Inwardly rectifying K^+ channel Kir7.1 is highly expressed in thyroid follicular cells, intestinal epithelial cells and choroid plexus epithelial cells: implication for a functional coupling with Na^+,K^+-ATPase

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A novel inwardly rectifying K^+ channel, Kir7.1, with unique pore properties, was cloned recently. Working in the field of osmoregulation, we have also identified the same human and rat channel and found that the channel is unique not only in its pore sequence but also in its dense localization in the follicular cells of the thyroid gland. Northern blot analysis revealed that the channel message was abundantly expressed in the thyroid gland and small intestine, and moderately in the kidney, stomach, spinal cord and brain. Immunohistochemistry of the rat thyroid, intestine and choroid plexus demonstrated the expression of the channel protein in the follicular cells and epithelial cells, suggesting a role in the regulation of the ion-transporting functions of these specialized cells. The unique pore properties of Kir7.1 make it a strong candidate for the hypothetical low-conductance K^+ channel that is functionally coupled with Na^+,K^+-ATPase by recycling K^+. We therefore further examined the co-localization of Kir7.1 and Na^+,K^+-ATPase and found that both are localized in the basolateral membrane of the thyroid follicular cell; in the choroid plexus, which is known to be unique in having Na^+,K^+-ATPase in the apical side of the epithelial cells, Kir7.1 was found in the apical membrane, implying a close functional coupling between the channel and Na^+,K^+-ATPase.

Key words: immunohistochemistry, intestine, K^+ channel, thyroid gland.

INTRODUCTION

Potassium channels have key roles in many aspects of cellular processes [1]. For example, in the nervous system they contribute to setting the membrane resting potential, shaping action potential waveforms, determining firing frequency and controlling neurotransmitter release; in non-excitable cells K^+ channels are involved in cell volume regulation and cell proliferation and differentiation. As expected from these multiple tasks, a large and increasing number of K^+ channels have been identified and shown to be classified into two distantly related families: voltage-gated (Kv) and inwardly rectifying (Kir) K^+ channels.

Kirs have a simpler structure than Kv channels [2]. The Kir proteins so far cloned all have two hydrophobic membrane-spanning domains and a pore-forming region that is similar to the P region of Kv channels with six transmembrane domains. Four such subunits assemble to form functional channels. Kirs form a large family; there are seven subfamilies, named Kir1 to Kir7, with differing electrophysiological properties and mechanisms of activation. For example, the recently identified Kir7.1 exhibits very low single-channel conductance because the positively charged arginine residue in the pore region, conserved among other Kir family members, is replaced with methionine [3]; the members of Kir1 and Kir6 are regulated by ATP; and the Kir3 family members are gated by G-proteins. K^+ ions flow through the Kirs when the membrane potential is negative to the Nernst K^+ equilibrium potential, E_K, but at more positive potentials outward currents are inhibited, permitting inward currents more readily than outward currents. This inward rectification is due to (1) a fast and highly voltage-dependent block of the channel pore by internal Mg^{2+} ions and (2) a much slower voltage-dependent block of the pore by cytoplasmic polyamines such as spermine and spermidine.

In our recent series of studies aimed at identifying differentially expressed genes between freshwater-adapted and seawater-adapted eels, we cloned a Kir whose expression is markedly elevated in seawater eel gills, where the Cl^--ion-transporting cells are densely localized [4]. Sequence analysis of the eel Kir cDNA suggested that it seems to belong to a novel Kir subfamily. We therefore initiated efforts to isolate the mammalian counterpart. A database search identified a related partial sequence in the GenBank expressed sequence tag (EST) database maintained by the National Center for Biotechnology Information. Northern blot analysis, performed after obtaining a full-length cDNA from a human and a rat cDNA library, revealed a very high expression of its mRNA in the thyroid gland and intestine. Because this localization is unique to the Kir family members and seemed to provide useful information for a better understanding of thyroid and intestinal physiology, we decided to determine the location of the channel at the cellular level. Immunohistochemistry revealed that the Kir is densely localized in the follicular cells of the thyroid gland and epithelial cells of the intestine, where the presence of a K^+ channel that is considered to be indirectly involved in the transports of ions and nutrients

Abbreviations used: EST, expressed sequence tag; HBSS, Hanks balanced salt solution; Kir, inwardly rectifying K^+ channel; Kv, voltage-gated K^+ channel; NHS, *N*-hydroxysuccinimide.

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such as I^-, Cl^- and glucose has been demonstrated by electrophysiological measurements but has not yet been cloned [5,6].

During the course of this study, Krapivinsky et al. [3] described the cloning, characterization and localization in the central nervous system of a novel human Kir, Kir7.1, identical to our human clone. Partiseti et al. [7] and Döring et al. [8] have also reported cloning and electrophysiological characterization of Kir7.1 and demonstrated high expression in the small intestine by Northern blot analysis and in the choroid plexus by *in situ* hybridization respectively. Here we therefore describe briefly the sequences of our human and rat clones to show how we obtained the clones and report in detail peripheral tissue distribution of Kir7.1 at the cellular level, together with the evidence for its co-localization and possible functional coupling with Na^+,K^+-ATPase.

