Cloning and characterization of a novel human inwardly rectifying potassium channel predominantly expressed in small intestine

Michel Partiseti*, Vincent Collura, Magali Agnel, Jean-Michel Culouscou, David Graham

Synthelabo Recherche, Department of Genomic Biology, 10 rue des Carrières, 92500 Rueil-Malmaison, France

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Abstract A new member of the two transmembrane domain potassium (K^+) channel family was identified and isolated from a human brain cDNA library. The cDNA clone contains an open reading frame which encodes a 360 amino acid sequence with a characteristic P domain flanked by two hydrophobic regions representing the membrane spanning segments. The closest homologue of this gene product is the inwardly rectifying potassium channel subunit, Kir1.2 (identity approximately 42%). Northern blot analysis of human tissues with a selective cDNA probe for this new K^+ subunit showed a single major transcript of 3.4 kb predominantly expressed at high levels in small intestine, with lower levels in stomach, kidney and brain. The main regions of expression in the central nervous system were medulla, hippocampus and corpus callosum. cRNA-injected oocytes and transiently transfected HEK293 cells expressed a K^+ conductance which displays an inward rectification. This conductance is blocked by cesium and barium but is insensitive to tolbutamide and diazoxide even upon co-transfection of this novel subunit with the plasmid encoding the sulfonylurea receptor SUR1. Taken together, these results demonstrate that we have isolated and characterized a novel K^+ channel subunit belonging to the inwardly rectifying K^+ (Kir) channel family to which, upon homology classification, we have given the nomenclature Kir7.1.

Key words: K^+ channel; Kir; *Xenopus* oocyte

1. Introduction

Inwardly rectifying potassium (Kir) channels are expressed in a wide variety of excitable and non-excitable cells and have been implicated in many different physiological processes with the common role of maintaining resting membrane potential near the K^+ equilibrium potential [1]. The activity of these channels can be regulated by hormones, neurotransmitters and intracellular factors linked to the metabolic state of the cell. The physiological importance of this K^+ channel family is supported by pharmacological studies which have shown that some of these channels could be major therapeutic targets to treat non-insulin-dependent diabetes mellitus and hypertension (for review see [2,3]).

The first cDNAs of this family were obtained by expression cloning techniques [4,5], and subsequently, other members were isolated by homology cloning [6–13]. These cloning efforts led to the distinction of six subfamilies (Kir1.0 to Kir6.0) based on their degree of homology [14,15]. The molecular dissection of all these subunits has revealed the basic motif to be a set of two membrane spanning domains flanking a P domain consensus sequence. Despite their homogeneity at the structural level, the Kir subunits display major heterogeneity in their tissue distribution and their functional properties. Differences in functional properties of these channels are reflected by their degree of inward rectification (strong or weak), as well as by modulatory changes as a result of interactions with G-proteins, intracellular ATP and pH.

In the present study, we report the cloning and tissue distribution of a novel Kir subunit that we have named Kir7.1. The electrophysiological properties of Kir7.1 were examined using the *Xenopus* oocyte system and transient cDNA transfection in the HEK293 cell line.

2. Materials and methods

2.1. Cloning

The Kir6.2 nucleotide and amino acid sequences were used to perform a BLAST search against an expressed sequenced tag (EST) database (Human Genome Sciences). Relevant ESTs were identified and a cDNA clone originating from a human brain cDNA library (SmithKline Beecham) was retrieved. This cDNA clone was sequenced on both strands to completion and used for further studies.

