

Cloning provides evidence for a family of inward rectifier and G-protein coupled K⁺ channels in the brain

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Abstract MbIRK3, mbGIRK2 and mbGIRK3 K⁺ channels cDNAs have been cloned from adult mouse brain. These cDNAs encode polypeptides of 445, 414 and 376 amino acids, respectively, which display the hallmarks of inward rectifier K⁺ channels, i.e. two hydrophobic membrane-spanning domains M1 and M2 and a pore-forming domain H5. MbIRK3 shows around 65% amino acid identity with IRK1 and rbIRK2 and only 50% with ROMK1 and GIRK1. On the other hand, mbGIRK2 and mbGIRK3 are more similar to GIRK1 (60%) than to ROMK1 and IRK1 (50%). Northern blot analysis reveals that these three novel clones are mainly expressed in the brain. *Xenopus* oocytes injected with mbIRK3 and mbGIRK2 cRNAs display inward rectifier K⁺-selective currents very similar to IRK1 and GIRK1, respectively. As expected from the sequence homology, mbGIRK2 cRNA directs the expression of G-protein coupled inward rectifier K⁺ channels which has been observed through their functional coupling with co-expressed δ -opioid receptors. These results provide the first evidence that the GIRK family, as the IRK family, is composed of multiple genes with members specifically expressed in the nervous system.

Key words: Inward rectifier K⁺ channel; G protein effector; *Xenopus* oocyte

1. Introduction

K⁺-selective channels represent the most diverse ion channel class known and numerous distinct members were identified by electrophysiology in the nervous system [1]. At the molecular level, the study of these channels has begun with the cloning of voltage-dependent K⁺ channels. Following the original cloning of the *Drosophila Shaker* gene, more than 16 mammalian genes were isolated encoding A-type and delayed-rectifier outward K⁺ channels (for reviews see [2–4]). More recently, expression cloning has led to the molecular identification of inwardly rectifying K⁺ channels in plant [5,6] and mammals (for reviews see [7,8]). In contrast with the outward K⁺ channels which display six putative membrane-spanning domains (S1 to S6), the mammalian inward rectifier K⁺ channels including the mild rectifying ROMK1 [9] and the more rectifying IRK1 [10] and G-protein coupled GIRK1/KGA [11,12], have two potential transmembrane domains (M1 and M2). Despite primary sequence differences, both families display a well conserved domain (H5) which is believed to form the K⁺-selective pore.

Molecular biology has helped to elucidate many of the mechanisms underlying the diversity of outward K⁺ currents in the brain such as gene duplication, alternative splicing, heteropolymerisation, β subunits, density of expression, etc. (for reviews see [2–4,13–15]). In order to elucidate the possibility that brain inward rectifier potassium channels are encoded by a multigenic family like the outward channels, we have screened under low stringency conditions a mouse brain cDNA library with IRK1 and GIRK1 probes. cDNAs encoding three novel channels essentially expressed in brain were isolated and designated mbIRK3, mbGIRK2 and mbGIRK3. The electrophysiological properties of mbIRK3 and mbGIRK2 were examined using the *Xenopus* oocyte system.

2. Materials and methods

2.1. cDNA cloning and sequencing

An oligo(dT)-primed cDNA λ ZAPII library derived from poly(A)⁺ RNA isolated from 20-day-old mouse brain (Stratagene) was screened sequentially with random primed ³²P-labeled DNA probes. The GIRK1 and IRK1 probes were amplified by polymerase chain reaction (PCR) using primer sequences corresponding to published sequences [10,11]. Filters were hybridized in 30% formamide, 5 $\times$ SSC, 4 $\times$ Denhardt's solution, 0.1% SDS, 100 μ g denatured salmon sperm DNA at 42°C for 18 h. Filters were then washed stepwise to a final stringency of 0.5 $\times$ SSC, 0.3% SDS at 45°C. The positive λ ZAPIIs were converted to plasmid cDNAs by rescue excision (Stratagene) and the cDNA inserts were sequenced on both strand by the dideoxy nucleotide chain termination method by using the dye terminator kit and automatic sequencing (Applied Biosystems model 373A). Deletion clones for sequencing were prepared with the Erase-A-Base system according to the manufacturer's protocol (Promega).