MATERIALS AND METHODS

Materials

Restriction enzymes, T4 polynucleotide kinase and Klenow DNA polymerase were obtained from Toyobo (Osaka, Japan) and Boehringer Mannheim (Mannheim, Germany); mRNA purification kit and Ready-To-Go DNA labelling kit were from Pharmacia (Uppsala, Sweden); DNA ligation kit version 2 was from Takara (Kyoto, Japan); human small-intestine 5′-stretch cDNA library, human multiple tissue Northern blots I, II and III and ExpressHyb hybridization solution were from Clontech (Palo Alto, CA, U.S.A.); nitrocellulose filters were from Schleicher & Schuell (Dassel, Germany); ^{32}P-labelled nucleotides were from Amersham (Little Chalfont, Bucks., U.K.); pBluescript II SK$^-$, *Taq* DNA polymerase, λZAP II and Gigapack III Gold *in vitro* packaging kit were from Stratagene (San Diego, CA, U.S.A.); SequiTherm Long-Read cycle sequencing kit was from Epicentre Technologies (Madison, WI, U.S.A.); Immobilon-P membrane was from Millipore Corp. (Bedford, MA, U.S.A.); alkaline phosphatase-conjugated goat anti-rabbit IgG antiserum was from Tago (Burlingame, CA, U.S.A.); Tissue-Tek OCT compound was from Miles (Elkhart, IN, U.S.A.); mouse monoclonal antibody against rabbit Na^+,K^+-ATPase α1 subunit was from Upstate Biotechnology (Lake Placid, NY, U.S.A.).

Cloning of cDNA

Through a BLASTN search of the GenBank EST database using, as a query, the eel Kir sequence (accession number AB009669), a human EST clone 260707 (accession number H97185) was found and obtained through the IMAGE Consortium (Lawrence Livermore National Laboratory). A human small-intestine 5′-stretch cDNA library (λgt10) was screened by standard methods with the insert of human EST clone (955 bp *Eco*RI/*Not*I fragment) as probe. Filters were prehybridized in a solution containing 50 % formamide, 5 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), 5 × Denhardt's solution and 0.1 % SDS for 1 h at 42 °C, then hybridized overnight at 42 °C in the same solution containing ^{32}P-labelled probe. Positive clones were plaque-purified; the cDNA inserts were subcloned into pBluescript II SK$^-$ with the use of the *Eco*RI restriction site for sequencing and further analysis.

For cloning rat cDNA, total RNA was isolated from rat kidney; poly(A)$^+$ RNA was purified with the guanidinium thiocyanate–CsCl method. A cDNA library was constructed by using a SuperScript Choice System for cDNA Synthesis (Life Technologies). First-strand synthesis was primed with random hexamers. After second-strand synthesis, the cDNA was ligated to *Eco*RI/*Not*I adaptors and inserted into the *Eco*RI site of λZAP II. The recombinant DNA was packaged with a Gigapack III Gold packaging kit and screened as above. Positive inserts were subcloned into pBluescript II SK$^-$ by the excision and recircularization process *in vivo*, as described by Stratagene, for sequencing and further analysis.

DNA sequence analysis

DNA was sequenced by the dideoxynucleotide chain-termination method [9] with a SequiTherm Long-Read cycle sequencing kit and an automated laser fluorescent DNA sequencer (model 4000; LI-COR).

Northern blot analysis

Human multiple tissue Northern blots [2 μg of poly(A)$^+$ RNA per lane] were hybridized with an [α-^{32}P]dCTP-labelled 1753 bp *Eco*RI fragment of human Kir7.1 cDNA or human β-actin cDNA (Clontech) in an ExpressHyb hybridization solution for 16 h at 65 °C. The membranes were washed twice in 2 × SSC containing 0.05 % SDS for 30 min at room temperature, in 0.1 × SSC containing 0.1 % SDS overnight at 50 °C and finally in 0.1 × SSC containing 0.1 % SDS overnight at 55 °C. The membranes were exposed to an imaging plate for 4 h and the results were analysed with a BAS-2000 image analyser (Fuji Film, Tokyo, Japan).

Antiserum against recombinant rat Kir7.1

To produce the histidine-tagged C-terminal cytoplasmic region of rat Kir7.1 protein, a cDNA encoding the rat Kir7.1 sequence from residues 270–360 was cloned into pTrcHisC (InVitrogen, San Diego, CA, U.S.A.). Recombinant protein was produced in *Escherichia coli* and purified with Ni^{2+}-nitrilotriacetate resin (Qiagen, Chatsworth, CA, U.S.A.) in accordance with the manufacturer's instructions. To make an anti-(rat Kir7.1) antiserum, purified protein was injected into a rabbit in the presence of Freund's adjuvant by using standard protocols.

Anti-peptide antiserum and Western blot analysis

An anti-peptide antiserum was made in a rabbit against the C-terminal peptide (GC)INGQSIDNFQISETGLTE (amino acid residues 343–360 of human Kir7.1 sequence with additional N-terminal glycine and cysteine residues). The extra glycine and cysteine residues were used to couple the peptide to a carrier protein, keyhole limpet haemocyanin, before immunization. For Western blotting, rat thyroid gland and small intestine were excised and homogenized in 200 μl of a sample buffer [4 % (w/v) SDS/20 % (v/v) glycerol/0.2 % Bromophenol Blue/100 mM Tris/HCl (pH 6.8)]. The tissue homogenates (10 μg of protein) were separated by SDS/PAGE [12.5 % (w/v) polyacrylamide] and electroblotted to Immobilon-P membrane. After blocking in TBST [150 mM NaCl/0.05 % (v/v) Tween 20/10 mM Tris/HCl (pH 8.0)] containing 5 % (v/v) non-fat milk for 1 h at room temperature, the blot was incubated with anti-peptide antiserum or preabsorbed antiserum at 1:1000 dilution overnight at 4 °C, incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG at 1:3000 dilution for 4 h at 4 °C, then developed with 5-bromo-4-chloroindol-3-yl phosphate/Nitro Blue Tetrazolium chloride as chromogenic substrates. Preabsorption was performed by incubating 1 μl of antiserum with 10 μg of antigenic synthetic peptide in 1 ml of TBST overnight at 4 °C.