2.2. Oocyte experiments

After linearization of the cDNA clone at the unique *Xho*I site in the polylinker region, capped cRNAs were synthesized using a T3 RNA polymerase kit (Stratagene). Stage V–VI oocytes from the ovary of anesthetized (in ice-cold water) *Xenopus laevis* frogs were defolliculated enzymatically with collagenase (Life Technologies, 5 mg/ml for 30–45 min), at room temperature, under gentle agitation. Oocytes were then stored 24 h in Barth medium (containing 88 mM NaCl, 10 mM HEPES, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 0.1 mg/ml gentamicin, pH 7.6 with Tris-OH). cRNAs were pressure-injected (20–40 nl, 0.7 μg cRNA/μl) in oocytes using a calibrated injection device (Inject+Matic, Geneva). Oocytes were then incubated for 3–6 days and then transferred to a home-made chamber superfused at 5 ml/min with a solution containing 96 mM NaCl, 2 mM KCl, 5 mM HEPES, 3 mM $MgCl_2$, pH 7.4 with NaOH. Current and voltage electrodes were filled with 3 M KCl (0.5–2 mΩ) and connected to a GeneClamp 500 amplifier (Axon). Currents were acquired and analyzed using the pClamp6 software.

2.3. Patch-clamp experiments

In order to perform a functional study in HEK293 cells, the Kir7.1 cDNA was subcloned in the pcDNA3 expression vector (Invitrogen). HEK293 cells (ATCC) were plated on coverslips (12 mm in diameter) in 24-well culture plates (Costar) at a density of 5×10^4 cells/well, then cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal calf serum (FCS, Life Technologies), 100 μg/ml streptomycin and 100 U/ml penicillin, and incubated at 37°C in 5% CO_2-containing atmosphere. Kir7.1 plasmid (500 ng/well) and the expression plasmid for pGreen Lantern-1 (Life Technologies, 100 ng/well) were transfected using the Fugene 6 reagent (Boehringer). Briefly, 24 h after plating on coverslips, the cells were bathed in the medium containing the Fugene 6 plasmids mixture during 4 h. After this time, cells were washed and incubated for a further 24–72 h before electro-

*Corresponding author. Fax: (33) (1) 41 39 13 04.
E-mail: mpartise@hol.fr

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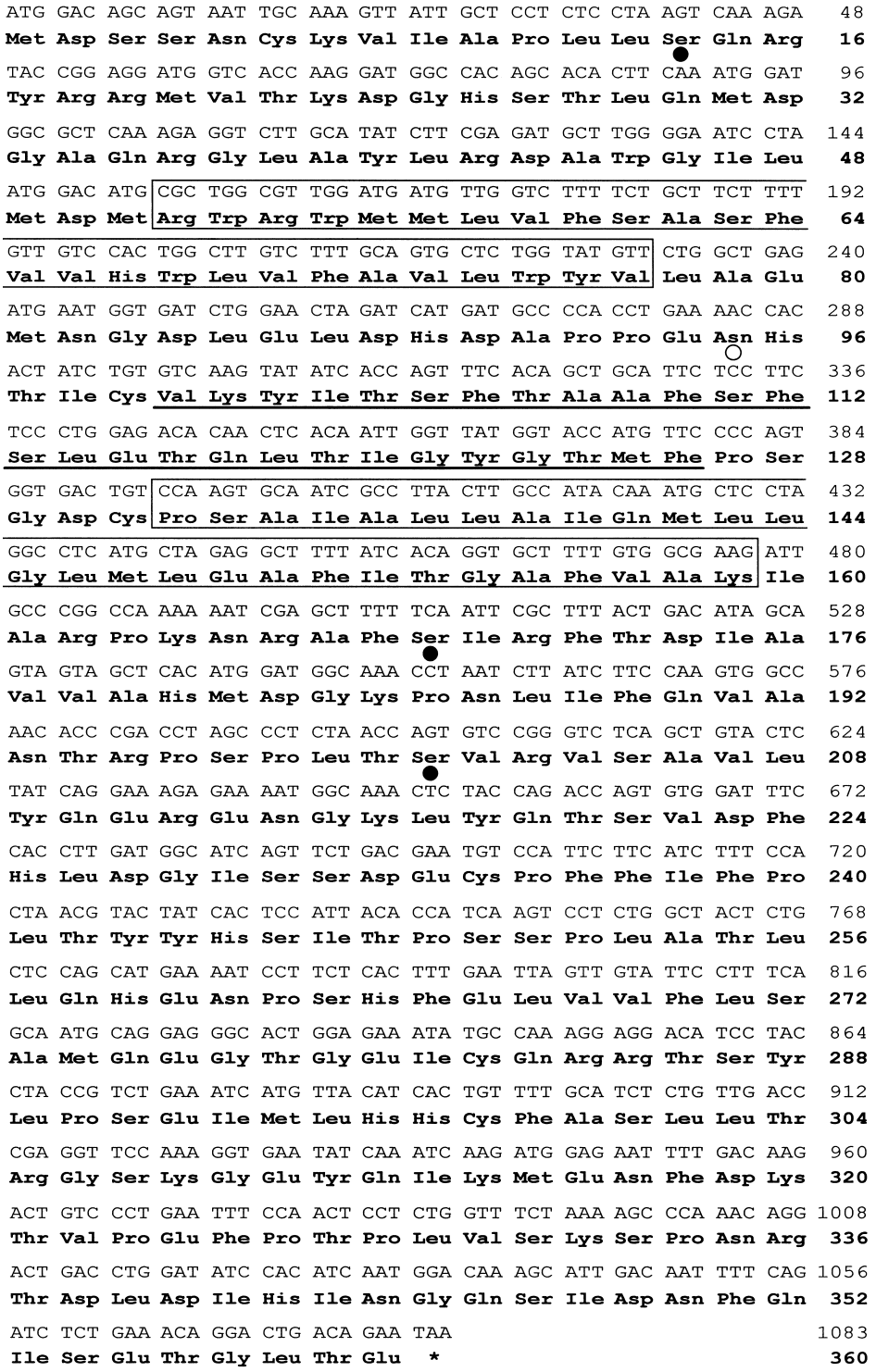

