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

CEREDWYN E. HILL, M. MARTHA BRIGGS, JUNJUN LIU, AND LESLIE MAGTANONG
Gastrointestinal Diseases Research Unit and Department of Physiology, Queen’s University, Kingston, Ontario K7L 5G2, Canada

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Hill, Ceredwyn E., M. Martha Briggs, Junjun Liu, and Leslie Magtanong. Cloning, expression, and localization of a rat hepatocyte inwardly rectifying potassium channel. Am J Physiol Gastrointest Liver Physiol 282: G233–G240, 2002. First published September 21, 2001; 10.1152/ajpgi.00256.2001.—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 subclass of inwardly rectifying potassium channels were found. The longer deduced isoform (4.2a) has 30 additional NH2-terminal amino acids, which identifies this as a new isoform. The short-form isoform 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 COOH-terminal fragment recognized bands between 40 and 45 kDa and at 90 kDa and recognized a high molecular mass band around 200 kDa in overexpressing HEK cells. Immunohistology of liver tissue shows hepatocellular plasma membrane localization. In hepatocyte coupled, Kir4.2 was predominantly localized to the basolateral membrane. Results demonstrate 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.

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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). Furthermore, the divalent organic anion 4,4'-disothiocyano stilbene-2,2'-disulfonic acid (DIDS) 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.

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 Ca2+ 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.

METHODS

Animals. Male Sprague-Dawley rats (200–225 g body wt) (Charles River Canada, Montreal, ON, Canada) were housed under a 12-h light/dark cycle with free access to water and rat chow according to the Canadian Council on Animal Care.

Hepatocyte couplet isolation. Livers were successively single-pass perfused at 40 ml/min with 250 ml HEPES-buffered

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Krebs saline (KH), pH 7.4, plus 0.5 mM EGTA and 100 units/100 μg penicillin/streptomycin per milliliter, and 100 ml of the same solution also containing 0.03% (wt/vol) collagenase (Liberase; Roche Biochemicals, Montreal, PQ, Canada) 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 a 25-μm nylon mesh, washed with KH, and used immediately for radioimmunoprecipitation, or plated out at 20–30% confluence in DMEM containing 10% fetal calf serum, 2 mM glutamine, and penicillin/streptomycin) onto sterile glass coverslips in 35-mm plates and plated out at 10% density into fresh medium (DMEM growth). Cell viability was assessed by trypan blue exclusion after 3 min, but this greatly impeded migration into the acrylamide gel. To increase mobility we incubated the membranes in the same solution also containing 0.03% (wt/vol) collagenase (Liberase; Roche Biochemicals, Montreal, PQ, Canada) 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 a 25-μm nylon mesh, washed with KH, and used immediately for radioimmunoprecipitation, or plated out at 20–30% confluence in DMEM containing 10% fetal calf serum, 2 mM glutamine, and penicillin/streptomycin) onto sterile glass coverslips in 35-mm plates and plated out at 10% density into fresh medium (DMEM growth). Cell viability was assessed by trypan blue exclusion.

RT-PCR. Total RNA was extracted (Trizol; GIBCO-BRL, Gaithersburg, MD) from 10 ml of packed hepatocytes followed by DNase (1 unit/μg RNA; Promega, Madison, WI) treatment to remove residual contaminating genomic DNA. Reverse transcription was performed by rapid amplification of cDNA ends (RACE) using 1–4 μg RNA, 200 units Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL) and cap and oligo-dT primers (SMART cDNA synthesis; Clontech, Palo Alto, CA) 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 (Oligoex; Qiagen, Valencia, CA). Proofreading (Platinum Pfx; Clontech Advantage or GIBCO-BRL) DNA polymerases were used for synthesis of PCR products destined for sequencing. Because 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 three-step PCR protocol using an MJ Research PTC-100 thermocycler was used, setting the annealing temperature 5°C lower than the primer melting temperature values, and extension times of 30 s/500 bp (minimum 30 s). Initial sequence information was obtained from primers designed against a 380-bp 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 open reading frame (ORF) was end modified with primers containing appropriate restriction sites for subcloning into the multiple cloning sites (MCS) of pCMS-EGFP (Clontech), a vector encoding enhanced green fluorescent protein (EGFP) under independent promoter control [pCMS; cytomegalovirus IE promoter, MCS, and SV40 promoter]. All fragments were cloned and sequenced by the automated dideoxy method.

