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Electrophysiological and molecular identification of hepatocellular volume-activated K^+ channels

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Abstract

Although K^+ channels are essential for hepatocellular function, it is not known which channels are involved in the regulatory volume decrease (RVD) in these cells. We have used a combination of electrophysiological and molecular approaches to describe the potential candidates for these channels. The dialysis of short-term cultured rat hepatocytes with a hypotonic solution containing high K^+ and low Cl^- concentration caused the slow activation of an outward, time-independent current under whole-cell configuration of the patch electrode voltage clamp. The reversal potential of this current suggested that K^+ was the primary charge carrier. The swelling-induced K^+ current (I_{Kvol}) occurred in the absence of Ca^{2+} and was inhibited with 1 μM Ca^{2+} in the pipette solution. The activation of I_{Kvol} required both Mg^{2+} and ATP and an increasing concentration of Mg–ATP from 0.25 through 0.5 to 0.9 mM activated I_{Kvol} increasingly faster and to a larger extent. The KCNQ1 inhibitor chromanol 293B reversibly depressed I_{Kvol} with an IC_{50} of 26 μM. RT-PCR detected the expression of members of the KCNQ family from KCNQ1 to KCNQ5 and of the accessory proteins KCNE1 to KCNE3 in the rat hepatocytes, but not KCNQ2 and KCNE2 in human liver. Western blotting showed KCNE3 expression in a plasma membrane-enriched fraction from rat hepatocytes. The results suggest that KCNQ1, probably with KCNE2 or KCNE3 as its accessory unit, provides a significant fraction of I_{Kvol} in rat hepatocytes.

Keywords: cDNA; Human; KCNE; KCNQ; Patch clamp; Rat; RVD

1. Introduction

Hepatocellular hydration, controlled by the uptake and metabolism of osmotically active substances [1], is a well-established signal regulating cellular metabolism, transport, gene expression and exocrine function [2,3]. Under hypotonic conditions that cause cell swelling, liver cell volume regulation is accomplished by an efflux of K^+, Cl^- and organic ions [4,5]. The ensuing flow of osmotically driven water out of the cell returns the cell to its initial volume and is referred to as a regulatory volume decrease (RVD).

Bile production, the exocrine function of the liver, is correlated with the hydrated status of the hepatocyte and with events associated with liver volume regulation including RVD and regulatory volume increase (RVI). In the isolated perfused rat liver hypotonic conditions are associated with choleresis [5] whereas RVI induced by hypertonic solutions reduce bile flow [6]. Cell swelling-induced choleresis is also paralleled by transient increases in passive K^+ flux [5]. Stimulated bile flow and K^+ flux also occurs upon the exposure of the isolated perfused rat liver to the cholephyllic disulfonates bromosulfophthalein (BSP) or diisothiocyanostilbenedisulfonate (DIDS) [7,8]. BSP, DIDS and a third disulfonate, indocyanine green hyperpolarize isolated rat hepatocytes by increasing K^+ conductance [9,10]. The combined results suggest that the choleretic disulfonate compounds may also stimulate the same K^+ channels activated by hypotonic exposure.

Several types of K^+ channels have been proposed as the molecular entities mediating the swelling-activated K^+ current (I_{Kvol}) associated with RVD in different tissue.

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These include members of each family of K^+ channel, such as the voltage-gated K^+ channels Kv 1.3 [11], Kv 1.5 [12] and the KCNQ/KCNE family [13,14], the two-pore domain channels (TASKs) [15] and large (BK), intermediate (IK) and small (SK) conductance Ca^{2+}-activated K^+ channels [16–18]. No functional evidence exists for the expression of the Kv channels in the mammalian hepatocyte and hepatoma though molecular cloning techniques have identified TASK3, SK2 and SK3, and Kir4.2 channel transcripts in these cells [17,19–22]. The molecular identification of the channel(s) responsible for volume regulation in the hepatocyte has not been established.