2.2. RNA isolation and analysis

Poly(A)⁺ RNAs were isolated and blotted onto nylon membranes as previously described [16]. The blots were probed with *EcoRI/XhoI* inserts of pBlueScript-IRK3 (pBS-IRK3), pBS-GIRK2 and pBS-GIRK3 in 50% formamide, 5 $\times$ SSPE (0.9 M sodium chloride, 50 mM sodium phosphate (pH 7.4), 5 mM EDTA), 0.1% SDS, 5 $\times$ Denhardt's solution, 20 mM potassium phosphate (pH 6.5) and 250 μ g denatured salmon sperm DNA at 55°C for 18 h and washed to a final stringency of 0.1 $\times$ SSC, 0.3% SDS at 65°C.

2.3. Synthesis of cRNA and functional expression in *Xenopus* oocytes

The coding sequence of mbIRK3, mbGIRK2 and mbGIRK3 were subcloned into the plasmid pBTG [17]. A DNA fragment corresponding to the coding sequence of the δ -opioid receptor was amplified by PCR and subcloned into pBS [18]. Capped cRNAs were synthesized using the T3 RNA polymerase (Stratagene). Preparation of oocytes, cRNA injections and electrophysiological measurements have been previously described [17]. The 98 mM K⁺ solution was made by substituting NaCl with KCl in the normal ND96 solution. Similarly, a 2 mM K⁺, sodium free solution, was obtained by substituting sodium with N-methyl-D-glucamine (NMDG). Both solutions were mixed in order to obtain solutions of intermediate K⁺ concentrations. Deltorphan I was purchased from Neosystem.

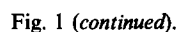
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Fig. 1. Nucleotide and deduced amino acid sequences of mbGIRK2 (A) and mGIRK3 (B) cDNA. Nucleotide residues are numbered from the first nucleotide of the initiating ATG codon. Amino acids are numbered beginning with the initiating methionine. The non-sense codons TGA at the end of ORFs are marked by an asterisk. The boxed areas delimit the two putative transmembrane domains M1 and M2 and the potential pore-forming domain H5. Putative protein kinase C (Δ), caseine kinase II (*) and Ca^{2+} -calmodulin kinase ($\circ$) phosphorylation sites are shown.

cytoplasmic M1-H5 interdomain and in the C-terminus. No homology was found between the C- and the N-termini of members of the GIRK family except in the C-termini of mbGIRK2 and mbGIRK3. Based upon the topology proposed for these channels, several potential sites for phosphorylation by protein kinase C, Ca²⁺ calmoduline-dependent kinase and casein kinase II are found in mbGIRK2 (Fig. 1A) and mbGIRK3 (Fig. 1B).

3.2. Tissue distribution and developmental expression of *IRK3* mRNA in the mouse brain

Using DNA fragments corresponding to the sequence of mbIRK3, mbGIRK2 and mbGIRK3 cDNA clones, we investigated the tissue distribution of these channels by Northern blot analysis (Fig. 3). The mbIRK3 probe strongly hybridized to a transcript of 2.9 kb and less intensively to a transcript of approximately 6.6 kb. MbIRK3 mRNA is highly expressed in the brain and only moderately in the skeletal muscle. No messenger was detected in the heart, lung, kidney or liver. The mbGIRK3 message is also highly expressed in brain RNA in