Fig. 1. Nucleotide and deduced amino acid sequences of human Kir7.1. The hydrophobic transmembrane segments and the P domain are boxed and underlined, respectively. The open circle (○) and filled circles (●) denote the consensus sequence for *N*-linked glycosylation and phosphorylation by protein kinase C, respectively. The plasmid containing this open reading frame was deposited in the American Type Culture Collection under the name 'plasmid Kir7.1' with the assigned number ATCC 209321.

physiological experiments. This protocol routinely showed that greater than 40–50% of the total population of cells were green fluorescent when illuminated on an epifluorescence inverted microscope (Nikon) used on the patch-clamp set-up. Only the fluorescent cells were selected for recording.

Standard patch-clamp methods were used to record whole-cell K^+ currents [16]. Prior to an experiment, a coverslip was transferred to a 400 μl recording chamber (Warner Instrument Corp.) and the cells were washed with the extracellular solution which consisted of 150 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.3 adjusted with NaOH. Pipettes were pulled from borosilicate glass capillaries (Clark Electromedical) and filled with a sol-

ution containing 130 mM KCl, 4 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM HEPES, pH 7.3 adjusted with KOH. The electrodes had a resistance of 3–5 MΩ. All chemicals were obtained from Sigma. Currents were recorded by an Axopatch 1D amplifier (Axon Instruments) and stored on a digital tape recorder (Biologic). Stimulation, data acquisition and analysis were done using pClamp 6 software (Axon). All experiments were performed at room temperature. Data are expressed as means ± S.D.

2.4. Northern blot analysis

Multiple tissue northern blots (2 μg RNA/lane, Clontech) were hybridized with a *Bam*HI-*Hin*dIII 2.0 kb fragment which was prepared from plasmid containing the novel Kir subunit and with glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA fragments (Clontech) using current molecular biology methods [17]. The probes were radiolabeled with ^{32}P-dCTP (NEN) using a random priming technique (Megaprime labeling kit, Amersham). The blots were scanned using a Molecular Dynamics Storm 860 Imager.

