

Molecular cloning and functional expression of a novel brain-specific inward rectifier potassium channel

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Abstract

We have cloned a novel brain-specific inward rectifier K⁺ channel from a mouse brain cDNA library and designated it MB-IRK3. The mouse brain cDNA library was screened using a fragment of the mouse macrophage inward rectifier K⁺ channel (IRK1) cDNA as a probe. The amino acid sequence of MB-IRK3 shares 61% and 64% identity to MB-IRK1 and RB-IRK2, respectively. *Xenopus* oocytes injected with cRNA derived from this clone expressed a potassium current which showed inward-rectifying channel characteristics similar to MB-IRK1 and RB-IRK2 currents, but distinct from ROMK1 or GIRK1 current. However, the single channel conductance of MB-IRK3 was ~10 pS with 140 mM extracellular K⁺, which was distinct from that of MB-IRK1 (20 pS). MB-IRK3 mRNA expressed specifically in the forebrain, which clearly differed from MB-IRK1 and RB-IRK2 mRNAs. These results indicate that members of the IRK family with distinct electrophysiological properties express differentially and may play heterogeneous functional roles in brain functions.

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

1. Introduction

Complementary DNAs of background or inward-rectifying 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 two transmembrane segments with one pore-forming region. The G protein-coupled muscarinic potassium channel (GIRK1), recently cloned from rat heart, also possesses essentially the same molecular structure [3]. 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]. These K⁺ channels can also be electrophysiologically distinguished from each other at the whole cell and single channel current levels.

We recently cloned the second member of IRK family from rat brain cDNA library and designated it RB-IRK2 [4]. Although RB-IRK2 currents expressed in *Xenopus*

oocytes show electrophysiological properties similar to IRK1 current, RB-IRK2 mRNA expresses highly in cerebellum, and moderately in forebrain, heart and skeletal muscle, which is distinct from the distribution of IRK1 [4]. Thus, the members of the IRK family may play multiple functional roles in various organs.

In the present study, to elucidate further diversity of the IRK family in the central nervous system and possible functional differences among the members, we have cloned from the mouse brain cDNA library, electrophysiologically characterized and examined organ distribution of a novel member of the IRK family and compared its electrophysiological properties and distribution with those of MB-IRK1 and RB-IRK2.

2. Materials and methods

2.1. Screening of mouse brain cDNA library and DNA sequencing

A mouse brain cDNA library was screened under a mild stringency conditions using a *Bst*XI–*Not*I digested IRK1 (~3.8 kb) as a probe. Mouse IRK1 cDNA was kindly provided by Dr. Lily Y. Jan (UCSF, San Francisco, CA) [2]. 4 × 10⁶ Phage clones were screened with a ³²P-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 screen. DNA sequencing was performed on both strands using a sequencing kit (USB, Cleveland, OH) by specific oligonucleotide primers as previously described [5].

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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.

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

The positive clone obtained was transcribed in vitro by T₃ RNA polymerase after digestion with *Xho*I [5]. This transcript was dissolved in sterile water, and injected 50 nl of 1 µg/µl to manually defoliated 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 MgCl₂, 5 mM HEPES (pH 7.4) and 150 µM niflumic acid to block endogenous chloride current. Oocytes were voltage-clamped at various holding potentials and voltage-steps of 1.5 s duration were applied to the cells in 10 mV increments every 5 s. Experiments were performed at room temperature (20–22°C). Single channel recordings were made in the cell-attached patch configuration

using a patch clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA). Both pipette and bath solutions contained 140 mM KCl, 1.4 mM MgCl₂ and 10 mM HEPES (pH adjusted to 7.4 with KOH). 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 (EP Analysis, Human Intelligence Inc., Rochester, MN).