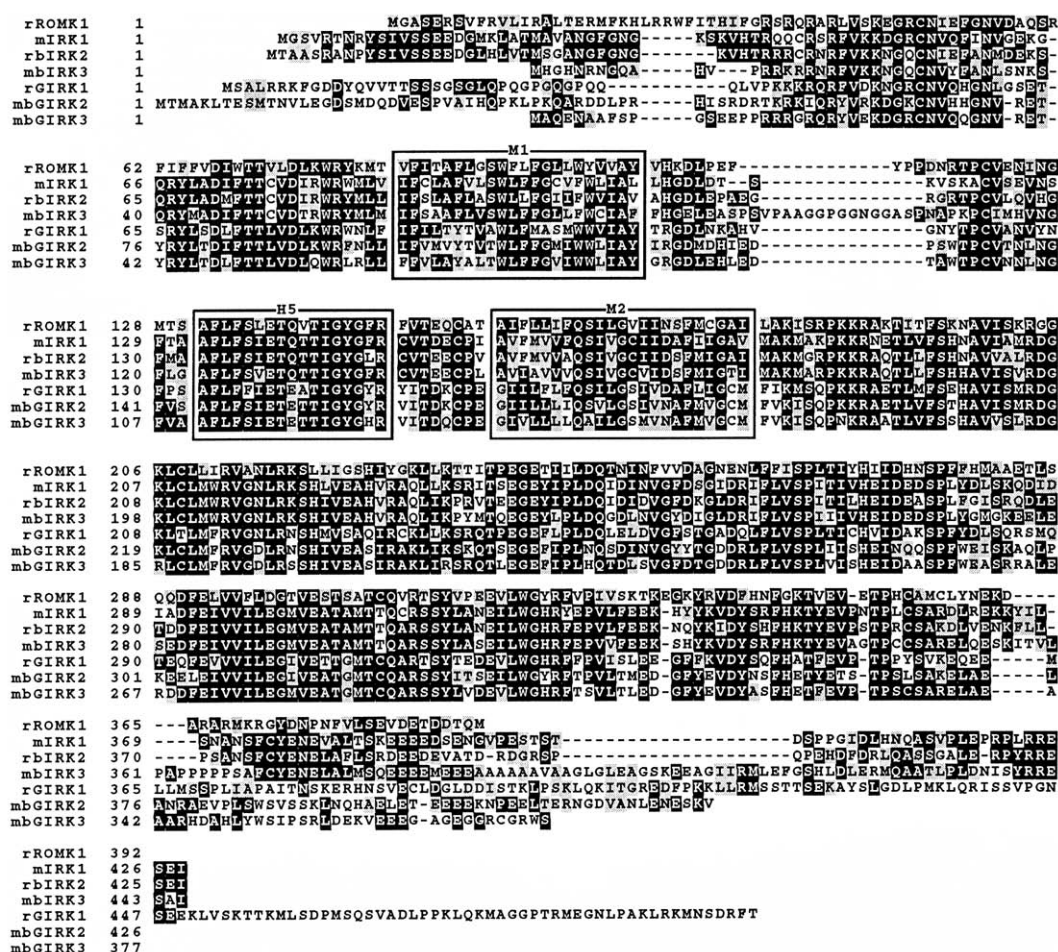


Fig. 2. Amino acid sequence alignment of the rat ROMK1, GIRK1 and IRK2, the mouse IRK1 K^+ channels and of the newly cloned mouse brain IRK3, GIRK2 and GIRK3 channels. Shaded amino acids represent fully conserved moieties between the three channels. The segments M1, H5 and M2 are boxed.

which a transcript of 4.2 kb is detected. A longer exposure of the autoradiogram revealed a low expression in the skeletal muscle and no expression in other examined tissues. Finally, mbGIRK2 is the less expressed of the three examined channels. A transcript of 6.8 kb was only detected in the brain. Because the size of the isolated cDNAs for GIRK2 and GIRK3 are shorter than those observed for mRNAs, it may be suggested that the sequences shown in Fig. 1 do not contain the entire untranslated sequences.

Since these K^+ channels are mainly expressed in brain, we have also examined their expression during brain development (at 2, 8, 15, 45 and 120 days post-partum). Northern blot analysis revealed that these three channels are moderately expressed at 2 days and that messages accumulate regularly from 2 to 45 days. Expressions are then stable from 45 to 120 days (data not shown).