Immunohistochemistry

Rat thyroid, small intestine and choroid plexus were fixed in 0.1 M PBS, pH 7.4, containing 2% (w/v) paraformaldehyde, immersed in PBS containing 30% (w/v) sucrose overnight at 4 °C and frozen in Tissue-Tek OCT compound. Frozen sections (thyroid, 2 μm; small intestine, 6 μm; choroid plexus, 5 μm) were cut in a cryostat at −20 °C and were mounted on gelatin-coated slides. Sections were washed in PBS, fixed with 2% (v/v) H_2O_2 in acetone for 1 h at room temperature and washed again in PBS. They were subsequently incubated with 5% (v/v) normal goat serum for 1 h at 4 °C, then overnight at 4 °C with anti-peptide antiserum [anti-(human Kir7.1)], anti-(Na^+,K^+-ATPase) monoclonal antibody, preimmune serum or preabsorbed antiserum at 1:1000 dilution, unless stated otherwise. Sections were treated with biotinylated goat anti-rabbit or anti-mouse IgG and avidin–biotin-conjugated peroxidase (Vectastain Elite ABC kit; Vector Laboratories) for 1 h, then detected with diaminobenzidine tetrahydrochloride in accordance with the manufacturer's instructions. Preabsorption was performed as described above.

Cell culture and Northern blot analysis

FRTL-5 cells (ATCC CRL 8305; American Type Culture Collection, Manassas, VA, U.S.A.), a strain of rat thyroid follicular cells, were grown in Coon's modified Ham's F-12 medium supplemented with 5% (v/v) calf serum, 10 μg/ml insulin, 10 ng/ml somatostatin, 10 nM hydrocortisone, 5 μg/ml transferrin, 10 ng/ml glycyl-L-histidyl-L-lysine acetate and 1 m-i.u./ml thyroid-stimulating hormone. For Northern blot analysis, total RNA was isolated from FRTL-5 cells by using the method described above. An aliquot (30 μg per lane) of the total RNA was subjected to electrophoresis and transferred to a Magna nylon membrane (Micron Separations, Westborough, MA, U.S.A.) and hybridized with a ^{32}P-labelled rat Kir7.1 cDNA prepared by random priming. Hybridization was performed in a solution containing 50% formamide, 5 × SSPE, 2 × Denhardt's solution, 0.5% SDS and 0.1 mg/ml heat-denatured salmon-sperm DNA at 42 °C for 16 h and the membrane was washed twice in 2 × SSC containing 0.05% SDS at room temperature for 20 min and once in 0.1 × SSC containing 0.1% SDS overnight at 55 °C. Bands on the Northern blot were detected as described above.

Cell surface biotinylation

FRTL-5 cells grown on 25 mm Transwell-Clear polyester filters (0.4 μm pore size; Costar Corp., Cambridge, MA, U.S.A.) were cooled to 4 °C and washed five times with cold Hanks balanced salt solution (HBSS). Apical or basolateral surfaces were selectively biotinylated by adding 0.5 mg/ml EZ-Link Sulfo-*N*-hydroxysuccinimide (NHS)-LC-Biotin (Pierce, Rockford, IL, U.S.A.) in HBSS to the inner (apical) or outer (basolateral) chamber, then incubated for 30 min at 4 °C. Cells were then washed six times in Ca^{2+}/Mg^{2+}-free HBSS, scraped from filters into HBSS and pelleted by centrifugation at 1000 ***g*** for 10 min. Pellets were resuspended in 500 μl of lysis buffer [10 mM sodium phosphate (pH 7.4)/1% (v/v) Nonidet P40/0.5% sodium deoxycholate/0.1% SDS/0.15 M NaCl/2 mM EDTA/50 mM NaF/1 mM benzamidine/10 mM leupeptin/1 mM pepstatin/1 mM PMSF] and homogenized rapidly. The extract was then centrifuged at 20000 ***g*** for 1 h. The resultant supernatant, containing solubilized FRTL-5 cell biotinylated membranes, was then precipitated by incubation overnight in 100 μl of a 50% (w/v) slurry of streptavidin–agarose beads (Pierce) at 4 °C. Beads were pelleted by centrifugation for 30 s at 14000 ***g*** and washed six times with lysis buffer. Biotinylated proteins were eluted by boiling for 5 min in 100 μl of Laemmli sample buffer. Samples were reduced by the addition of 5% (v/v) 2-mercaptoethanol, then separated by SDS/PAGE and transferred to Immobilon-P membranes. The membranes were probed with an anti-(human Kir7.1) peptide antiserum (1:1000 dilution), preimmune serum (1:1000 dilution) and a mouse monoclonal antibody against rabbit Na^+,K^+-ATPase α1 subunit (50 ng/ml).