3. Results

3.1. Isolation of a new putative inwardly rectifying potassium channel subunit

A search for novel potassium channels was initiated by performing a homology comparison (BLAST alignment program) using Kir6.2 nucleotide and amino acid sequences against EST databases. The result of this search pointed to a relevant sequence showing significant similarity to Kir channels. Upon sequencing of the corresponding cDNA clone, a nucleotide sequence of 2931 bp with a single open reading frame of 1080 nucleotides was identified. This clone encodes a polypeptide of 360 amino acids (Fig. 1). Similar to other Kir subunits, hydrophobicity analysis of this protein indicates the presence of two transmembrane segments, namely TM1 and TM2, flanking a P domain consensus sequence. These hydrophobic domains were predicted using the TMAP software [18] which takes into account the hydrophobicity as well as multiple alignment analyses of the Kir sequences. Moreover, within the P domain, the characteristic Gly-Tyr-Gly motif of potassium channels is present. One potential *N*-glycosylation site (Asn-95) is located in the extracellular sequence and three consensus phosphorylation sites for protein kinase C are found in the N-terminal (Ser-14) and C-terminal (Ser-169 and Ser-201) cytoplasmic domains. A comparison of the amino-acid sequence with representative members of the K^+ channel superfamily reveals that it is most similar to the Kir subfamily. However, it shares only 29–42% identity and 37–47% similarity with them, warranting placement within a distinct subfamily (Fig. 2A). For comparison, Fig. 2B displays a pairwise alignment of the new Kir and Kir1.2 amino acid sequences, the latter being the most closely related. Therefore, we have chosen to designate this new Kir protein Kir7.1.

3.2. Functional expression of Kir7.1 subunit in Xenopus laevis oocytes and human embryonic kidney (HEK293) cells

Injection of in vitro transcribed cRNA for Kir7.1 in *Xenopus* oocytes produced inwardly rectifying currents as shown in the left column of Fig. 3A. Transient transfection in

A

	identity (%)	similarity (%)
Kir1.1	35	43
Kir1.2	42	47
Kir1.3	38	46
Kir2.1	31	40
Kir2.2	33	43
Kir2.3	36	44
Kir3.1	30	40
Kir3.2	32	40
Kir3.3	32	39
Kir3.4	30	39
Kir4.1	41	47
Kir5.1	30	37
Kir6.1	29	38
Kir6.2	30	39

B

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1   MDSSNCKVI----------APLLSQ--RYRR  KIR7-1
1   MTSVA-KVYYSQTTQTESRPLMGPGIRRRR  KIR1-2

