Cloning, expression and localization of a rat hepatocyte inwardly rectifying potassium channel.

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ABSTRACT

Bile formation involves anion accumulation within the apical lumen of hepatocytes. Potassium flux through hepatocellular basolateral membrane channels may provide the counterion for apical anion efflux. Here we cloned a molecular candidate for maintaining charge balance during bile secretion. Two transcripts resembling the Kir4.2 sub-class of inwardly-rectifying potassium channels were found. The longer deduced isoform (4.2a) has 30 additional N-terminal amino acids, identifying this as a new isoform. The short form shared 86-91% identity with the mouse, human and guinea-pig channels. Whole-cell currents of either rat isoform expressed in HEK293T cells demonstrated time independence and inward rectification. Antibodies against a C-terminal fragment recognized bands between 40 and 45 kDa, 90 kDa, and a high molecular weight band around 200 kDa in overexpressing HEK cells. Immunohistology of liver tissue shows hepatocellular plasma membrane localization. In hepatocyte couplets Kir4.2 was predominantly localized to the basolateral membrane. The results demonstrate the expression of a new Kir4.2 isoform in the rat hepatocyte, whose functional properties are compatible with a role in maintaining electrical integrity of bile-generating hepatocytes.

KEY WORDS

liver, inward rectifier, Western blot, antibodies, heterologous expression, HEK293, patch clamp
INTRODUCTION

Bile, the exocrine secretion of the hepatocyte, is generated through the accumulation of organic and inorganic anions in the biliary canaliculus. The molecular processes underlying bile acid-independent exocrine activity of the liver are not well understood. By analogy with other secretory epithelia, basolateral $K^+$ channels would provide the counterion, and potentially some of the driving force, for anion secretion and transepithelial water flow into the apical lumen (bile canaliculus) adjoining pairs of hepatocytes. In support of this contention, blockers of $K^+$ flux inhibit bile generation (8;14). Further, the divalent organic anion DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonate) stimulates $K^+$ efflux into the portal space in proportion to choleresis (13), suggesting a functional coupling between sinusoidal $K^+$ and canalicular anion efflux. The amplitude of DIDS-stimulated $K^+$ flux as a function of perfusate $K^+$ concentration suggests inward rectification (13). Additionally, weakly rectifying $K^+$ currents have been identified in isolated rat and chick hepatocytes (12), and a Kir4.2 has been cloned from whole mouse liver (22). Although the properties of Kir4.2 channels render them good candidates for the basis of a hepatocyte basolateral (sinusoidal) potassium conductance involved in bile formation, it has yet to be determined whether such channels are expressed in hepatocytes and, if so, where they are localized within the cell.

The electrophysiological properties of Kir channels make them well-suited candidates for coupling basolateral $K^+$ efflux with luminal secretion in cells, like hepatocytes, that do not undergo the oscillations in membrane potential that are requisite for opening of voltage-gated $K^+$ channels (Kv), and/or do not couple increases in cytosolic $Ca^{2+}$ to secretion (20). In contrast to the Kv class, Kir channels are open at the resting potential of most cells, and thus are their major
hyperpolarizing influence (21). This is important for maintaining the driving force for anion secretion and water flow.

Here we have cloned the rat homologue of Kir4.2 and a new isoform of an inwardly rectifying potassium channel (Kir4.2a) from rat hepatocytes. We have produced a specific antibody to these proteins, and localized their expression to the hepatocyte plasma membrane. Additionally, functional expression in HEK293T cells reveals properties supporting the proposal that this channel may provide the major K⁺-selective current component in the rat hepatocyte.

**EXPERIMENTAL PROCEDURES**

*Animals* - Male Sprague-Dawley rats (200-225 g body weight) (Charles River Canada, Montreal) were housed under a twelve hour light/dark cycle with free access to water and rat chow according to the Canadian Council on Animal Care.

*Hepatocyte Couplet Isolation* - Livers were single-pass perfused, at 40 ml/min, successively with 250 ml Hepes-buffered Krebs saline (KH) pH 7.4 plus 0.5 mM EGTA and 100 U/100 µg penicillin/streptomycin per ml, and 100 ml of the same solution also containing 0.03% (w/v) collagenase (Liberase, Roche Biochemicals, Montreal) and 5 mM CaCl₂. The partially digested liver was minced and incubated for a further 10 min with fresh collagenase. The cell suspension was filtered through 25 µm nylon mesh, washed with KH, and used immediately for RNA isolation, or plated out at 20-30 % confluency in DMEM containing 10 % fetal calf serum, 2 mM glutamine, 5 µg/ml insulin and 1 µM dexamethasone and used for immunohistochemical analysis after 24 hours culture.