RESULTS

Properties of rat Kir7.1

Figure 1 shows an amino acid sequence comparison between rat and human Kir7.1, which are composed of 360 amino acid residues. The rat channel exhibits 95% identity with the human counterpart. As pointed out by Krapivinsky et al. for the human sequence [3], the pore sequence or the H5 region of the rat channel is also well conserved with regard to other Kirs except for the replacements of Leu-111 with Ser and of Arg-125 with Met. Of special interest is the replacement of the arginine residue with methionine because the positively charged arginine residue has been shown to be conserved in other Kirs and to act as an external barrier for cationic blockers and its modification, to result in greatly decreased single-channel conductances [10].

As mentioned in the Introduction section, inward rectification of the Kirs is due to the blocking of outward currents by intracellular Mg^{2+} and polyamines. The molecular determinants of the rectification, namely critical sites of the channel that mediate the blocking by Mg^{2+} and polyamine, have been elucidated by site-directed mutagenesis and shown, for the Kir2 family, which exhibits strong rectification, to be Asp-172 in the M2 region and Glu-224 in the cytoplasmic C-terminal tail (the numbering corresponds to rat Kir2.1 [11,12]). These residues are replaced with Glu and Ser/Thr (human/rat) respectively in Kir7.1.

A potential N-glycosylation site, Asn-His-Thr (residues 95–97), is present in the extracellular loop between the M1 and H5 segments. Similar consensus sequences are also present in the members of the closest subfamily (Kir1) at the same position but they are followed by a proline residue that often prevents N-glycosylation. The following consensus sites for protein kinase C

Figure 1 Primary structure of rat Kir7.1 (rKir7.1) and a comparison with that of human Kir7.1 (hKir7.1)

Identical residues are shown with black shading. The asterisk indicates the replacement of the arginine residue conserved in all other Kirs with methionine in Kir7.1. Two transmembrane regions (M1 and M2) and the pore region (H5) are indicated by labelled bars. The antigenic peptide sequence is indicated by a bar at the C-terminus. The filled circle indicates potential phosphorylation site based on consensus motifs for cAMP-dependent protein kinase and open circles indicate those for protein kinase C. The closed triangle indicates a potential N-glycosylation site.

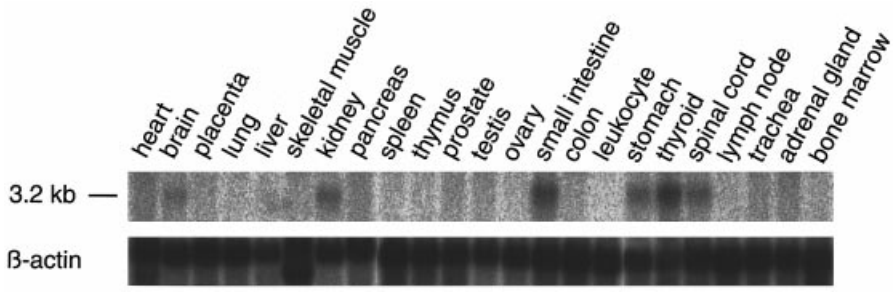

Figure 2 Tissue distribution of human Kir7.1 mRNA

Human multiple-tissue Northern blots [2 μg of poly(A)$^+$ RNA per lane] were hybridized with an [α-^{32}P]dCTP-labelled human Kir7.1 cDNA probe (upper panel). Blots were stripped and rehybridized with a human β-actin cDNA probe (lower panel). The size of the human Kir7.1 mRNA was 3.2 kb, as indicated in the upper panel. Human β-actin transcripts were present as 2.0 kb (ubiquitous) and 1.8 kb (specific for skeletal muscle) species.

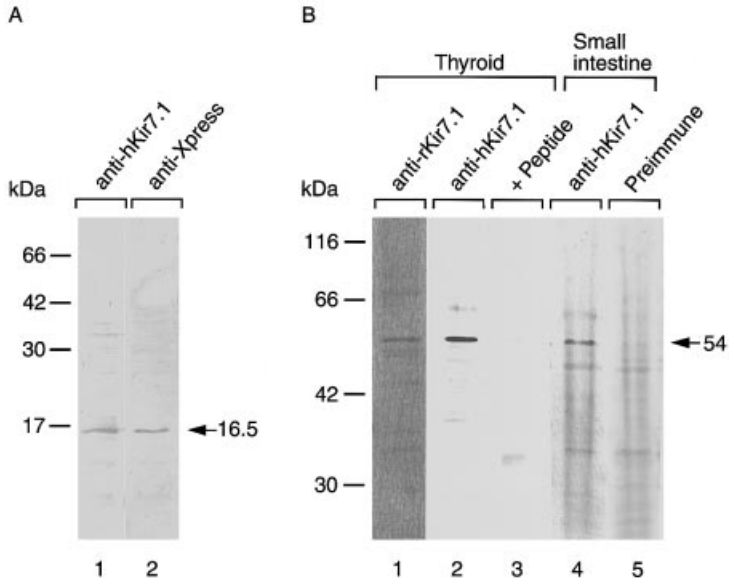

Figure 3 Western blot analysis of Kir7.1 in rat thyroid gland and small intestine