20  MVTKDGHSTLQMDG-AQRGLAYLRDAWGIL  KIR7-1
30  VLTKDGRSNVRMEHIADKRFLYLKDLWTTF  KIR1-2

49  MDMRWRWMMLVFSASFVVHWLVFAVLWYVL  KIR7-1
60  IDMQWRYKLLLFSATFAGTWFLFGVVWYLV  KIR1-2

79  AEMNGDLELDHDAPPENHTICVKYITSFTA  KIR7-1
90  AVAHGDL-LELD-PPANHTPCVVQVHTLTG  KIR1-2

109 AFSFSLETQLTIGYGTMFPSGDCPSAIALL  KIR7-1
118 AFLFSLESQTTIGYGFRYISEECPLAIVLL  KIR1-2

139 AIQMLLGLMLEAFITGAFVAKIARPKNRAF  KIR7-1
148 IAQLVLTTILEIFITGTFLAKIARPKKRAE  KIR1-2

169 SIRFTDIAVVAHMDGKPNLIFQVANTRPSP  KIR7-1
178 TIRFSQHAVVASHNGKPCLMIRVANMRKSL  KIR1-2

199 LTSVRVSAVLYQER-----ENGKLYQTSVD  KIR7-1
208 LIGCQVTGKLLQTHQTKEGENIRLNQVNVT  KIR1-2

224 FHLDGISSDECPFFIFPLTYYHSITPSSPL  KIR7-1
238 FQVD--TASDSPFLILPLTFYHVVDETSPL  KIR1-2

254 ATLLQHENPSHFELVVFLSAMQEGTGEICQ  KIR7-1
266 KDLPLRSGEGDFELVLILSGTVESTSATCQ  KIR1-2

284 RRTSYLPSEIMLHHCFASLLTRGSKGEYQI  KIR7-1
296 VRTSYLPEEILWGYEFTPAISLSASGKYIA  KIR1-2

314 KMENFDKTVP-EFPTPLVSKS-----PNRT  KIR7-1
326 DFSLFDQVVKVASPSGLRDSTVRYGDPEKL  KIR1-2

338 DLDIHINGQSI-DNFQISETGLTE        KIR7-1
356 KLEESLREQAEKEGSALSVRISNV        KIR1-2
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Fig. 2. Sequence analysis of Kir7.1 with respect to different members of the Kir channel family. A: Pairwise comparisons using GAP from GCG (Genetics Computer Group) with the default parameters. The full-length coding sequences for the listed subunits were used. The gap penalty was 12, and the gap extension penalty was 4. Specific members of subfamilies and their accession numbers are as follows: Kir1.1 (ROMK1, U12541), Kir1.2 (U73191), Kir1.3 (U73193), Kir2.1 (U12507), Kir2.2 (X78461), Kir2.3 (U07364), Kir3.1 (U01071), Kir3.2 (U11859), Kir3.3 (U11860), Kir3.4 (CIR, X83584), Kir4.1 (BIR10, X83585), Kir5.1 (BIR9, X83581), Kir6.1 (D42145) and Kir6.2 (D50581). Note that the Kir1.2 sequence was defined as the human homologue of the rat sequence Kir4.1 [15]. B: Sequence comparison between Kir7.1 and Kir1.2. Identical amino acids are boxed in black. The alignment was performed with MEGALIGN (version 3.10a) from DNASTAR.