*RT-PCR* - Total RNA was extracted (Trizol, GIBCO/BRL, Gaithersburg) from 10 ml packed hepatocytes followed by DNase (1 unit/µg RNA, Promega, Madison)
treatment to remove residual contaminating genomic DNA. Reverse transcription was performed by RACE (rapid amplification of cDNA ends) using 1-4 µg RNA, 200 units MMLV reverse transcriptase (GIBCO/BRL) and cap and oligo-dT primers (SMART cDNA synthesis, Clontech, Palo Alto) to generate end-modified cDNA representing full-length mRNA. Standard RT-PCR was also conducted on hepatocellular mRNA isolated from total RNA using immobilized oligo-dT (Oligotex, Qiagen, Valencia). Proof-reading (Clontech Advantage or GIBCO/BRL Platinum Pfx) cDNA polymerases were used for synthesis of PCR products destined for sequencing. Since K+ channels represent low copy number message, single-stranded cDNA was normally subjected to two rounds of PCR, 30 cycles each round, with a first pair of primers followed by reamplification with nested primers (Table 1). A standard 3-step PCR protocol using an M&J Research PTC-100 thermocycler was used, setting the annealing temperature 5°C lower than the primer Tm values, and extension times of 30 s per 500 bp (minimum 30 s). Initial sequence information was obtained from primers designed against a 380bp fragment encoding the pore region to beyond the M2 domain in the human and guinea-pig sequences (Set A followed by Set B, Table 1). The sequence coding for the deduced ORF was end-modified with primers containing appropriate restriction sites for subcloning into the multiple cloning site (MCS) of pCMS-EGFP (Clontech), a vector encoding GFP under independent promoter control. All fragments were cloned and sequenced by the automated dideoxy method.

Cell Culture and Transfection - HEK293T cells at 85 % confluency were dissociated with 0.05 % trypsin and 0.02 % EDTA and plated out at 10 % density into fresh medium (DMEM containing 10 % fetal calf serum, 2 mM glutamine and penicillin/streptomycin) onto sterile glass coverslips in 35 mm Petri dishes essentially
as described by Hamid et al (9). After 24 hours the cells were transfected with a complex of Fugene6 (Roche Biochemicals) and plasmid (3 µg/plate) and 20 hours later the cells were transferred to 28°C to stimulate protein synthesis and decrease cell proliferation. Cells were used for electrophysiological experiments 1 to 3 days later.

**Electrophysiology** – Patch clamp recordings were made in the whole-cell configuration using an Axopatch 200A amplifier (Axon Instruments, Foster City) and Clampex 7 software as described previously (15). Whole-cell currents were digitized (Digidata 1200B) at 5 kHz. Cells were bathed in normal physiological saline (in mM, 5 KCl, 140 NaCl, 5 glucose, 5 Hepes, 1 CaCl₂, 1 MgSO₄, pH 7.4), or one in which K⁺ was increased to 115 mM with an equimolar reduction in NaCl. Patch pipettes (2-5 MΩ) were filled with, in mM, 145 KCl, 1 MgSO₄, 1 EGTA and 5 HEPES, pH 7.6.