(**A**) Recognition of rat Kir7.1 sequence by anti-(human Kir7.1) (anti-hKir7.1). The C-terminal cytoplasmic region (residues 270–360) of rat Kir7.1 was produced in *E. coli* as a recombinant protein and the bacterial extract containing 10 μg of the recombinant protein was analysed by Western blotting with anti-hKir7.1 (1:1000 dilution; lane 1). The blots were treated with alkaline phosphatase-conjugated goat anti-rabbit IgG as secondary antibody and then developed with 5-bromo-4-chloroindol-3-yl phosphate/Nitro Blue Tetrazolium chloride as substrates. Attempts to produce full-length Kir7.1 in *E. coli* failed because its induction and accumulation were lethal to the bacterial cell. The histidine-tagged recombinant protein was also stained with anti-Xpress (1:5000 dilution; lane 2), an antiserum against histidine-tagged protein expressed with the pTrcHis system (InVitrogen). (**B**) Specificity of antiserum and immunoblot analysis of Kir7.1 in the rat thyroid and intestine. Total cell homogenates of rat thyroid and small intestine (10 μg of protein) were separated by SDS/PAGE and subjected to Western analysis with an anti-recombinant protein antiserum (anti-rKir7.1, 1:500) and an anti-peptide antiserum against human Kir7.1 (1:1000). The arrow indicates the 54 kDa immunoreactive band of rat Kir7.1. Lane 1, thyroid homogenate probed with anti-rKir7.1; lane 2, thyroid homogenate probed with anti-hKir7.1; lane 3, thyroid homogenate probed with preabsorbed anti-hKir7.1; lane 4, small-intestine homogenate probed with anti-hKir7.1; and lane 5, small-intestine homogenate probed with preimmune serum. The positions of molecular mass markers are indicated at the left of each panel.

phosphorylation were found in the N-terminal and C-terminal cytoplasmic domains: Ser-Gln-Arg (residues 14–16), Ser-Ile-Arg (residues 169–171) and Ser-Val-Arg (residues 201–203). A putative cAMP-dependent protein kinase phosphorylation site, Arg-Arg-Thr-Ser (residues 284–287), is located near the C-terminal end.

Tissue distribution

Northern blot analysis of poly(A)$^+$ RNA isolated from various human tissues revealed mRNA species of 3.2 kb predominantly in the thyroid gland and small intestine and to a smaller extent in the kidney, stomach, spinal cord and brain (Figure 2).

Western blot analysis and immunohistochemical localization

To examine further the localization of Kir7.1 at the cellular level, we raised antisera, characterized them and performed immunohistochemical staining of the rat thyroid and small intestine. Two antisera were produced in rabbits: one against rat recombinant Kir7.1 and the other against a synthetic peptide corresponding to

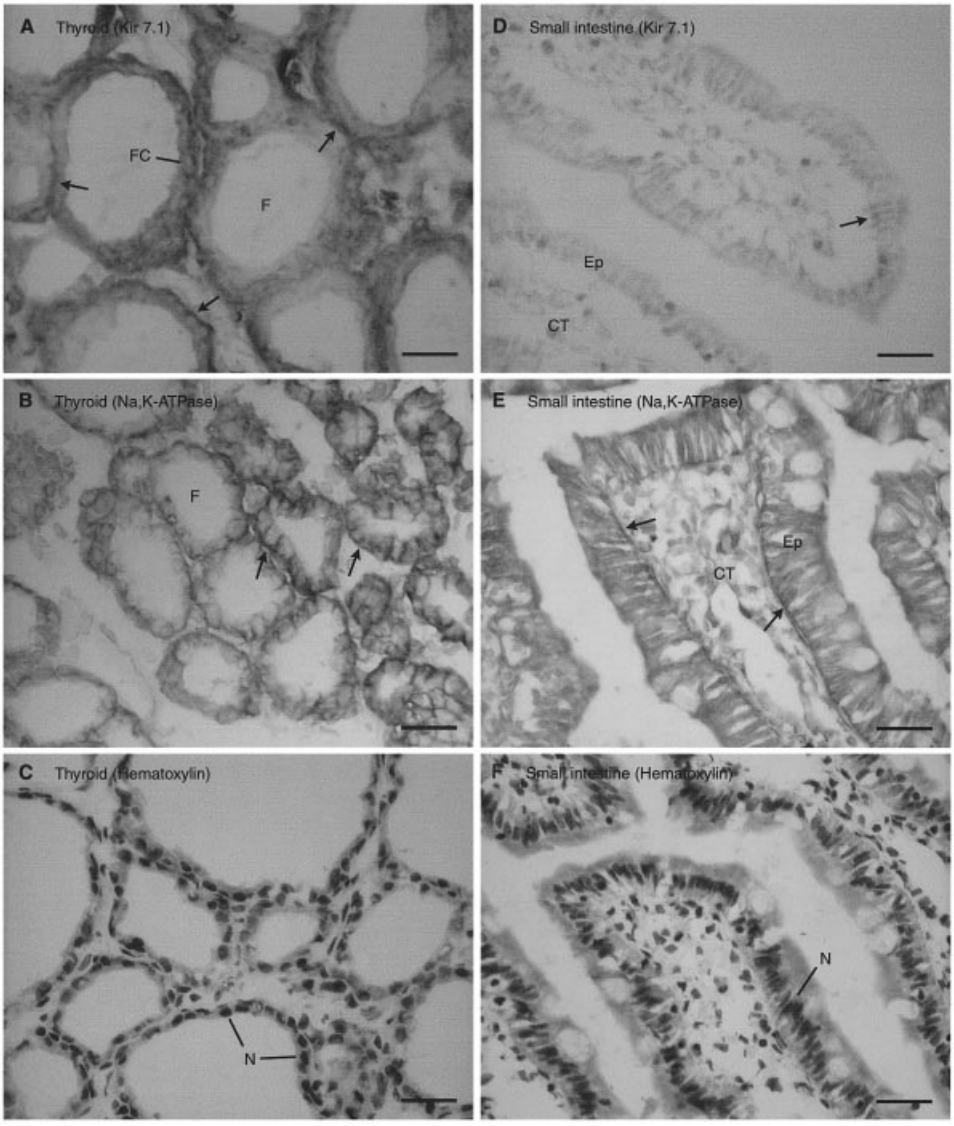

Figure 4 Immunohistochemistry of rat thyroid (A–C) and small intestine (D–F)

