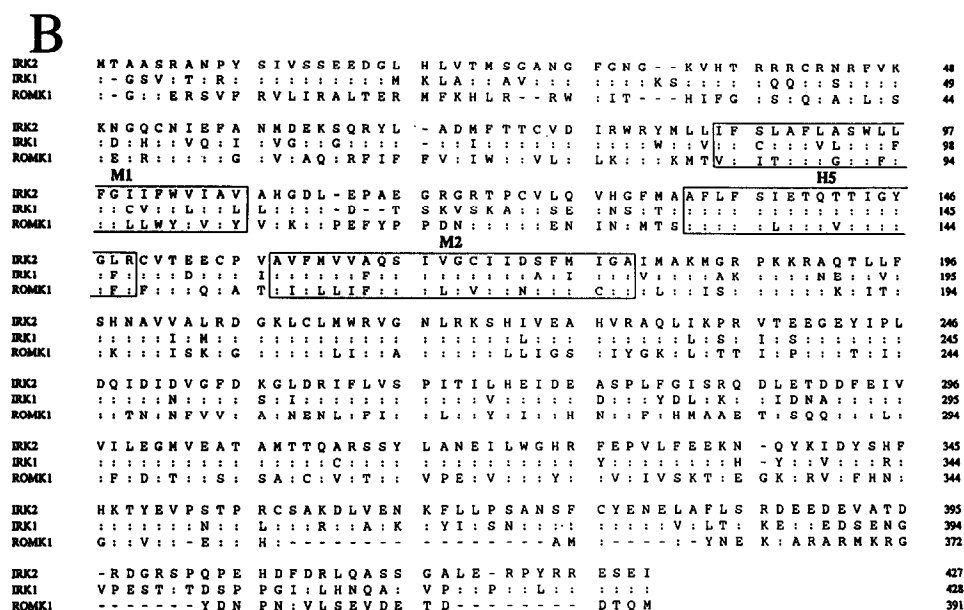


Fig. 1. (B) Alignment of the amino acid sequences (single letter code) of RB-IRK2, mouse IRK1 and rat ROMK1. :, amino acids identical with those in RB-IRK2. Dashes indicate gaps introduced into the sequence to improve alignment. The proposed transmembrane regions, M1 and M2, and the H5-like segments are boxed.

potential membrane spanning hydrophobic segments (M1 and M2) with a pore forming region (H5), a structure common to IRK1, ROMK1 and GIRK1 (data not shown) [1–3].

### 3.2. Electrophysiological characteristics of RB18-15 current

Fig. 2 illustrates the results obtained from a *Xenopus* oocyte which had been injected with cRNA derived from a RB-IRK2 clone 96 h before the recording. Under control conditions 90 mM of extracellular  $K^+$  ( $[K^+]_o$ ) (top traces in Fig. 2A, C and E), hyperpolarizing voltage steps from a holding potential of 0 mV revealed rapid activation ( $<10$  ms) of large inward currents which showed slight voltage-dependent inactivation at extreme hyperpolarizing voltages. Upon depolarization the current showed a clear inwardly rectifying property.

The effect of  $[K^+]_o$  on the RB-IRK2 current is depicted in Fig. 2A and B. As  $[K^+]_o$  was lowered from 90 mV to 45, 20 and 10 mV, the slope conductance of RB-IRK2 current was decreased. The activation potential defined as the potential at which the slope conductance changes noticeably was in good agreement with  $E_K$  values predicted from the Nernst equation at various  $[K^+]_o$ . Outward currents at potentials positive to  $E_K$  were considerably less than that predicted by a linear current-voltage relationship. These properties are consistent with those of the conventional type of inward rectifier potassium channels [8] and IRK1 [2,9].

External  $Ba^{2+}$  (Fig. 2C and D) concentration-dependently induced a time- and voltage-dependent block of the inward currents expressed by RB-IRK2. At 5 and

50  $\mu\text{M}$ ,  $\text{Ba}^{2+}$  clearly induced the time- and voltage-dependent block with a comparatively small effect upon the instantaneous current (Fig. 2C), but a marked influence upon the steady-state current (Fig. 2D). At 500  $\mu\text{M}$ ,  $\text{Ba}^{2+}$  essentially abolished the oocyte inward current, at both instantaneous and steady state. All three concentrations of  $\text{Ba}^{2+}$  slightly reduced the outward currents recorded by voltage steps to positive membrane potentials.