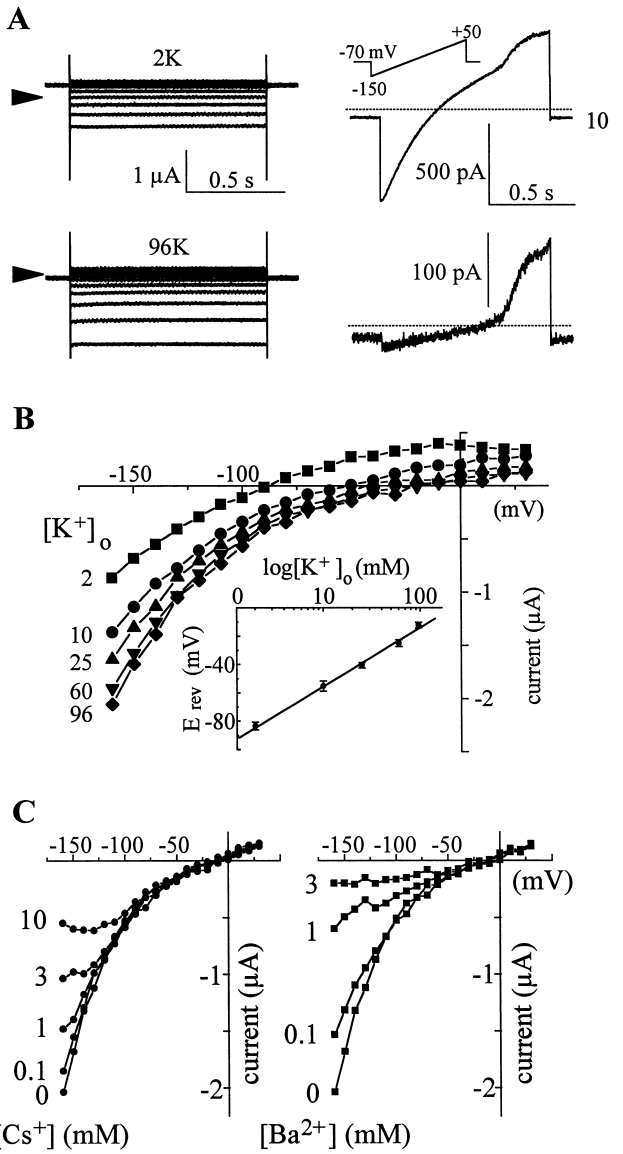

Fig. 3. Inwardly rectifying currents from *Xenopus* oocytes injected with cRNA transcribed from Kir7.1 cDNA clone and from transiently transfected HEK293 cells. A: Representative recordings collected from a Kir7.1 cRNA injected oocyte (left column), and two HEK293 cells (right column) transfected either with Kir7.1+GFP cDNAs (upper trace) or with GFP cDNA (lower trace). HEK293 cells were recorded in an external medium containing 30 mM K^+. The currents were monitored during pulses incremented by 20 mV from −150 mV with a holding potential of −60 mV for oocyte experiments and during voltage ramps (−150 to +50 mV, 1 s duration) for patch-clamp experiments. B: Current-voltage (I-V) relationship recorded from an oocyte injected with Kir7.1 cRNA in an extracellular solution containing 2, 10, 25, 60 or 96 mM of potassium ($[K^+]_o$). Inset: zero current (E_{rev}) potentials as a function of $[K^+]_o$ (logarithmic scale). The solid line is a linear regression fit to the data measured on five different oocytes. C: I-V relationships recorded from an oocyte in control solution (96 mM $[K^+]_o$) and increasing concentrations of cesium ([Cs^+] mM) and barium ([Ba^{2+}] mM).

HEK293 cells also induced an inwardly rectifying current elicited by voltage ramps. These currents were not seen in water-injected oocytes and cells transfected with green fluorescent protein alone. To determine whether this expressed current is K^+ selective, the current-voltage relationship was measured at different external K^+ concentrations (Fig. 3B). The shift in reversal potential (E_{rev}) was 42 ± 3 mV and 52 ± 2 mV per 10-fold change of $[K^+]_o$ in oocytes ($n = 5$, inset of Fig. 3B) and HEK293 cells ($n = 5$, data not shown), confirming the high K^+ selectivity for the Kir7.1 channel. Pharmacological studies revealed that this current was inhibited by increasing concentrations of the extracellular cations cesium and barium as visualized in Fig. 3C. The block induced by Cs^+ is voltage-dependent whereas that produced by Ba^{2+} shows less voltage dependence. A recent study has described that members of different subunits of Kir subfamilies (Kir6.0 and Kir1.0) could

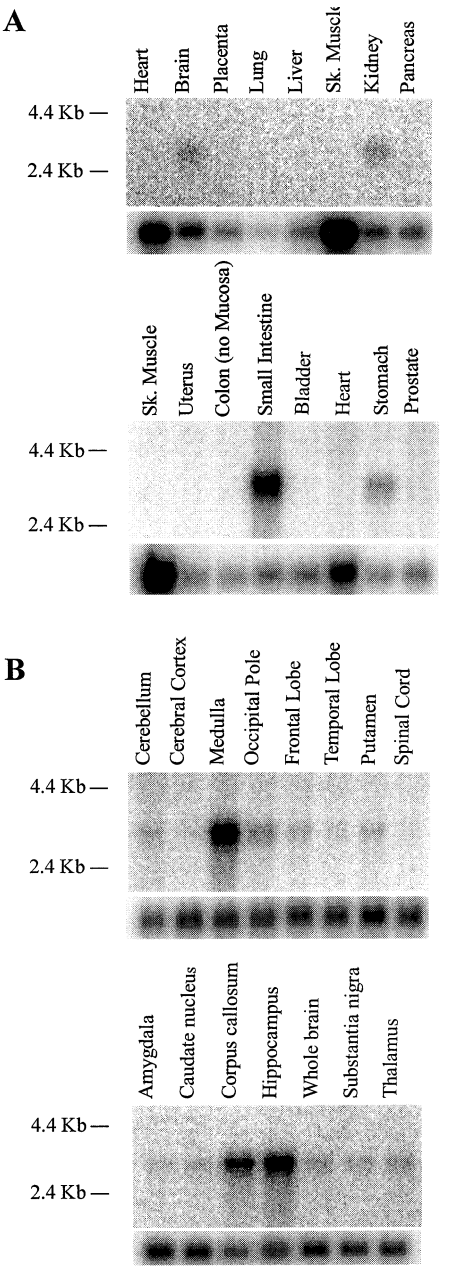

Fig. 4. Kir7.1 mRNA expression in human peripheral and brain tissues (A and B). The Northern blots were sequentially probed with Kir7.1 (upper panel) and G3PDH (lower panel) probes under stringent conditions. The position of molecular markers is indicated on the left of the figures. The time of exposure was 3 days.