**Fusion Protein Synthesis and Antibody Generation** – A maltose binding protein-Kir4.2 fusion protein was synthesized in *E.coli* using the pMALc2X vector (New England Biolabs, Beverly). A fragment corresponding to bases coding for the 170 amino acid residues completing the C-terminus of the Kir4.2a transcript was ligated into the MCS of pMAL. The purified protein was emulsified with TiterMax agent (CytRx, Norcross). Antibody production was monitored in test bleeds after four and six weeks with essentially identical results. The IgG fraction was isolated by chromatography on DEAE Affi-Gel (BioRad, Hercules). Western analysis of the fusion protein and its proteolytic cleavage products demonstrated a strong reaction of the fusion protein with a clear signal at the predicted size of the C170 peptide (calculated mass of 18.4 kD) (not shown). Specific antibodies were purified by affinity chromatography on an MBP-C170-immobilized carbonyldiimidazole-activated agarose column (Reacti-gel, Pearce Biochemicals, Rockford).
**Western Blotting of Recombinant Protein** - HEK293T cells were grown in T-75 flasks. At 50% confluency they were transfected with Fugene6 and 12 µg of either pCMS-EGFP or the same vector containing either the rat hepatocyte Kir4.2a or Kir4.2 ORF. After 48 hours the cells were washed twice with PBS pH 7.4 and permeabilized in 1 ml 5 mM HEPES/KOH pH 7.4 containing a protease inhibitor cocktail (Complete-Mini, Roche Biochemicals). The suspension was then drawn through a 22 and a 27 gauge needle (10 and 5 times respectively). Membranes were collected by centrifuging at 10,000xg for 30 min, resuspended in permeabilization buffer, and solubilized with SDS-PAGE sample buffer containing 100 mM DTT and 2% SDS. Initially the samples were heated at 94°C for 3 min, but this greatly impeded migration into the acrylamide gel. To increase mobility we incubated the membranes in the same sample buffer for at least 24 hr at room temperature, followed by heating, successively, at 50°C for 15 min, 20°C for 30 min, and 50°C for 15 min as has been reported for an SDS-resistant inward rectifier (4). Proteins were separated on 12% SDS-PAGE equilibrated with running buffer containing 0.1% SDS, eluted onto PVDF membrane, blocked for 16 hours with 5% non-fat milk, and probed with the affinity-purified primary antibody (4 µg/ml) followed by an alkaline phosphatase-conjugated goat-antirabbit secondary antibody (2 µg/ml). Preabsorbed antibody was incubated (16 hours, 4°C) with a 20 times (mass) excess of purified fusion protein. Alkaline phosphatase was detected with the chromogenic substrate, NBT/BCIP.

**Immunohistochemistry of Liver and Hepatocyte Couplets** - Livers were perfused with 100 ml normal KH. 5-8 mm³ pieces were protected in sucrose, frozen in liquid N₂, and stored at -70°C prior to thin sectioning (12 µm). Sections were fixed in neutral-buffered formalin, blocked with 2 % horse serum (1 h) prior to treating with
1:50 dilution of the polyclonal antiserum or pre-immune serum (16 h) and, following this, a Cy3-conjugated goat-antirabbit secondary antibody (1:2000 dilution, Molecular Probes, Eugene). Cultured cells were fixed in ice-cold methanol (10 min, -20°C), air dried and rinsed with PBS prior to incubating them successively with 10% goat serum, affinity-purified AbC170-MBP (4 µg/ml) and a tetramethylrhodamine-conjugated goat-antirabbit secondary antibody (2 µg/ml, Molecular Probes). Fluorescence was visualized with an IMT2 inverted microscope (Olympus, Melville) and images were digitized using ImagePro software (Media Cybernetics, Baltimore).

**RESULTS**

*Modified RT-PCR reveals two transcripts for a rat hepatocyte Kir4.2 channel*

First strand cDNA was synthesized using RACE technology. This results in cDNA having both 5’ (cap) and 3’ (oligo-d[T]) end-modified fragments complementary to full-length mRNA, with specific transcripts present in approximately the same number as in the original mRNA pool. Initially, gene-specific sequence data was obtained by cloning and sequencing a 380bp PCR product from two consecutive rounds of PCR using nested oligonucleotide primers homologous to regions in the pore and C-terminus of the mouse and human homologues (Set A & B, Table 1). To obtain sequence data for the full-length transcript, the forward and reverse primers of Set A were coupled, respectively, with d[T]30 and cap oligonucleotides for amplifying the cDNA out to the 3’ poly-A and the 5’ cap termini (Set C & E). These products were used as templates for a second round of PCR coupling the appropriate primers from Sets D and F (Table 1). Two major bands above 800 bp were observed with the cap and gene-specific primers. These were extracted, tested for the presence of the 380 bp Kir4.2 fragment, and positives were
cloned and sequenced. Since we could not identify distinct bands in the second round of gene-specific and d[T]30 primer PCR, we designed a degenerate reverse primer based on a conserved sequence within the 3’-untranslated region (3’UTR) of the sequenced Kir4.2 transcripts and paired this with the Kir4.2-specific forward primer (Set F). Two bands longer than 800 bp were extracted. PCR with Set B produced a 380 bp band using the 1.6 kb fragment as template. Sequencing revealed this fragment to have a 400 bp overlap with the 5’ cap sequence-containing fragment.