Cs<sup>+</sup> (Fig. 2E and F) exhibited less of a time-dependent effect, when compared to Ba<sup>2+</sup>, upon the RB-IRK2 current, yet showed a clear voltage-dependence of the block in a concentration-dependent manner (5–500 μM). Even 500 μM Cs<sup>+</sup> was essentially without effect at potentials positive to –70 mV (Fig. 2F).

### 3.3. Distribution of RB-IRK2 mRNA in various tissues

We conducted Northern blot analysis of RB-IRK2 in various tissues. RB-IRK2 mRNAs (about 2.4 and 4.4 kb) was detected in heart atrium, ventricle, forebrain, cerebellum, kidney and skeletal muscle, but not in uterus, liver and pancreas (Fig. 3). The transcript was most abundant in cerebellum and was very weak in heart, where no significant difference existed between atrium and ventricle.

## 4. Discussion

In the present study, we described the amino acid sequence, electrophysiological characteristics and localization of a novel inward rectifier potassium channel cloned from rat brain. The electrophysiological characteristics

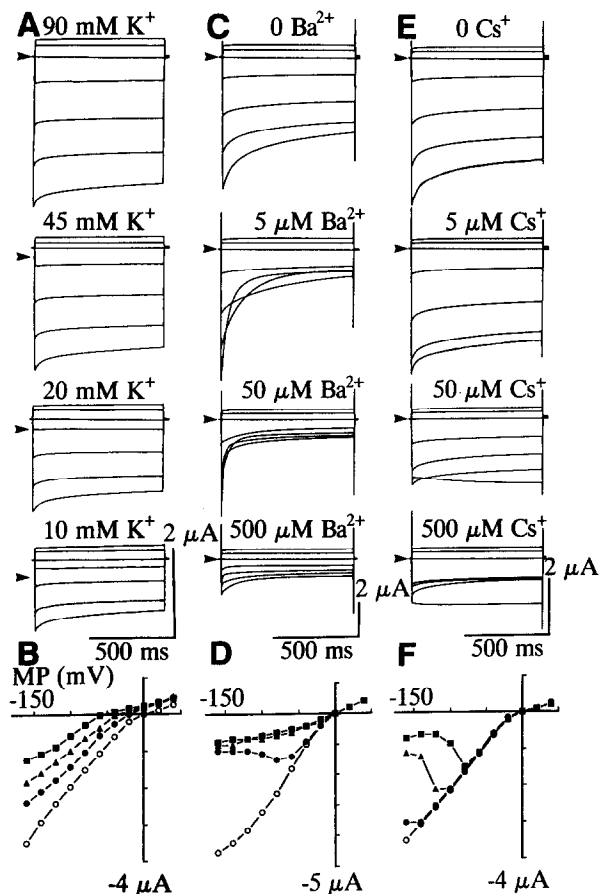


Fig. 2. Cell currents recorded from a *Xenopus* oocyte expressing the RB-IRK2 clone. A, C and E illustrate currents induced by voltage steps from 0 mV to, in descending order, +80, +40, 0, -40, -80, -120 and -160 mV (the full sequence of voltage steps is not shown for clarity). Upper traces in each column show records obtained under control conditions. (A) The effect of external  $K^+$  on the RB-IRK2 induced current. (B) Current-voltage relationship of the peak currents (10 ms after the start of voltage pulses) recorded from this oocyte at various  $K^+$  concentrations. (C) The effect of external  $Ba^{2+}$ . (D) Current-voltage relationships of the steady-state currents recorded from this oocyte in the presence of  $Ba^{2+}$ . (E) The effects of external  $Cs^+$ . (F) Current-voltage relationships recorded in the presence of  $Cs^+$ . The time and cell current scales are applicable to all traces which were recorded from a single oocyte. Labels above each family of traces indicate the concentration of cations which had been included in the solution which bathed the oocytes.  $\circ$ , control;  $\Delta$ , 5  $\mu M$  cation;  $\blacktriangle$ , 50  $\mu M$  cation;  $\blacksquare$ , 500  $\mu M$  cation. Arrows indicate the zero current level.