be promiscuously coupled to the sulfonylurea receptor (SUR) upon transient co-expression in the HEK cell-line [19]. Thus, we performed co-transfection of the Kir7.1 cDNA with SUR1 cDNA in HEK293 cells. Addition of diazoxide (200 μM) or tolbutamide (200 μM), two compounds known to respectively activate and inhibit Kir channels coupled to SUR1 [2], did not change the amplitude of the Kir7.1 conductance in our conditions, thus suggesting that this subunit is not associated with SUR1.

3.3. Tissue distribution of the Kir7.1 subunit

To determine the distribution of Kir7.1, multiple Northern blot analysis of poly(A)$^+$ RNA from different human tissues was performed at high stringency with a specific Kir7.1 probe. To compare relative levels of expression, these Northern blot were rehybridized to evaluate G3PDH expression. The results indicate a major Kir7.1 transcript of approximately 3.4 kb which is predominantly expressed in small intestine but also at significant levels in stomach, kidney and brain (Fig. 4A). No signal was detected in skeletal muscle, placenta, lung, liver, pancreas, uterus, bladder, heart, prostate and colon without mucosa. Moreover, Northern blots from immune tissues such as spleen, thymus, lymph nodes, peripheral blood leukocytes, bone marrow and fetal liver did not display any signal when hybridized with the Kir7.1 probe (data not shown). In order to evaluate the expression levels of Kir7.1 in different areas of the central nervous system, similar experiments were done on Northern blots from various human brain regions (Fig. 4B). A 3.4 kb band was detected in all central nervous system regions tested, with the exception of the spinal cord: the highest amounts were in medulla, hippocampus and corpus callosum.

4. Discussion

In the present report, we describe the isolation and characterization of a new putative inward rectifier K^+ channel. The novel sequence encodes a potassium channel subunit protein which we have called Kir7.1 since it cannot be classified on a structural basis into any of the previously described (Kir1.0 to Kir6.0) subfamilies. The Kir1.0 subfamily, which includes Kir1.1, Kir1.2, Kir1.3 and now Kir4.1 [15,20], appears to be the most closely related to Kir7.1. However, these proteins share less than 42% identity with Kir7.1. Interestingly, to our knowledge, the Kir7.1 protein is the first Kir subunit which contains a methionine residue (Met-125) in the P domain region, in place of the conserved asparagine present in all known Kir subunits. This asparagine residue has recently been implicated in ion selectivity and pore permeation of the Kir channel [21]. The presence of the methionine in Kir7.1 could not be attributed to a mutation in the cDNA clone because partial sequence information on two other cDNA libraries (human hippocampus and PC3 prostatic cell-line) also revealed Kir7.1 clones with a methionine in the same position.

The assignment of Kir7.1 to the Kir family was functionally supported by expression in *Xenopus* oocytes and mammalian cells. Kir7.1 cRNA injections and cDNA transfections induced the expression of selective K^+ currents displaying weak inward rectification and a block by the cations Cs^+ and Ba^{2+}. However, our results do not exclude that this subunit could form heteromeric channels with (an)other Kir subunit(s) as previously described for the G-protein coupled Kir3.0 subfamily [9,22–24].

The variety of Kir subunit sequences and their distinctive specific tissue distribution patterns have been linked to different physiological roles. In this respect, Kir7.1 mRNA transcripts are restricted to brain (mostly medulla, hippocampus and corpus callosum), kidney, stomach as well as small intestine where a particularly high level of expression is observed. Thus, it is tempting to hypothesize that Kir7.1 channels could be implicated in K^+ transport in the epithelium of this tissue, especially those K^+ conductances linked to Na^+-coupled absorption of nutrients. Such a K^+-selective current, displaying inward rectification and sensitivity to Ba^{2+}, has been characterized in mammalian enterocytes [25]. Further studies, such as cellular localization and co-expression with others Kir subunits, will help to elucidate more precisely the physiological role of Kir7.1.

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