2.3. Northern blot analysis

Total RNAs from various organs were extracted by the guanidine thiocyanate method [7], and poly(A)⁺ RNAs were isolated using Oligotex-dT mRNA kit (QIAGEN, Chatsworth, CA). Equal amounts (3 µg) of poly(A)⁺ RNAs, as determined by absorbance at 260 nm, were separated in 1% agarose-formaldehyde gel and transferred to a Hybond-N nylon membrane (Amersham, Arlington Heights, IL). Hybridization using the random primer labeled probe was performed in 50% formamide hybridization buffer with *Not*I digested fragment (1.4

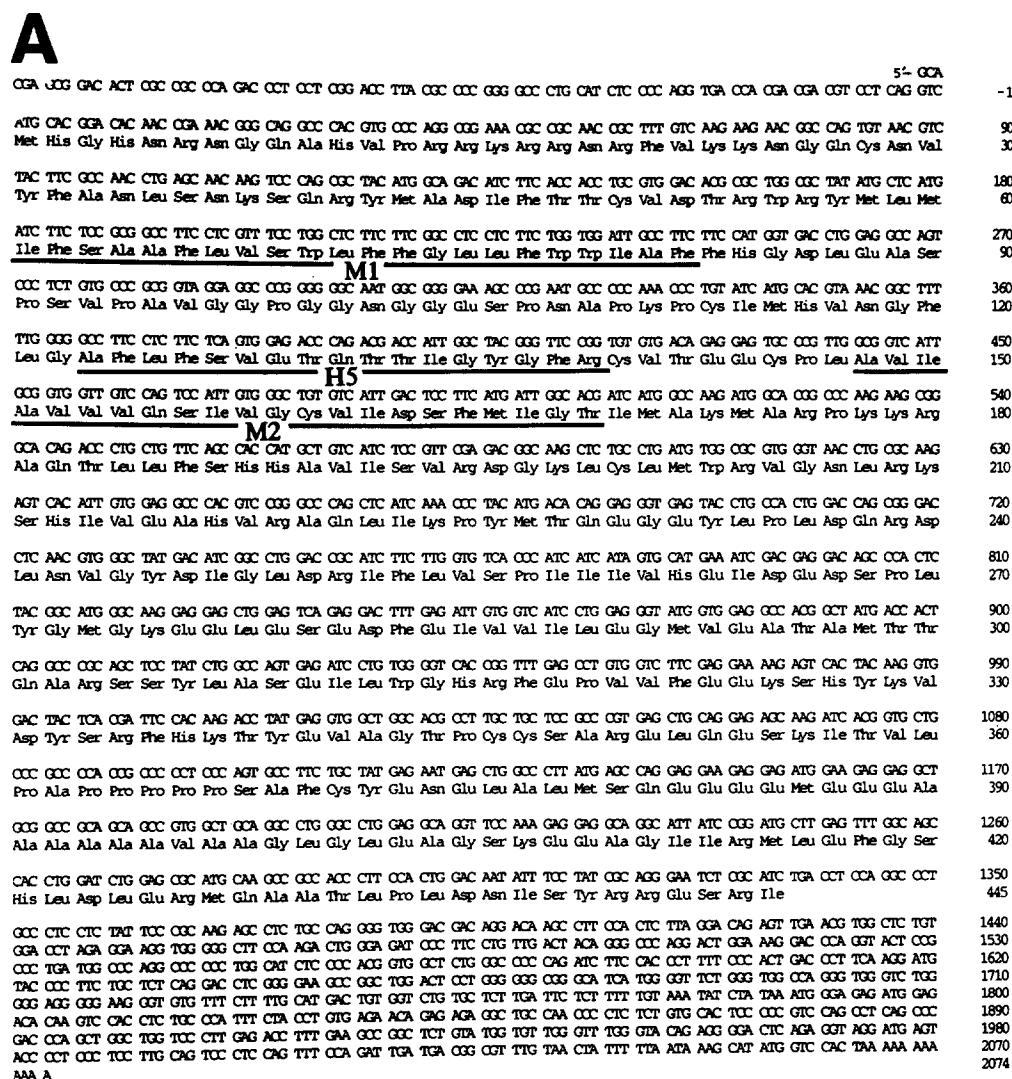


Fig. 1. (A) The nucleotide sequence and deduced amino acid sequence of the MB-IRK3 cDNA. The amino acids are numbered starting at the Met of the predicted amino terminus. The nucleotides are numbered at the side of the sequence. Positive numbers are assigned to nucleotides 3' to the A of the initiation codon, which is assigned as +1. Nucleotides 5' to this codon are assigned negative numbers. There is one potential cAMP and cGMP-dependent protein kinase phosphorylation site (443S), five potential protein kinase C phosphorylation sites (36S, 39S, 193S, 348S and 438S) and no potential tyrosine kinase phosphorylation sites. Possible membrane spanning regions (M1 and M2) and potential pore-forming region (H5) are indicated by lines below the sequence. (B) Alignment