Rat thyroid and small intestine sections were treated with anti-peptide antiserum specifically recognizing Kir7.1 (**A** and **D**) or anti-Na^+,K^+-ATPase antiserum (**B** and **E**); the immune complexes were detected by exposure to a peroxidase-conjugated secondary antibody and diaminobenzidine. Primary antisera were used at a dilution of 1:1000 (**A**, **B** and **E**) or 1:10000 (**D**). (**C**, **F**) Haematoxylin staining of thyroid (**C**) and small intestine (**F**) sections. In both tissue sections, no significant staining was observed with preabsorbed anti-hKir7.1 antiserum (results not shown). Arrows point to the basal membranes. Scale bars, 25 μm. Abbreviations: F, follicle; FC, follicular cell; CT, connective tissues; Ep, epithelial cells; N, nucleus.

the C-terminal cytoplasmic region of human Kir7.1 in which only one amino acid residue was different between the rat and human sequences (Figure 1). As expected, anti-hKir recognized the rat antigen (Figure 3A). Both antisera specifically stained a band of approx. 54 kDa when rat thyroid and small-intestine homogenates were analysed by Western blotting (Figure 3B). Because anti-(human Kir7.1) recognized the rat antigen in addition to the human antigen and it exhibited a higher titre and specificity (Figure 3B, lanes 1 and 2), we used anti-(human Kir7.1) in the following experiments. The molecular size estimated by the Western analysis was larger than the calculated size (40636 Da). At present the reason for this discrepancy is not clear but it might be due to an anomalous electrophoretic mobility and to post-translational modifications such as glycosylation and phosphorylation; similar anomalous mobility on SDS/PAGE has been reported for Kir2.2 [48.4 kDa (calculated) compared with 60–64 kDa (observed)] [13].

In immunohistochemistry, plasmalemmal staining was observed in the thyroid follicular cells and intestinal epithelial cells (Figures 4A and 4D). Such staining was absent from the cryo-sections treated with preimmune serum or immuno-neutralized antiserum (results not shown). The staining patterns were very similar to those of Na^+,K^+-ATPase (Figures 4B and 4E), which is known to localize to the basolateral membrane of the thyroid and small-intestine epithelial cells.

The follicular cell localization of Kir7.1 was confirmed with the Fischer rat thyroid follicular cell line FRTL-5. A Northern blot analysis of RNA samples from the FRTL-5 cell line revealed that Kir7.1 mRNA is expressed abundantly in the follicular cell line, so that it can be detected with total RNA preparations

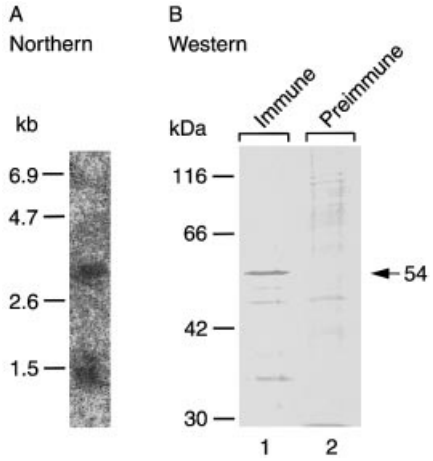

Figure 5 Presence of Kir7.1 in cultured thyroid follicular cells

FRTL-5, a cell line established from Fischer rat thyroid follicular cells, was cultured and subjected to Northern and Western blot analyses for the rat Kir7.1 message and protein respectively. (**A**) Northern blot analysis. In contrast with a single band of human Kir7.1 mRNA (3.2 kb; Figure 2), rat transcript yielded two bands at 3.2 and 1.4 kb; a similar two-band pattern was reported recently by Döring et al. [8]. (**B**) Western blot analysis. The arrow indicates rat Kir7.1 protein detected with anti-hKir (lane 1 in **B**). The positions of molecular mass markers are indicated at the left of each panel.