Two additional nested primer pairs (G and H) amplified the deduced open reading frame (ORF), which, when the clones were sequenced, demonstrated two possible transcripts with amino acid sequences of 375 and 405 residues. Of fifteen positive clones derived from three separate amplifications of the initial end-modified cDNA, three encoded the 405 residue protein while the remainder represented the shorter isoform. A Blastp search (NCBI, Bethesda) showed the 375 residue isoform shares 91%, 89% and 87% identity with the mouse, human and guinea-pig isoforms respectively (Fig 1) and therefore we have named it rKir4.2. The 405 residue protein, called rKir4.2a, arises from a nucleotide sequence in which 12 bp are absent, resulting in loss of a stop codon in the 5’-UTR of the 375 residue isoform (Fig 2A). This sequence has been deposited with GenBank (Accession # AY028455).

Conventional RT-PCR was used to confirm the presence of Kir4.2 transcripts in rat hepatocyte mRNA. The product of a PCR reaction between the first strand cDNA and primer set G (Fig 2B ‘PCR #1’) were used in a second amplification with primers encompassing the 5’ deletion site (Set I and Fig 2A). Separation of the products on agarose revealed a single band (Fig 2B ‘PCR #2 +RT’) that migrated to the same position as the 241 bp product of a PCR reaction containing primer Set I and the rat Kir4.2 transcript. In contrast, the 229 bp product of the same primer set and Kir4.2a
migrated further, corresponding to its smaller size (Fig 2B). Sequencing of the mRNA-derived, cloned PCR product identified it as Kir4.2. Three separate RT reactions were used as templates for six different PCR amplifications, and of the latter only one generated a detectable product. This outcome may have resulted from a less efficient RT reaction as compared to the larger number of full-length transcripts expected to be generated by the RACE technology. The combined results of the RACE and conventional RT-PCR suggest that Kir4.2 may be expressed at a higher frequency in rat hepatocyte mRNA than the longer isoform, Kir4.2a.

A C-terminal antibody recognizes the over-expressed protein and a sinusoidal signal in rat hepatocytes

A maltose-binding protein/Kir4.2 fusion protein containing the final, carboxy-terminal 170 amino acid residues of the channel was synthesized and used to produce rabbit polyclonal antibodies. The specificity of the affinity-purified antibody (AbC170) was determined by performing Western blots of HEK293T cell membrane extracts from nontransfected cells, or those transfected with Kir4.2a in pCMS-EGFP. Membranes were solubilized in 2% SDS followed by, initially, a three minute incubation at 94°C. Western blotting revealed a high molecular weight band (about 180kD, Fig 3A, right-hand panel) in the transfected cells (‘4.2a’) that was absent in nontransfected cells (‘NT’) or when the antibody was preabsorbed with the fusion protein (left-hand panel). Weak bands were also observed near the size of the monomer (about 42 kDa) and dimer (near 80 kDa), suggesting that the monomer undergoes significant aggregation into dimeric and tetrameric states that are heat-sensitive and SDS-resistant, as with many integral membrane proteins. This pattern also resembled the mobility of two other K⁺ channels claimed to be SDS resistant,
GIRK4 and KcsA (4;5;11). To determine the nature of this apparent aggregation state we followed an incubation protocol used for GIRK4 (see Methods). Fig 3B (right lane) shows that this more gentle treatment caused less aggregation, with the majority of the signal appearing near the putative monomeric mass. Further, three bands that differ by 3-4 kDa each are clearly visible in the membranes from Kir4.2a-transfected cells. When HEK cells were transfected with Kir4.2 the slowest migrating band was not detected (Fig 3B, left lane), suggesting that the latter band represents the full-length monomer containing the thirty N-terminal residues encoded by the Kir4.2a transcript. Since the antibody reacts with protein only in the transfected cells (lanes labeled ‘4.2’ or ‘4.2a’) it is concluded that we have generated a highly specific antibody, suitable for immunohistochemical studies in the liver.

Fig 4A shows the reaction of the polyclonal antiserum with thin cryosections of rat liver. Specific binding is localized to the circumference of hepatocytes, since this signal was diminished to background levels in tissue treated with preimmune serum (not shown). To confirm the plasma membrane localization of this reaction in hepatocytes, and to determine whether this was sinusoidal in nature, we treated isolated, short-term cultured rat hepatocyte couplets with affinity-purified C-170-MBP antibody. Again, specific signal was localized to the periphery of the cells, consistent with basolateral localization of the channel. This was completely lost when the cells were treated with antibody preabsorbed with C170-MBP (Fig 4B). From these studies it is concluded that Kir4.2 is expressed in the sinusoidal membrane of the rat hepatocyte, although more defined techniques are required to determine whether expression is excluded from the canalicular domain.