of the amino acid sequences (single letter code) of MB-IRK3 and MB-IRK1. Colons indicate amino acids identical with those in MB-IRK3. Dashes indicate gaps introduced into the sequence to improve alignment. The proposed transmembrane regions (M1 and M2) and potential pore-forming region H5 are boxed.

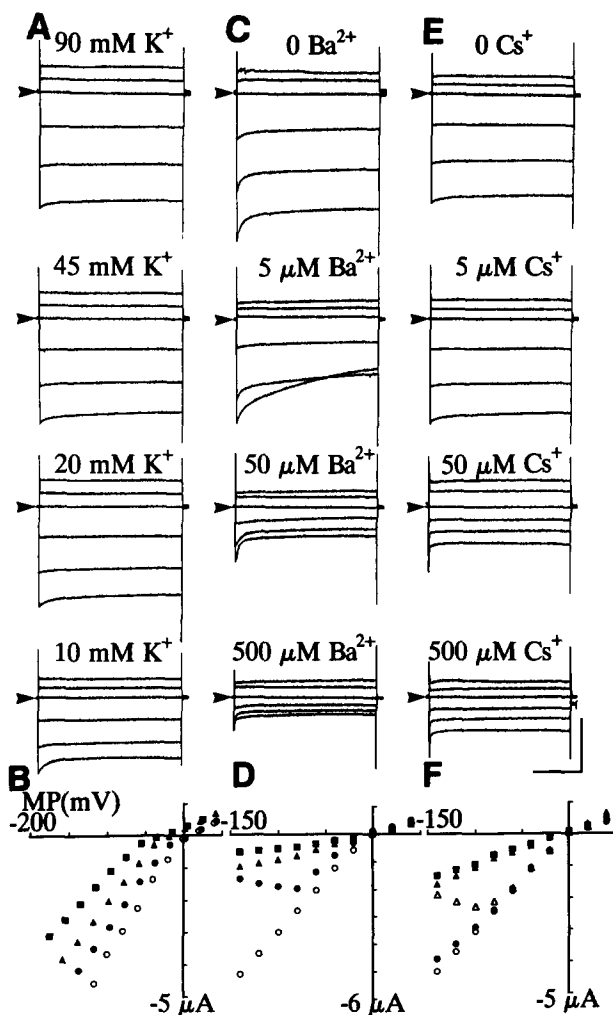


Fig. 2. Cell currents recorded from *Xenopus* oocytes expressing the MB-IRK3 clone. (A) The effect of varying external K^+ concentration. The holding potential was set at the zero current level in each solution, i.e. at 0 mV in 90 mM K^+ , at -17.4 mV in 45 mM K^+ , at -37.9 mV in 20 mM K^+ , at -55.3 mV in 10 mM K^+ and at -78.4 mV in 4 mM K^+ ; the values correspond to the equilibrium potential for K^+ at each concentration of external K^+ , with an assumption that the intracellular K^+ concentration of oocytes is 90 mM. Upper traces in each column show records obtained under control conditions. (A) The effect of external K^+ on the MB-IRK3 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. open circles: control; closed circle: 5 μM cation; open triangles: 30 μM , filled triangles: 50 μM cation; squares: 500 μM cation. Arrows indicate the zero current level.

potentials for K^+ (E_K) 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 [9], including MB-IRK1 [2,6] and RB-IRK2 [4].