Since volume-activated K^+ channels play an integral role in hepatocellular volume regulation and bile secretion, the aim of our study was to identify and characterize these channels at the functional and molecular levels. Our results demonstrate that hypotonic solutions activate a weakly outwardly rectifying K^+ current that is sensitive to the KCNQ family blocker chromanol 293B. RT-PCR identified transcripts for multiple members of the KCNQ and KCNE families of K^+ channels in the rat hepatocyte and human liver. We conclude that the KCNQ1/KCNE3 heterotetrameric channel mediates a significant fraction of I_{Kvol} in the rat hepatocyte.

2. Materials and methods

2.1. Materials

Female Sprague–Dawley rats (200–225 g) were obtained from Charles River (Montreal, QC) and maintained on a 12 h light/dark regime with access to water and rat chow ad libitum according to the Canadian Council on Animal Care. Human liver cDNA was purchased from Ambion, Inc. (Austin, TX). Unless otherwise noted, chemicals were purchased from Sigma-Aldrich Chemicals (St. Louis, MO) or British Drug Houses (Toronto, ON) and were of highest grade available. DIDS (4,4′ -diisothiocyanatostilbene-2,2′ -disulfonate) and foetal calf serum and Taq DNA polymerase were supplied by Toronto Biochemicals (Toronto, ON) and GIBCO/Invitrogen Corporation (Burlington, ON) respectively. Oligotex Direct mRNA and Expand reverse transcriptase were purchased from Qiagen, Inc. (Mississauga, ON) and Roche Biochemicals (Montreal, QC). Chromanol 293B (*trans*-6-cyano-4-(*N*-ethylsulfonyl-*N*-methylamino)-3-hydroxy-2,2-dimethyl-chromane), a generous gift from Aventis Pharma Deutschland GmbH (Frankfurt, Germany), was dissolved in dimethylsulfoxide (DMSO) to make a 100 mM stock solution and was diluted at a final concentration of 1–100 μM in the external solution (DMSO concentrations ≤0.1%). In preliminary experiments, we confirmed that DMSO alone did not have any appreciable effect on K^+ currents at concentrations of up to 0.5%.

2.2. Cell isolation and culture

Rat hepatocytes were isolated using a collagenase (Liberase, Roche Biochemicals, Montreal, QC, 0.35 mg/ml) perfusion technique and plated at 1×10^5 cells per ml on glass coverslips in 35 mm Petri dishes. Cells were cultured in DMEM solution containing 0.15% $NaHCO_3$, 10 mM Hepes, 10% (v/v) foetal calf serum, 2 mM glutamine, 5 μg/ml insulin, 1 μM dexamethasone and 100 U/100 μg per ml penicillin/streptomycin, pH 7.4 in a humidified atmosphere of 95/5% air/CO_2 at 37 °C. Electrophysiological experiments were carried out on cells cultured between 12 and 72 h.

2.3. Electrophysiology

Patch clamp recordings were made at room temperature in the whole-cell configuration using an Axopatch 200A amplifier (Axon Instruments, Foster City) and Clampex 7 software as described previously [22]. Patch pipettes were made from borosilicate glass (Warner Instrument Corp., Hamden, CT, # G85165T-4) with a resistance of 2 to 4 MΩ when filled and immersed in K^+-containing solutions. Whole-cell currents were digitized (Digidata 1200B) at 5 kHz. Sampled data were analyzed by Origin 6.0 software (Origin Lab, Northampton, MA). The time course of swelling-induced current development was monitored from a holding potential of 0 mV by repetitively applying (0.03 Hz) voltage steps of 1 s duration to −80, 0 and 80 mV. To measure the activation and deactivation kinetics, voltage steps of −120 to +120 mV (in 20 mV increments, 2.5 s duration) were applied from a 1 s pre-pulse to −40 mV. To minimize swelling-activated anion currents, whole-cell recordings were carried out using low Cl^--containing pipette and bath solutions, and DIDS (100 μM), a blocker of these currents in rat hepatocytes [23,24], was included in the bath solution. Cells were bathed in solutions containing, in mM, 5 glucose, 5 Hepes, 1 $CaCl_2$, 1 $MgCl_2$ and where noted, 0.5, 5 or 50 mM potassium-gluconate. Total gluconate was maintained at 145 mM with sodium-gluconate (pH adjusted to 7.4 with NaOH). The pipettes were filled with solutions containing, in mM, 1 $MgCl_2$, 1 $CaCl_2$, 5 Hepes, 4 ATP, 11 EGTA and with either 140 mM K^+-gluconate or 140 mM Na^+-gluconate in the presence or absence of 80 mM raffinose to activate I_{Kvol} (pH adjusted to 7.2 with KOH). In some experiments $CaCl_2$ was adjusted to give an ionized Ca^{2+} of 0, 13 nM and 1 μM, and in others $MgCl_2$ was increased to give Mg–ATP concentrations of 0, 0.25, 0.5 and 0.9 mM. Ca^{2+} and Mg–ATP concentrations were calculated using EQCAL software (Biosoft, Cambridge, UK). Data are presented as representative recordings or as means±S.E. of n observations, in which n is the number of samples.