*Functional expression shows inward rectification in transfected HEK cells.*
The functional activity of Kir4.2a was assayed under voltage clamp using the whole-cell configuration of the patch electrode technique. Since HEK293 cells express endogenous voltage-gated K⁺ channels (30), we used a clamp protocol which inactivated the majority of these channels so that they would not obscure outward current through Kir4.2a. To further enhance Kir4.2a currents we dialyzed the cells with a relatively high pH (7.6) buffer since there is evidence that other Kir4.x homologues are pH sensitive (22;26;29), and set the K⁺ equilibrium potential close to 0 mV by bathing and dialyzing the cells with K⁺-rich solutions (see Methods). In pCMS-EGFP-Kir4.2a transfected cells a voltage step protocol from a holding potential (Vh) of 0 mV resulted in large, time-independent inward currents at potentials negative to Vh, and relatively smaller outward currents at equivalent positive potentials (Fig 5A, lower traces). Every GFP-expressing cell assayed possessed significant inwardly rectifying whole-cell currents. In contrast, fluorescing cells transfected with vector alone, or non-fluorescing cells, displayed small, non-rectifying currents (Fig 5A, upper traces). Since there was no significant time dependence to the currents, we also performed voltage ramps from positive to negative potentials, to eliminate Kv channel activation. The resulting current-voltage relationships (Fig 5B), illustrated with negative current and voltage plotted in the lower right quadrant, were used to calculate the mean conductance of Kir4.2a-expressing and nontransfected cells. The mean inward, whole-cell conductance, normalized to cell capacitance, of Kir4.2a expressing cells (2.09 ± .25 nS/pF (n=4)) was 17 times that of nonexpressing cells (0.12 ± 0.01 nS/pF (n=4)). To demonstrate that the expressed protein is a K⁺-selective channel, ramp currents were generated in cells bathed in a Na⁺-rich solution (see Methods). The resulting current-voltage relationship shifted 67 mV along the voltage axis, closely corresponding to the 74 mV
change in the Nernst potential for K⁺ while the Cl⁻ equilibrium was maintained at 0 mV.

To determine whether the rat hepatocyte Kir4.2 transcript could be expected to produce functional channels in vivo we transfected HEK293T cells with the same amount of pCMS-EGFP containing Kir4.2 as we had used for the Kir4.2a experiments just described. We observed a similar lack of time dependence and current densities between the two isoforms. Specifically, Fig 6 shows the mean current densities at –120 mV and +100 mV of nontransfected HEK293T cells, or those transfected with the rat Kir4.2 or Kir4.2a.

**DISCUSSION**

In the present study we cloned, sequenced, generated specific antibodies for immunolocalization, and assayed functional activity of two closely related homologues of Kir4.2 that are expressed in rat hepatocytes. These results extend the recent cloning of a Kir4.2 from whole mouse liver (22). Although Kir4.2 mRNA was not detected on Northern blots of embryonic mouse liver (27), this likely resulted from an inability to detect rare messages such as K⁺ channels in non-excitatory tissue, and/or the tissue developmental stage. Non-conducting human and guinea-pig transcripts have also been cloned (6;25). The overall sequence identity at the amino acid level is 90%. The biophysical properties of Kir4.2 make it a suitable candidate for the functional data accumulated regarding K⁺ fluxes in the intact liver and isolated hepatocytes. Further, whole-cell currents of short-term cultured rat hepatocytes exhibit characteristics of moderately inwardly rectifying K⁺ channels (12) such as Kir4.2.

The Kir4.X family consists of three genes, two of which are known to code for multiple, tissue-specific isoforms (25). Kir4.1 was cloned first, originally from rat
brain and human kidney, and called BIR10 and Kir1.2 respectively (3;25). The Kir4.2 (initially called 1.3) gene has been cloned from cDNA from human and guinea-pig kidney and mouse liver, and human DNA (6;7;22;25) and localized to the recently fully sequenced human chromosome 21 at q22.2, in the middle of the Down’s Syndrome Critical Region (7;10). As with Kir4.1, it is composed of (at least) two exons, the first one being located within the 5'-UTR of the transcript, and the ‘core’ exon encoding the majority of the ORF (7). This 5’ heterogeneity is similar to the more fully characterized rat kidney gene product ROMK1 (Kir1.1), which also has splice variants containing up to 19 additional amino acid residues at the start of the ORF (1;2;17;25).