3.3. Functional expression of inward rectifier K^+ channels in *Xenopus* oocytes

Large K^+ -selective currents were recorded in mbIRK3 cRNA injected oocytes. The electrophysiological properties of

these currents are essentially identical to those reported by Morishige et al. [19] and are not illustrated. Briefly, mbIRK3 shows a strong rectification and is sensitive to Ba^{2+} ($IC_{50} = 6.4 \mu M$ at -150 mV) and to Cs^+ ($IC_{50} = 10.9 \mu M$ at -150 mV). A slope conductance of 16 ± 4 pS ($n = 12$), estimated between -50 and -120 mV, was found in the cell-attached configuration (140 mM K^+ in the pipette).

The electrophysiological properties of mbGIRK2 were characterized in oocytes injected with mbGIRK2 and δ -opioid receptor cRNAs. Application of an external solution containing 98 mM K^+ instead of the external normal solution (ND96) revealed an inward current of 511 ± 52 nA ($n = 5$) at -80 mV (Fig. 4B). This current represented the basal activity of inward rectifier K^+ channels. The application of a δ -opioid agonist (deltorhin I) in 98 mM K^+ solution increased the inward current by 190 ± 16 nA ($n = 5$) (Fig. 4B). The $I-V$ characteristics of the deltorhin activated mGIRK2 current showed strong inward rectification. The current amplitude and the reversal potential were both dependent on the external K^+ concentration (Fig. 4C). The reversal potential of the mbGIRK2 current changed as a function of the external K^+ concentration indicat-

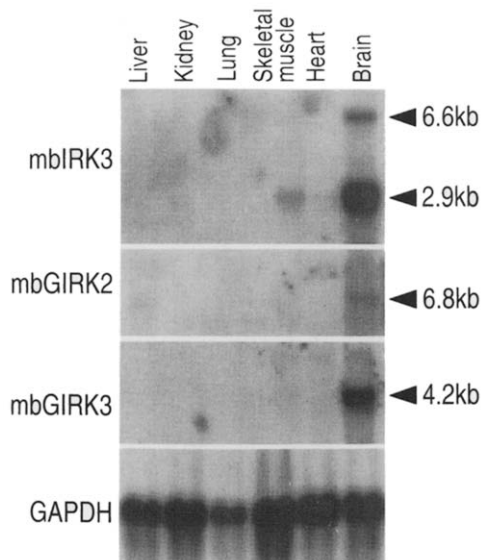


Fig. 3. Northern blot analysis of mbIRK3, mbGIRK2 and mbGIRK3 expressions in adult mouse. Each lane represents 5 μ g of poly(A)⁺RNA. The autoradiographs of mbIRK3 and mbGIRK3 were exposed for 24 h at -70°C and mbGIRK2 for 96 h. A blot was hybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe for relative quantification. The exposure time was 2 h at -70°C .

ing that the current was mainly carried by K^{+} (Fig. 4C). In comparison, the control oocytes injected with the δ -opioid receptors showed only low endogenous inward currents (9 ± 4 nA ($n = 5$)) during application of a solution containing 98 mM K^{+} and deltorphin I (Fig. 4A).

Using the same electrophysiological protocols as for mbIRK3 and mbGIRK2 channels, we failed to characterized any exogenous K^{+} current in oocytes injected with mbGIRK3 cRNA alone or co-injected with the δ -opioid receptor cRNA.

4. Discussion

In order to isolate and characterize new brain inward rectifier K^{+} channels, we have screened at low stringency a mouse brain cDNA library with IRK1 and GIRK1 probes. This resulted in the isolation of three novel sequences. One of these encodes a protein identical to the channel recently cloned by Morishige et al. [19] and designated mbIRK3. With 60 to 70% amino acid identity with the mouse macrophage IRK1 and the rat brain IRK2 and less than 50% with ROMK1 and GIRK1 channels, mbIRK3 is expected to belong to the functional IRK subclass of inward rectifier channels. Effectively, in *Xenopus* oocytes, mbIRK3 cRNA directs the expression of K^{+} -selective currents with properties typical of the previously expressed IRK1 and rbIRK2 isoforms, i.e. a strong rectification (channels are open at potentials more negative than E_{K}) and a block by Cs^{+} and Ba^{2+} . However, the mbIRK3 channel has a unitary conductance (16 pS) different of that of IRK1 (20 pS) [10] and rbIRK2 (35 pS) [20]. Another major difference between these different isoforms is their tissue distribution. While they are all expressed in the brain, IRK1 is also detected in macrophage, heart and skeletal muscle and rbIRK2 in heart, skeletal muscle and kidney. Only mbIRK3 is highly and almost exclusively expressed in the brain. This diversity of sequences and distribu-