2.4. RT-PCR

Rat mRNA was isolated from freshly purified rat hepatocytes and reverse transcribed according to the

manufacturer's directions. Controls were conducted in the absence of enzyme. 20 μl PCR reactions contained either human (0.5 ng) or rat (from 75 ng mRNA) cDNA and 0.5 U Taq DNA polymerase, 1.5 mM $MgCl_2$, 0.2 μM dNTPs and 0.5 μM primers. When nested PCR was performed 1 or 2 μl first round PCR product for rat or human respectively was used in the reaction. Gene-specific forward and reverse primers were designed for KCNQ and KCNE gene sequences obtained from GenBank DNA sequence database (see Table 1). PCR products were cloned into an A/U cloning vector (pDrive, Qiagen) and sequenced by the automated dideoxy method for identity confirmation.

2.5. Western blotting of KCNE3 in rat plasma membranes

Rat liver membranes were isolated on step gradients of sucrose following perfusion and homogenization in ice-cold 1 mM $NaHCO_3$ [25]. Membranes were cleaned of associated protein by incubating (0.5 mg/ml) in 100 mM Na_2CO_3 pH 11.5 for 30 min at 0 °C [26]. The suspension was centrifuged at 100,000×*g* for 1 h at 4 °C and the pellet was solubilized in 10% SDS, 62.5 mM Tris pH 6.8 and dialyzed overnight against 2% SDS, 62.5 mM Tris pH 6.8. Samples were mixed with Laemmli buffer and incubated at 50 °C for 15 min, 20 °C for 30 min and a final 15 min at 50 °C. 50 μg protein was loaded on SDS-PAGE in 10% acrylamide. Proteins were transferred to PVDF membrane by wet transfer. Membranes were blocked with 5% milk powder in Tris-buffered saline plus 0.25% Tween (TBST) overnight, followed by a 4 h incubation with KCNE3 antibodies (2 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA) in TBST. After four 15 min washes with TBST, membranes were incubated with alkaline phosphatase-conjugated anti-goat IgG for 60 min and visualized with NBT/BCIP. Western blotting was performed four times with different preparations and representative results are illustrated.

3. Results

3.1. Biophysical properties of hepatocellular I_{Kvol}

Hypotonic conditions are associated with the activation of a large Cl^- conductance in the hepatocyte [23,24]. To isolate K^+ current (I_K) from Cl^- current (I_{Cl}), experiments were carried out using low Cl^- concentration (4 mM) in both bath and pipette solutions and included DIDS (100 μM) in the bath solution. Therefore, the current–voltage relation for I_K can be obtained with little contamination from I_{Cl}. To establish the time course of activation of volume-activated K^+ currents (I_{Kvol}) repetitive voltage steps of 1 s duration to E_K (−80 mV), through E_{Cl} (0 mV) to 80 mV were used to monitor I_{Cl} and I_K respectively. Fig. 1A (left panel) shows representative current records evoked by the repetitive cycle voltage protocol from two different cells, one dialyzed with isotonic solution ('Control') and the other with hypotonic solution ('Raffinose'). In the control cells, I_K decayed within 5 min of obtaining the whole-cell configuration. Conversely, hypotonic solutions caused the

Table 1
Primers for the PCR amplification of KCNQ and KCNE in rat hepatocyte and human liver cDNA