The putative novel, longer isoform (Kir4.2a) cloned here from rat hepatocytes apparently arises from a twelve base pair deletion in the 5' UTR of Kir4.2, resulting in the loss of a stop codon upstream of its predicted start site. The simplest explanation for the loss of this short sequence is the presence of multiple exons and/or alternate splicing of these. Splice site prediction analysis, conducted through the Berkley Drosophila Genome Project web site, revealed a possible donor site in Kir4.2 corresponding to the 5’ end of the ‘deletion’ in Kir4.2a. However, a classical acceptor site is not present in the 5’ UTR of Kir4.2. The putative first or alternative start codon in rKir4.2a is flanked by a sequence (5’-agccccauugg) that conforms weakly to the Kozak consensus sequence (5’-gcc(a/g)ccaugg, (18)), as does the methionine following the 5’ UTR stop codon (5’-cugaggaugg). The mouse kidney cDNA sequence (NCBI Accession # AJ012368) is also missing an in-frame stop codon in a similar position as rKir4.2a. Translation from the first methionine would result in a 402 residue protein whose first 27 amino acids are conserved between it and the rat N-terminus. It remains to be determined what functional translation product(s) are synthesized in the hepatocyte.
Sequence comparison indicates that the rat isoforms bear closest resemblance to the ‘non-conductive’ guinea-pig and human homologues (see Fig 1). However, we show here that expression of either Kir4.2 or Kir4.2a in HEK293 cells produce conductive channels. Expression of the mouse isoform in *Xenopus* produced functional channels with inwardly rectifying properties similar to both rat isoforms (22). The regions of highest heterogeneity between the different species exists in the N-terminus and the external M1-pore linker (see Fig 1), with the mouse channel having the largest net negative charge across the latter region, possibly accounting for the difference in $K^+$ conductance between the mouse and human/guinea-pig isoforms. However, the rat hepatocyte isoforms resemble the non-conductive homologues rather than the mouse channel. Thus some additional differences must exist in the rat isoforms to account for the different conduction properties. Mutagenesis of a single lysine residue to arginine in the M1-pore linker of the guinea-pig Kir4.2 results in significantly increased conduction as compared to the wild type isoform, with the authors concluding that this residue is important in channel function (6). However, both the conducting mouse Kir4.2 and the rat hepatocyte isoforms have a lysine at this position. Alternatively, heterologous expression with other channel subunits may differentially affect the conductive properties of Kir4.2 homologues. In support of this, both the Kir4.1 and the mouse Kir4.2 proteins generate significantly larger whole-cell currents when co-expressed in stoichiometric amounts with the non-conducting Kir5.1 as compared with the homologous system (22;23;26;28;29).

Members of the Kir4.x class of inward rectifiers are routinely found to be distributed in epithelia in a vectorial fashion. Kir4.1 has been demonstrated in the basolateral membranes of renal distal tubular and retinal pigment epithelium (16;19). Conversely, Kir4.2 immunoblots (Western) or histochemistry have not been reported.
In the present study we prepared affinity-purified polyclonal antibodies to the C-terminal 170 residues of Kir4.2 and observed a specific signal in (at least) the basolateral (sinusoidal) membranes of hepatocytes. This localization would permit the efflux of $\text{K}^+$ from the hepatocyte surface in a similar fashion as has been seen in classical exocrine secretory tissues (24). Minimally, this efflux would serve as a mechanism for maintaining electroneutrality in the face of organic anion and $\text{Cl}^-$ efflux across the apical (canalicular) membrane. This is requisite for maintenance of production of bile and disruption of this process could result in depressed bile formation, or cholestasis.

In conclusion, we have cloned two new isoforms of Kir4.2 from rat hepatocytes, demonstrated localization at least to the basolateral membrane of these cells, and shown that both isoforms generate functional channels when expressed in HEK293 cells. The time independence and inwardly rectifying properties of these channels would provide a low, steady level of outward $\text{K}^+$ flux that may underlie a significant component of passive $\text{K}^+$ release in the liver.


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FIGURE LEGENDS

Fig 1  Homology comparison of the two rat hepatocyte isoforms with human, guinea-pig and mouse primary sequences.