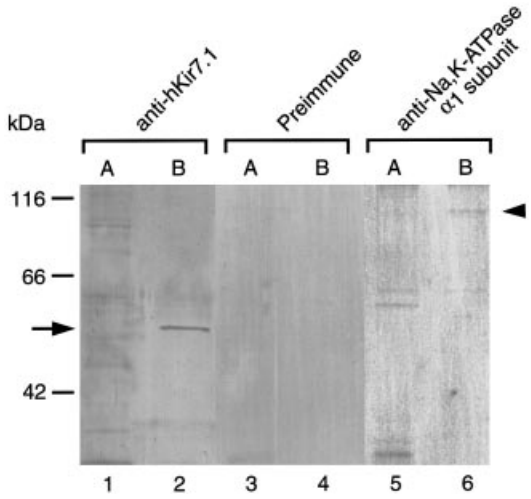

Figure 6 Basolateral localization of Kir7.1 protein in FRTL-5 cells

FRTL-5 cells cultured on Transwell filters were biotinylated on either the apical (lanes A) or basolateral (lanes B) surface with EZ-Link Sulfo-NHS-LC-Biotin. Cells were extracted with Nonidet P40 lysis buffer; biotinylated proteins were precipitated with streptavidin–agarose beads. Precipitates were separated by SDS/PAGE under reducing conditions and subjected to Western analysis. Kir7.1 (54 kDa band, arrow) was detected predominantly on the basolateral surface with anti-(human Kir7.1) (anti-hKir7.1) (lane 2). Na^+,K^+-ATPase α1 subunit (110 kDa band, arrowhead) was also detected on the same surface with monoclonal antibody against rabbit Na^+,K^+-ATPase α1 subunit (lane 6). The positions of molecular mass markers are indicated at the left.

(Figure 5A). The presence of Kir7.1 in FRTL-5 was also demonstrated at the protein level by Western blot analysis with Triton X-100 extracts of the cultured FRTL-5 cells (Figure 5B).

Basolateral localization of Kir7.1 in FRTL-5 cells

The cell-surface biotinylation method was used to determine the polarity of the distribution of Kir7.1 in FRTL-5 cells. The cells were grown on Transwell filters and labelled by adding an impermeant biotinylating reagent, NHS-biotin, to either the apical or basolateral medium compartments. The biotinylated cell surface proteins were then isolated with streptavidin–agarose gel and analysed by Western blotting. Biotinylation of Kir7.1 occurred only when NHS-biotin was added to the basolateral compartment of the Transwell chamber (Figure 6), indicating that there is a highly polarized distribution of Kir7.1 to the basolateral plasma membranes of the thyroid follicular FRTL-5 cells.

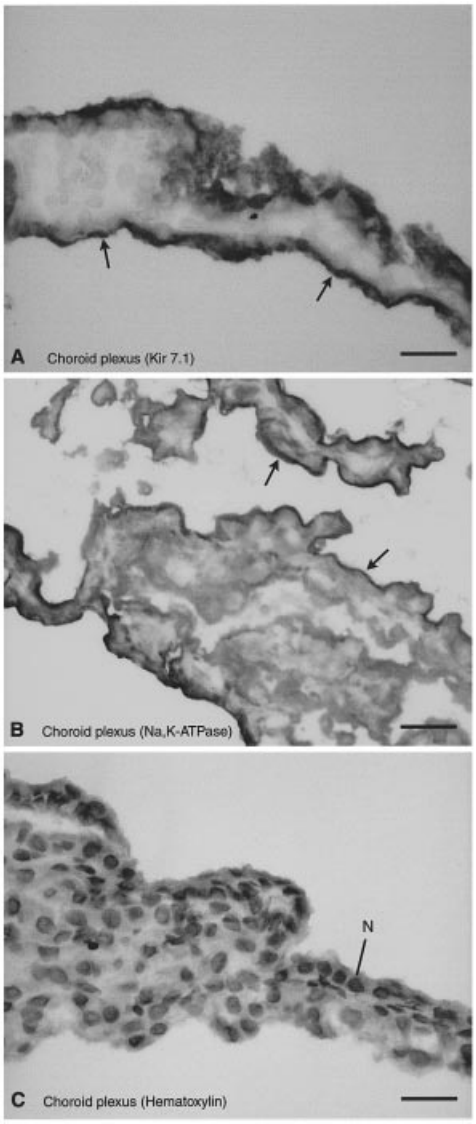

Figure 7 Immunohistochemical localization of Kir7.1 in choroid plexus

Rat choroid plexus was perfusion-fixed and embedded in Tissue-Tek OCT compound. Tissue sections were incubated with either anti-hKir7.1 (**A**) or antiserum directed against the Na^+,K^+-ATPase α1 subunit (**B**) and processed further as described in the Materials and methods section. Reflecting the reverse polarity of the choroidal epithelial cell, both Na^+,K^+-ATPase and Kir7.1 were detected on the apical membrane of the choroid plexus epithelial cells. (**C**) Staining with haematoxylin. No significant staining was observed with preimmune serum or preabsorbed anti-hKir7.1 antiserum (results not shown). Arrows point to the apical membranes.

Co-localization of Kir7.1 and Na^+,K^+-ATPase: implication for their functional coupling

Kir7.1 has uniquely low single-channel conductance [3], which makes it a strong candidate for the hypothetical low-conductance K^+ channel that is functionally coupled with Na^+,K^+-ATPase by recycling K^+ and therefore absolutely required for the continued Na^+-pumping activity of Na^+,K^+-ATPase. To obtain anatomical evidence for this possible coupling, we determined their co-localization and polarity of distribution. Immunohistochemistry demonstrated almost identical staining patterns for Kir7.1 and Na^+,K^+-ATPase in the thyroid (Figure 4B) and small intestine (Figure 4E); in both cases, basolateral membranes seemed to be decorated. Furthermore, cell-surface biotinylation revealed a basolateral polarity for Kir7.1 and Na^+,K^+-ATPase in the thyroid follicular FRTL-5 cells (Figure 6).