Cell culture and transfection. HEK293T cells at 85% confluence were dissociated with 0.05% trypsin and 0.02% EDTA at 50% confluence in 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 h the cells were transfected with a complex of Fugene 6 (Roche Biochemicals) and plasmid (1 μg/plate) and 20 h later, the cells were transferred to 28°C to stimulate protein synthesis and decrease cell proliferation. Cells were used for electrophysiological experiments 1–3 days later.

Electrophysiology. Patch clamp recordings were made in the whole cell configuration using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) 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 consisting of (in mM) 5 KCl, 140 NaCl, 5 glucose, 5 HEPES, 1 CaCl₂, and 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 EGFP, 5 HEPES, pH 7.6.

Fusion protein synthesis and antibody generation. A maltose binding protein-Kir4.2 fusion protein was synthesized in Escherichia coli using the pMALc2X vector (New England BioLabs, Beverly, MA). A fragment corresponding to bases coding for the 170 amino acid residues completing the COOH-terminus of the Kir4.2a transcript was ligated into the MCS of pMAL. The purified protein was emulsified with TiterMax agent (CytRx, Norcross, GA). Antibody production was monitored in test bleeds after 4 and 6 wk with essentially identical results. The IgG fraction was isolated by chromatography on DEAE Affi-Gel (Bio-Rad, Hercules, CA). 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 MBB-C170-immobilized carbonyldimidazole-activated agarose column (Reacti-gel; Pierce, Rockford, IL).

Western blotting of recombinant protein. HEK293T cells were grown in T-75 flasks. At 50% confluence, 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 h, the cells were washed twice with PBS pH 7.4 and permeabilized in 1 ml/5 mM HEPES pH 7.4 containing a protease inhibitor cocktail (Complete-Mini; Roche). The suspension was then drawn through a 22- and a 27-gauge needle (10 and 5 times, respectively). Membranes were collected by centrifuging at 10,000 g 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 h 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 62.5 mM Tris-HCl, pH 8.8, and 2% SDS. Whole cell currents were recorded with cell-attached patches formed by aspiration of the pipette along the edge of a glass coverslip or watching for well-defined leak current at 20–25 mV in the absence of any other current. Whole cell currents were recorded with cell-attached patches formed by aspiration of the pipette along the edge of a glass coverslip or watching for well-defined leak current at 20–25 mV in the absence of any other current.

Table 1. Primer pairs used to clone rat hepatocyte Kir4.2 isoforms

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0.1% SDS, eluted onto polyvinylidene difluoride membrane, blocked for 16 h with 5% nonfat milk, and probed with the affinity-purified primary antibody (4 µg/ml) followed by an alkaline phosphatase-conjugated goat-anti-rabbit secondary antibody (2 µg/ml). Preabsorbed antibody was incubated (16 h, 4°C) with a 20 times (mass) excess of purified fusion protein. Alkaline phosphatase was detected with the chromogenic substrate NBT/BCIP.

**RESULTS**

**Immunohistochemistry of liver and hepatocyte couplets.** Livers were perfused with 100 ml normal KH. Pieces (5–8 mm³) were protected in sucrose, frozen in liquid N₂, and stored at −70°C before thin sectioning (12 µm). Sections were fixed in neutral-buffered formalin and blocked with 2% horse serum (1 h) before treating with 1:50 dilution of the polyclonal antiseraum or preimmune serum (16 h) and next a Cy3-conjugated goat-anti-rabbit secondary antibody (1:2,000 dilution; Molecular Probes, Eugene, OR). Cultured cells were fixed in ice-cold methanol (10 min, −20°C), air dried, and rinsed with PBS before incubating them successively with 10% goat serum, affinity-purified AbC170-MBP (4 g/ml), air dried, and rinsed with PBS before incubating them successively with 10% goat serum, affinity-purified AbC170-MBP (4 g/ml) and the 5′ cap termini. M1 and M2, integral membrane helices; P, pore-forming domain.

**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) was used in a second amplification with primers encompassing the 5′-UTR of the Kir4.2 transcript (Fig. 2A).

Sequencing revealed two possible transcripts with amino acid sequences of 375 and 405 residues. Of 15 positive clones derived from three separate amplifications of the initial end-modified cDNA, three encoded the 405 residue protein, whereas the remainder represented the shorter isoform. A BLAST (blastp) search [National Center for Biotechnology Information, Bethesda, MD] 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 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 no. AY028455).