The rat isoforms share 91, 89 and 87 % identity with the mouse (AF085696), human (U73191) and guinea-pig (AF049076) isoforms. The putative longer rat isoform (Kir4.2a) has an additional 30 residues at its N-terminus. Lack of identity between all homologues exists within the M1-pore linker and the N-terminus. M1 and M2 represent the integral membrane helices, P is the pore-forming domain.

Fig 2  RT-PCR of hepatocyte mRNA shows Kir4.2 is the major liver isoform.

(A) PCR primers (Set I, shown within the boxes) bridging the 12 bp deletion in Kir4.2a were used to amplify a fragment from a first round of PCR designed to amplify the ORF (Set G). Putative start codons for each isoform are underscored with a single line; the in-frame stop codon in Kir4.2 is doubly underlined. (B) Separation of the PCR reactions on 2.5% agarose revealed a single band after the second round of PCR (‘PCR #2’). No products were detectable after the initial 30 cycles (‘PCR #1’), or in the absence of reverse transcriptase in the RT reaction (‘-RT’). Plasmids containing the Kir4.2 or Kir4.2a cDNA were also amplified with the same primers to generate positive controls (‘4.2a’ and ‘4.2’). Size markers (‘M’) are a 25 bp ladder ranging from 25 to 300 bp.

Fig 3  A C-terminal antibody detects monomeric and oligomeric forms of Kir4.2a in membranes of HEK293 cells expressing the channel.

Membrane extracts were prepared from HEK293T cells transfected with the pCMS-EGFP vector alone (‘NT’) or with pCMS-EGFP containing Kir4.2a or Kir4.2 (‘4.2a’, ‘4.2’). Proteins were separated by 12% PAGE, and blotted with affinity-purified
C170-MBP antibody ((A) right-hand panel, (B)), or antibody preabsorbed with a 20X excess of fusion protein ((A) left-hand panel). Membranes were solubilized in SDS-PAGE sample buffer and heated at either (A) 94°C for 3 min, or (B) successively at 50, 20 and 50°C for 15, 30 and 15 min respectively, prior to loading.

**Fig 4** Immunohistochemistry with the C170-MBP antibody demonstrates a hepatocellular signal localized to the basolateral membrane.

Frozen tissue slices (A) or 24 h cultured hepatocytes (B) were treated with either the polyclonal antiserum (A), or the affinity-purified antibody alone or preabsorbed with fusion protein (B), respectively. Antibody was detected by immunofluorescence using (A) Cy3 or (B) tetramethylrhodamine-conjugated secondary antibodies.

**Fig 5** Functional expression of rat hepatocyte Kir4.2a reveals a time-independent, inwardly rectifying K⁺ conductance.

Currents resulting from voltage steps between −140 and +120 mV in HEK293T cells transfected with vector alone (‘pCMS-EGFP’) or vector with the 4.2a ORF (‘pCMS-EGFP-Kir4.2a’). Currents are normalized to cell capacitance; dotted line = 0 current level. (B) Mean (±SD, n=4 for each condition), capacitance-normalized, current-voltage relationships derived from voltage ramps from +100 to −120 mV in cells transfected with vector alone (‘NT’), or with Kir4.2a (‘Kir4.2a’). Cells were bathed in 5 mM or 115 mM K⁺-containing solutions to shift Eₚ between −80 (●) and −6 mV (■, ▲). Axes converge at 0 pA/pF and 0 mV.

**Fig 6** Both rat hepatocyte Kir4.2 and Kir4.2a are functionally active following expression in HEK293T cells.
Cells were transfected with vector alone (black bars), or pCMS-EGFP containing either Kir4.2 (white bars) or Kir 4.2a (shaded bars). Mean current densities at -120 and +100 mV were collected from voltage ramps generated as illustrated in Fig 5B (n=4 for each condition). Cells were bathed in 115 mM K+-containing solutions.
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Figure 1
Figure 2

A

rKir4.2a

<table>
<thead>
<tr>
<th>PCR #1</th>
<th>PCR #2</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>RT</td>
</tr>
</tbody>
</table>

B

- 1800 bp
- 300
- 200
- 100

M

- RT

+RT

4.2a

4.2
Figure 3
Figure 5

A

PCMS-EGFP

PCMS-EGFP-Kir4.2a

50 pA/pF

50 ms

B

+100

0 mV

-120

E_k (mV)

100 pA/pF

50 mV

Kir4.2a -6

Kir4.2a -80

NT -6