External Ba^{2+} (Figs. 2C,D) concentration-dependently induced a time- and voltage-dependent block of the inward currents expressed by MB-IRK3. At 5 and 50 μM , 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 μM , Ba^{2+} essentially abolished the inward currents, both instantaneous and steady state. All three concentrations of Ba^{2+} slightly reduced the outward currents recorded by voltage steps to positive membrane potentials.

Cs^+ (Fig. 2E,F) exhibited less of a time-dependent effect, when compared to Ba^{2+} , upon the MB-IRK3 current. It, however, showed a clear voltage-dependence of the block in a concentration-dependent manner (5–500 μM).

3.3. Single channel currents of MB-IRK3

Single channel currents flowing through MB-IRK3 were recorded in the cell-attached configuration from oocytes which had been injected with cRNA derived from MB-IRK3, 48–96 h before recording (Fig. 3). Currents passing through these channels were observed only in the inward direction and, thus, showed a strong inwardly rectifying property (Fig. 3A,B). As shown in Fig. 3B, the mean slope conductance of MB-IRK3 channel was 13.4 ± 2.1 pS (mean \pm S.E.M., $n = 5$), significantly different from MB-IRK1 (~ 20 pS) and RB-IRK2 (~ 35 pS, unpublished data) [2, 6]. Thus, these three clones clearly expressed distinct inward-rectifying K^+ channels in *Xenopus* oocytes, although the difference was not clear at the whole cell current level.

3.4. Distribution of MB-IRK3 mRNA in various tissues

We conducted Northern blotting analysis of MB-IRK3 in various tissues. MB-IRK3 mRNAs (about 2.7 kb) was detected specifically in forebrain (cerebral hemispheres and diencephalon) and not in any other tissues, such as cerebellum, heart, skeletal muscle or kidney (Fig. 4). In addition, a very weak band of 5.5 kb was also detected in forebrain, which may result from alternative polyadenylation.

4. Discussion

In the present study, we described the amino acid sequence, electrophysiological characteristics and organ distribution of the third class of IRK family cloned from the mouse brain. The electrophysiological characteristics 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

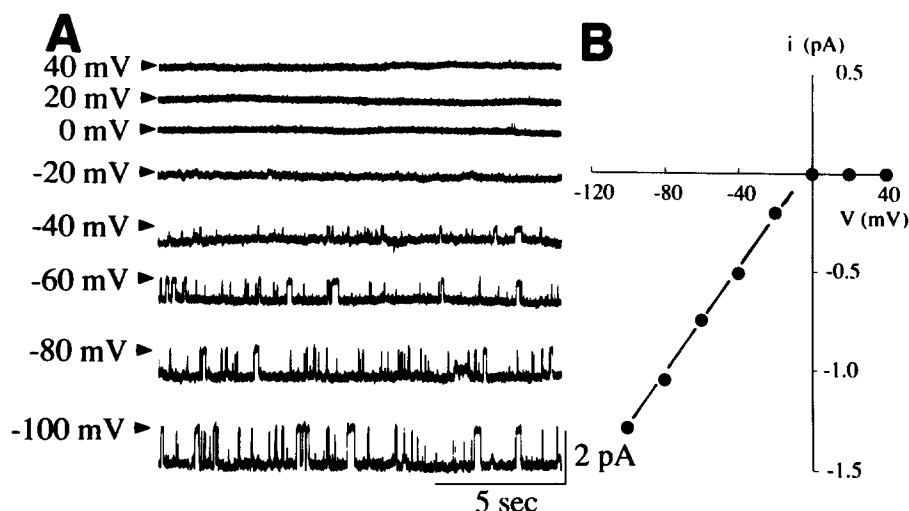


Fig. 3. Single channel recordings from cell-attached membrane patches of *Xenopus* oocytes expressing the MB-IRK3. (A) Membrane current traces recorded at the membrane potential values indicated to the left of each trace. The arrows to the right of certain traces indicate the patch current level recorded when all channels were closed. Each of these patches appeared to contain one inwardly rectifying potassium channel. The cut off frequency of the recording was 1 kHz (at -3 dB). (B) The current-voltage relationships of the channel records shown in (A).