**Fig. 1.** Homology comparison of the two rat hepatocyte isoforms with human, guinea pig and mouse homologues (sets A and 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′ polyA and the 5′ cap termini (sets C and E). These products were used as templates for a second round of PCR by 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. Because 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 a 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 ORF, which, when the clones were sequenced, demonstrated two possible transcripts with amino acid sequences of 375 and 405 residues. Of 15 positive clones derived from three separate amplifications of the initial end-modified cDNA, three encoded the 405 residue protein, whereas the remainder represented the shorter isoform. A BLAST (blastp) search [National Center for Biotechnology Information, Bethesda, MD] 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 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 no. AY028455).

**Fig. 2.** Homology comparison of the two rat hepatocyte isoforms with human, guinea pig and mouse homologues (sets A and 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′ polyA and the 5′ cap termini (sets C and E). These products were used as templates for a second round of PCR by 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. Because 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 a template. Sequencing revealed this fragment to have a 400-bp overlap with the 5′-cap sequence-containing fragment.

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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 compared with 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 COOH-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, COOH-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 blot analyses of HEK293T cell membrane extracts from nontransfected (NT) cells, or those transfected with Kir4.2a in pCMS-EGFP. Membranes were solubilized in 2% SDS initially followed by a 3-min incubation period at 94°C. Western blot analysis revealed a high molecular mass band (about 180 kDa, Fig. 3A, right) in the transfected cells (4.2a) that was absent in NT cells or when the antibody was preabsorbed with the fusion protein (Fig. 3A, left). 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). Figure 3B (right lane) shows that this more gentle treatment caused less aggregation, with the majority of the signal appearing near the putative monomeric mass. Furthermore, 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), suggest-
ing that the latter band represents the full-length monomer containing the NH$_3$-terminal residues encoded by the Kir4.2a transcript. Because 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.

Figure 4A shows the reaction of the polyclonal antiserum with thin cryosections of rat liver. Specific binding is localized to the circumference of hepatocytes, because 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, a 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. Because HEK293 cells express endogenous voltage-gated K$^+$ channels (30), we used a clamp protocol that inactivated the majority of these channels so 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, because there is evidence that other Kir4.x homologues are pH sensitive (22, 26, 29) and we 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 nonfluorescing cells displayed small nonrectifying currents (Fig. 5A, upper traces). Because there was no significant time dependence to the currents, we also performed voltage ramps from positive to negative Fig. 5. Functional expression of rat hepatocyte Kir4.2a reveals a time-independent, inwardly rectifying K$^+$ conductance. A: 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: 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. Cells were bathed in 5 mM or 115 mM K$^+$-containing solutions to shift K$^+$ equilibrium potential (E_K) between −80 (●) and −6 mV (●, ▲). Mean ± SD, n = 4 for each condition. Axes converge at 0 pA/pF and 0 mV.
The expressed protein is a 

Kir4.2a expressing cells 

The mean conductance of Kir4.2a-expressing and NT cells. The mean currents 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.

Fig. 6. Both rat hepatocyte Kir4.2 and Kir4.2a are functionally active after expression in HEK293T cells. Cells were transfected with vector alone (filled bars), or pCMS-EGFP containing either Kir4.2 (open bars) or Kir 4.2a (hatched 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.

potentials to eliminate Kv channel activation. 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 NT cells. The mean inward, whole cell conductance, normalized to cell capacitance, of Kir4.2a expressing cells [2.09 ± 0.25 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+, whereas the Cl- equilibrium potential 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 NT HEK293T cells or those transfected with the rat Kir4.2 or Kir4.2a.

**DISCUSSION**

In the present study, we cloned, sequenced, and 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 blot analyses of embryonic mouse liver (27), this likely resulted from an inability to detect rare messages such as K+ channels in nonexcitable tissue and/or the tissue developmental stage. Nonconducting 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. Furthermore, 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 12-bp 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 exons. 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’-agccccaggg) that conforms weakly to the Kozak consensus sequence [5’-gcc(a/g)ccaugg, (18)], as does methionine after the 5’ UTR stop codon (5’-cugagg-gaugg). The mouse kidney cDNA sequence (NCBI Accession no. 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 that has its first 27 amino acids conserved between it and the rat NH2-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 nonconductive 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. Expres-
sion of the mouse isoform in *Xenopus laevis* 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 NH3-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 nonconductive homologues rather than the mouse channel. Thus some additional differences must exist in the rat isoforms to account for the different conductance 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 compared with 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 coexpressed in stochiometric amounts with the nonconducting Kir5.1 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 COOH-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 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 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 K+ flux that may underlie a significant component of passive K+ release in the liver.

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