Recently, Döring et al. [8] have reported a dense localization of Kir7.1 mRNA in the epithelium cells of the choroid plexus, which is located within the ventricles of the brain and is responsible for the formation of cerebrospinal fluid. We considered that this tissue might provide us with a unique opportunity to test the co-localization and possible functional coupling of Kir7.1 and Na^+,K^+-ATPase because its epithelia exhibit a reverse polarity of the distribution of Na^+,K^+-ATPase compared with other transporting epithelia such as those of the thyroid, kidney and intestine: in most cells involved in transepithelial transport of ions, Na^+,K^+-ATPase is located in the basolateral plasma membranes, but in the choroid plexus it is expressed on the apical membrane of the secretory epithelial cells [14–18]. Immunohistochemical staining demonstrated an apical localization of Kir7.1 as well as of Na^+,K^+-ATPase in the choroid plexus epithelial cells (Figure 7).

DISCUSSION

Since the cloning of an ATP-regulated Kir in 1993 [19], an expanding number of Kirs have been cloned and divided, according to their phylogenetic relationships, into seven subfamilies (Kir1 to Kir7), significantly increasing our understanding of the molecular diversity, subunit composition and functional properties of the Kirs. The recently cloned Kir7.1 is similar in overall structure to other known members of the Kir family but is clearly distinguishable in its H5 pore sequence; it has a methionine residue at position 125, where all other members have arginine [3,7,8]. The conserved arginine residue has been shown to be important in the channel activity [10,20]. This replacement was strongly suggested as a characteristic signature of the Kir7 subfamily by Krapivinsky et al. [3] on the basis of the human Kir7.1 sequence and is now firmly established as such by the present cloning of the rat sequence. As predicted by this alteration of an arginine residue to methionine, the channel activity has been demonstrated to be very weak [3]; in rat Kir7.1, the channel activity was almost undetectable (results not shown). The lack of significant levels of channel activity suggests that in its native state the channel is made by a heteromeric subunit assembly as in the heteromultimers between Kir1.2 and Kir5.1 [21] and between Kir3.1 and Kir3.4 [22]. It is also possible that the activity of Kir7.1 is regulated by an associated protein as in the ATP-sensitive Kir in the β-cells of the pancreas that is formed from two distinct subunit proteins: Kir6.2 and the sulphonylurea receptor SUR1, a member of the ATP-binding cassette superfamily [23]. With regard to a role in the central nervous system, in which considerable quantities of Kir7.1 are present, Krapivinsky et al. [3] have suggested, on the basis of its uniquely low single-channel conductance, that the channel would provide a steady background K^+ current to help set the membrane potential. In contrast, Döring et al. [8] have demonstrated by *in situ* hybridization that the Kir7.1 message is essentially absent from neurons and glia but strongly expressed in the secretory epithelial cells of the choroid plexus; those authors suggested its involvement in the transepithelial transport of potassium. We confirmed the choroid plexus localization of Kir7.1 by immunohistochemistry and used this location to test our hypothesis that Kir7.1 co-localizes with Na^+,K^+-ATPase. Although circumstantial, supporting evidence for this possibility was obtained by immunohistochemical co-localization studies with tissues containing abundant levels of Kir7.1 (thyroid, intestine and choroid plexus). We considered the choroid plexus an attractive tissue on which to test this hypothesis because in striking contrast with most other transporting epithelia, in which Na^+,K^+-ATPase is expressed basolaterally, the choroid plexus epithelial cells display Na^+,K^+-ATPase on the apical membrane [14–18]. Consistent with this polarity reversal of choroid plexus Na^+,K^+-ATPase is the observation that Kir7.1 also exhibited apical polarity in the choroid plexus epithelial cells.

Immunohistochemistry also revealed the localization of Kir7.1 in thyroid follicular cells and intestinal epithelial cells. Like the secretory epithelial cells of the choroid plexus, these cells are actively involved in ion transport. For example, in follicular cells there are many transporters and channels involved directly or indirectly in the uptake of I^- from the basolateral membrane and the excretion through the apical membrane into the lumen; key elements of this transport system include electrogenic Na^+,K^+-ATPase and a Na^+/I^- symporter [24] on the basolateral membrane and an I^- channel on the apical membrane [25]. In addition, a K^+ channel is required on the basolateral side to recycle K^+; this recycling results in membrane hyperpolarization, which in turn serves as the driving force for I^- secretion into the lumen. Kir7.1 could be a candidate for this channel. Similarly, in the intestinal epithelial cells, as reviewed by Dawson and Richards [26], salt absorption and secretion are coupled to basolateral K conductance; for example, in salt secretion the epithelial cells actively transport Cl^- from blood via the basolateral Na^+, K^+, Cl^- co-transporter, which is driven by the Na^+ gradient generated by Na^+,K^+-ATPase in the basolateral membrane. Activation of the hypothetical basolateral K^+ channel results in hyperpolarization of the membrane potential and maintains the driving force for Cl^- secretion across the apical membrane into the lumen. In addition, in salt absorption or Na^+-coupled nutrient absorption the recycling of K^+ through the basolateral K^+ channel is essential. The ion transport functions of the intestinal epithelial cells and thyroid follicular cells are known to be highly regulated by cellular signals including changes in cAMP levels. With the sequence characterization presented here, which shows putative cAMP-dependent protein kinase phosphorylation sites and the cell-type localization of Kir7.1, the plausible involvement of Kir7.1 in this and other regulatory activities of ion transport in the epithelial and follicular cells can now be tested.

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