Cs⁺, were the same as those of classical inward rectifier potassium channels in a variety of cell types, including MB-IRK1 and RB-IRK2 [2,4,6]. The ROMK1 current expressed in oocytes does not rectify prominently [1]. Activation of G proteins is essential for the GIRK1 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 MB-IRK1 and RB-IRK2, but is distinct from ROMK1 or GIRK1. Consistently with this notion, the amino acid sequence of the clone shows 61% identity with mouse macrophage IRK1 [2], 64% with RB-IRK2 [4], 38% with rat kidney ROMK1 [1] and 43% with rat heart GIRK1 [3]. Therefore, we have designated it MB-IRK3.

Like all previously cloned inward rectifier potassium channels, the MB-IRK3 channel possesses two transmembrane domains with a putative pore-forming region (H5). The H5 region of MB-IRK3 comprises virtually identical amino acids with those present in MB-IRK1 (with the exception of residue 128) and RB-IRK2 (with the exception of residues 128 and 138). These differences in the amino acid sequences of the H5 region might be responsible for the difference in the unitary conductance among these channels, since MacKinnon and Yellen [10] showed that one amino acid substitution in the H5 region alters the single channel conductance of *Shaker*-related potassium channels. However, it was also indicated that the regions outside of H5 are involved in the regulation of single channel conductance of *Shaker*-related potassium channels [11,12]. Therefore, further studies such as site-directed mutagenesis of the amino acids are necessary for understanding the precise roles of the H5 region and other regions on the single channel conductance of IRK.

One of the most specific features of MB-IRK3 is its distribution among various organs which is distinct from those of MB-IRK1, RB-IRK2, GIRK1 and ROMK1;

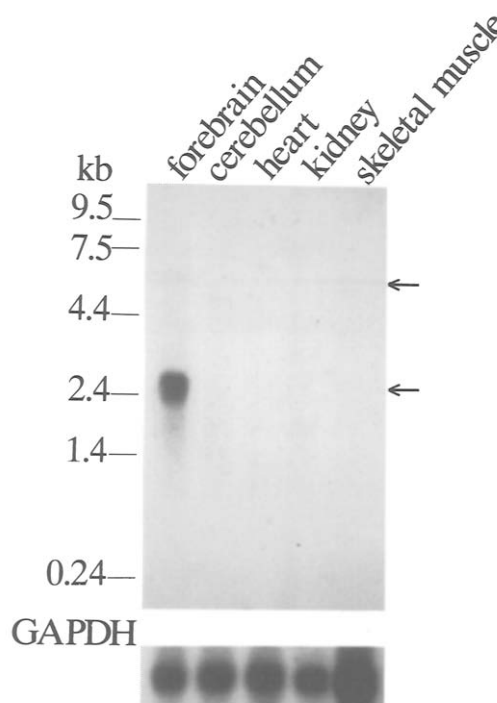


Fig. 4. Distribution of MB-IRK3 mRNA in various tissues by Northern blot analysis. The lane represent poly(A)⁺ RNA from mouse forebrain, cerebellum, heart, kidney and skeletal muscle. *NotI* digested fragment (1.4 kb) of pMB-IRK3 was used for a probe. One major band of ~2.7 kb and the other weak band of ~5.5 kb mRNAs 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 reprobating the same blot with a labelled cDNA for GAPDH (lower panel).

i.e. MB-IRK3 mRNA is expressed only in the forebrain, whereas IRK1, RB-IRK2, GIRK1 and ROMK1 are expressed not only in the forebrain, but also in other peripheral tissues. This result further supports the notion that the MB-IRK3 is a novel member of IRK family.

This study provides an evidence that the IRK family is composed of at least three genes. There may exist other clones belonging to this family. The members of IRK family may distribute differentially in various cells and play multiple functional roles. Further studies are needed to elucidate these possibilities.

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