Gene	Primer sequences		Accession number	T_m (°C)	Product length (bp)
	1st round primers	2nd round primers			
rKCNQ1	F-CTCCATCTACAGTACGCGTC	F-CCATCATCGACCTCATCGTG	NM_032073	50	305
hKCNQ1	R-ATCTGCGTAGCTGCCAAAC	R-GCGTAGCTGCCAAACTCGAT	NM_181798		304
rKCNQ2	F-AGTGCGGATCAGAGTCTC	F-GCTTTCCGTATCAAGGGCG	NM_133322	50	138
hKCNQ2	R-GCTCTGATGCTGACTTTGAGGC	R-TGCTGACTTTGAGGCCAGG			136
rKCNQ3	F-CAGCAAAGAACTCATCACCG	F-CATCGGCTTCCTGACACTCA	NM_031597		121
hKCNQ3	R-ATGGTGGCCAGTGTGATCAG	R-GGGCATCAGCATAGGTCTCAA	NM_004519	50	122
rKCNQ4	F-CCCTCCAAGCAGCATCTG	F-ATGCCTGCTGTGAAGACGG	XM_233477		147
hKCNQ4	R-TTGATTCGTCCCAGCATGTCCA	R-TTCGTCCCAGCATGTCCA	NM_172163	55	148
rKCNQ5	F-GGAACCCAGCTGCCAACCTCAT	F-TGCCAACCTCATCCAGTGTG	XM_347246		96
rKCNQ5	R-CTTTCTTGGTAGGGCTGCAG	R-GGTGTGCAAGGCCTTCAGA	NM_019842	50	97
rKCNE1	F-GGATGGCCCTGTCCAATTC	–		50	214
	R-CCAGCTTCTTGGATCGGATG				
rKCNE2	F-CAGCTGGAGGAGGAACACAAC	–		50	207
	R-TGCCAATCCTCCACGATGT				
rKCNE3	F-CAACGGGACTGAGACCTGGT	–	NM_022235	50	275
	R-TGCGTGAACGGGTATATCCC				
hKCNE1	F-TGGTACTGGGATTCTTCGGC	–		50	204
	R-AGGAAGGTGTGTGTTGGGTTG				
hKCNE2	F-GCTGAGGCTTGTGTGCAACC	–		50	431
	R-GGATGGTGGCCTTCGATTC				
hKCNE3	F-ACCAATGGAACGGAGACCTG	–	NM_005472	50	238
	R-ACTACGCTTGTCCACTTTGCG				

T_m=melting temperature as given by the supplier; r=rat, h=human. F and R represent the 5′ and 3′ primer, respectively.