tions of IRK channels is expected to be associated with multiple functional roles of these different channels particularly in the brain.

The isolation of two cDNAs encoding proteins with sequences more similar to GIRK1 than to IRKs and ROMK1 and designated mbGIRK2 and mbGIRK3 argues for the existence of a subfamily of G protein-modulated inward rectifier K^{+} channels. The observation that mbGIRK2 cRNA directs in oocytes the expression of a strongly rectifying K^{+} channel which is modulated by the δ -opioid receptor probably via an endogenous protein G confirms this view. It is interesting to note that mbGIRK2, like GIRK1/KGA [12], displays a basal activity independent of the G protein coupled receptor. This basal activity probably contributes to the resting membrane potential even in the absence of an activated receptor. For unknown reasons, mbGIRK3 failed to give a functional expression in *Xenopus* oocytes and experiments are being carried out to express this putative channel in other cell types and/or with other G coupled receptors. MbGIRK2 and mbGIRK3 are specifically expressed in the central nervous system. In contrast, GIRK1/KGA is expressed as well in the heart as in the brain [11,12].

In conclusion, this work presents further evidence that inward rectifier K^{+} channels form a large multigenic family. Cloning of these channels provides molecular probes to explore and define the physiological roles of these different inward rectifier

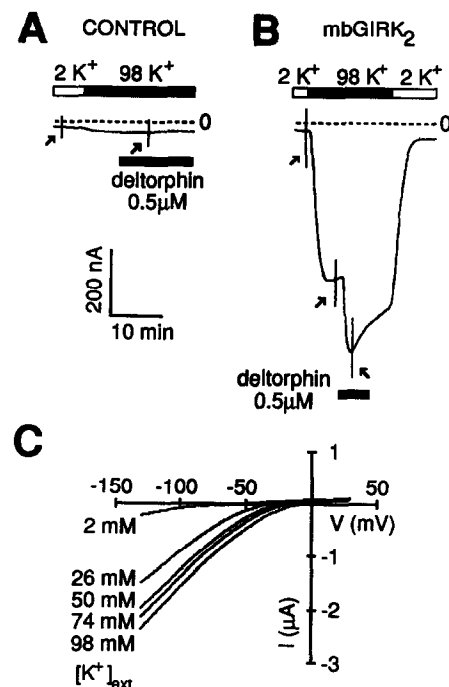


Fig. 4. Functional characterization of mbGIRK2 in *Xenopus* oocytes by the two electrodes voltage clamp technique. Inward currents evoked by external application of 98 mM K^{+} and 0.5 μM deltorphin in δ -opioid receptor cRNA injected oocyte (A) and in δ -opioid receptor and mbGIRK2 cRNAs injected oocyte (B). The holding potential was -80 mV. External K^{+} concentrations and deltorphin application are shown by horizontal bars (A,B), voltage ramps are indicated by arrows on current traces (reduced for clarity of the plot). (C) I - V curves were recorded in various concentrations of K^{+} by using voltage ramps (between -130 to $+30$ mV) lasting 500 ms. Ramps were recorded at the peak of the deltorphin-evoked current.

K⁺ channels in the brain. On the other hand, the characterization of functional G coupled IRK channels (1 and 2) as well as related but uncoupled IRKs channels (1, 2 and 3) will probably provide useful elements for further study of structure-function relationships of G protein-K⁺ channel interactions.

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