of this clone, i.e. such as absence of current at potentials positive to  $E_K$ , the dependence of the slope conductance on  $[K^+]_o$ , rapid activation upon hyperpolarizing pulses, and the time- and voltage-dependent block by  $Ba^{2+}$  and  $Cs^+$ , were the same as those of classical inward rectifier potassium channels in a variety of cell types, including IRK1. The ROMK1 current expressed in oocytes does not rectify prominently [1]. This is probably because ROMK1 does not possess the activation gating mechanism which may be majorly responsible for the inward rectification of the whole cell IRK current in addition to

the  $Mg^{2+}$  block of the outward-flowing current [10,11]. Activation of G proteins is essential for the GIRK current, which is also characterized with a slow activation time-course upon hyperpolarizing pulses [3]. Thus, the obtained clone may belong to the same family as IRK1, but is distinct from ROMK1 or GIRK1. Consistent with this notion, the amino acid sequence of the clone shows 70% identity with mouse macrophage IRK1 [2], 40% with rat kidney ROMK1 [1] and 45% with rat heart GIRK1 [3]. Therefore, we designated it RB-IRK2. Like all previously cloned inward rectifier potassium channels, the RB-IRK2 channel possesses two transmembrane domains with a putative pore-forming region (H5). The H5 region comprises virtually identical amino acids as that of mouse IRK1 with the exception of residue 148. To examine whether this difference may influence the conductance of this  $K^+$  channel, a single channel recording experiment will be considered.

One of the most specific features of RB-IRK2 is its localization which is distinct from those of IRK1, GIRK1 and ROMK1; i.e. RB-IRK2 mRNA is most prominent in cerebellum and also expressed in forebrain, kidney, heart and skeletal muscle, whereas IRK1 and GIRK1 are not expressed in kidney and ROMK1 is not expressed in either heart, cerebellum or skeletal muscle. This result further supports the notion that RB-IRK2 is

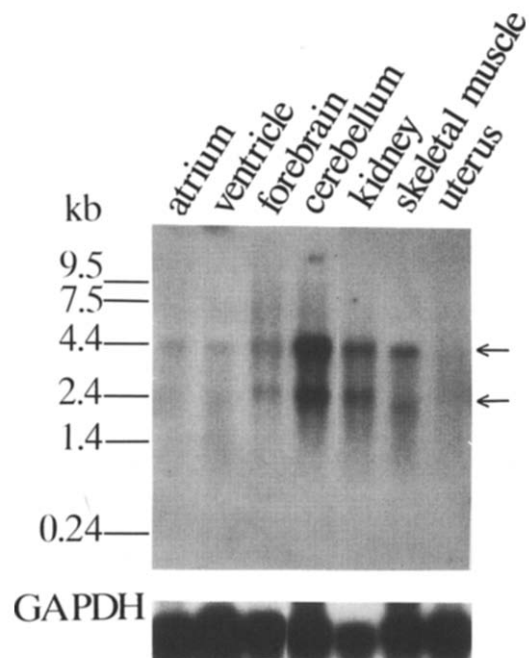


Fig. 3. Distribution of RB-IRK2 mRNA in various tissues by Northern blot analysis. The lanes represent poly(A)<sup>+</sup> RNA from rat heart atrium, ventricle, forebrain, cerebellum, kidney, skeletal muscle and uterus. An *Apal*-digested fragment (2.1 kb) of pRB-IRK2 was used for a probe. Two major bands of 4.4 and 2.4 kb RNA that hybridized with the probe are indicated with arrows. The positions of RNA size markers are shown on the left side. The quantity of RNA samples were standardized by re-probing the same blot with a labelled cDNA for GAPDH (lower panel). No signal was detectable using RNA from pancreas and liver.

a novel member of the inward rectifier potassium channel family.

This study provides for the first time evidence that the IRK family is composed of multiple genes. There may exist other clones belonging to this family. The members of the IRK family may distribute differentially in various cells and play multiple functional roles.

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