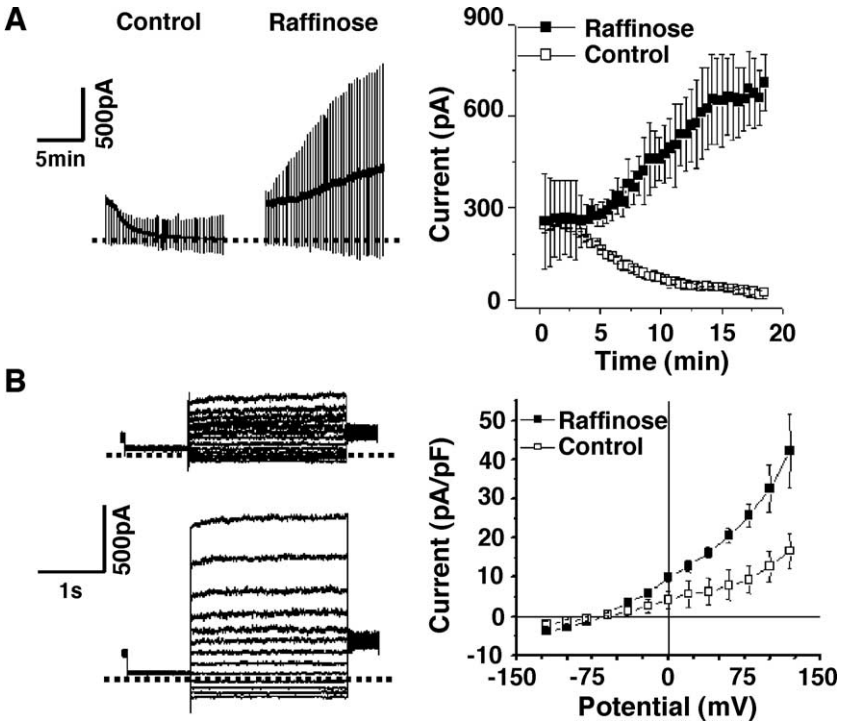

Fig. 1. Swelling-induced currents in rat hepatocytes. (A) Left panel — Representative current traces from two different rat hepatocytes dialyzed with or without 80 mM raffinose during the application of voltage steps (from −80 through 0 to +80 mV). Under the control conditions currents decayed whereas raffinose activated outward currents at 0 and +80 mV. Right panel — Mean currents at 0 mV from cells recorded as in the left panel (±S.E.M., n=5). (B) Left panel — Representative current traces from cells dialyzed as in Panel (A) except that the voltage steps were applied 12 min after whole-cell attainment, in 20 mV increments from −120 to +120 mV following a pre-pulse to −60 mV (holding potential was 0 mV). Right panel — Mean current–voltage relationships from control (±S.E.M., n=6) and raffinose dialyzed cells (±S.E.M., n=6). Hypotonic conditions (raffinose dialysis) increase outward currents. Dotted lines represent zero current level.

slow activation of I_K. Under these two conditions, currents at E_K (−80 mV) were smaller and relatively stable compared with those at E_{Cl} (0 mV). Fig. 1A (right panel) shows that currents decreased from 240±30 to 20±20 pA (n=4) in the control, but increased from 250±150 to 700±90 pA (n=5) in rat hepatocytes dialyzed with raffinose for 20 min. Thus under the experimental conditions used here we were able to isolate I_K from I_{Cl}. To determine the activation kinetics of the resting and swelling-activated currents we applied a series of voltage steps between −120 and +120 mV. Fig. 1B (left panel) shows the records from a control (upper) and a raffinose-dialyzed (lower) hepatocyte. The currents are essentially time independent and, especially in hypotonic solution, weakly outwardly rectifying. Normalized current–voltage relationships derived from voltage steps applied 12 min following whole-cell attainment are plotted in Fig. 1B (right panel). The pooled data confirms the outwardly rectifying nature of the whole-cell currents and that conditions causing cell swelling enhance these currents.

3.2. K^+ selectivity of I_{Kvol}

Since K^+, Cl^- and non-selective cation channels are activated under hypotonic conditions, we changed the extracellular K^+ concentration ($[K^+]_o$) but kept concentrations of gluconate$^-$ and Cl^- constant to determine whether the swelling-activated currents are K^+ selective. Fig. 2A shows that the exposure of a hepatocyte to different concentrations of $[K^+]_o$ changed the amplitude of the current consistent with K^+ being the major charge carrier. Specifically, outward currents at 0 mV were larger when E_K was made more negative by decreasing $[K^+]_o$, and inward currents at −80 mV were larger when E_K was made more positive by increasing $[K^+]_o$. The mean current–voltage relationships under these three conditions were generated from digitized voltage ramp data as detailed in Fig. 1 (Fig. 2B, left panel). When $[K^+]_o$ was increased from 0.5 to 5 and 50 mM, the reversal potential (E_{rev}) shifted in the positive direction. E_{rev} shifted about 30 mV per 10-fold change in $[K^+]_o$ indicating a partial selectivity to K^+ (Fig. 2B, right panel). We determined the contribution of non-selective cation channels to the swelling-activated current by replacing all of the K^+ in the pipette solution with Na^+ and calculating the relative permeabilities of the whole-cell current to K^+ and Na^+ using the Goldman-Hodgkin-Katz equation. In these experiments the cells were bathed in 5 mM K^+. As seen in Fig. 2C, Na^+ could pass through the cation channel and relative permeability (P_{Na}/P_K) was 0.06. The combined results suggest that K^+ channels are the major contributors to the swelling activated cation current in hepatocytes with

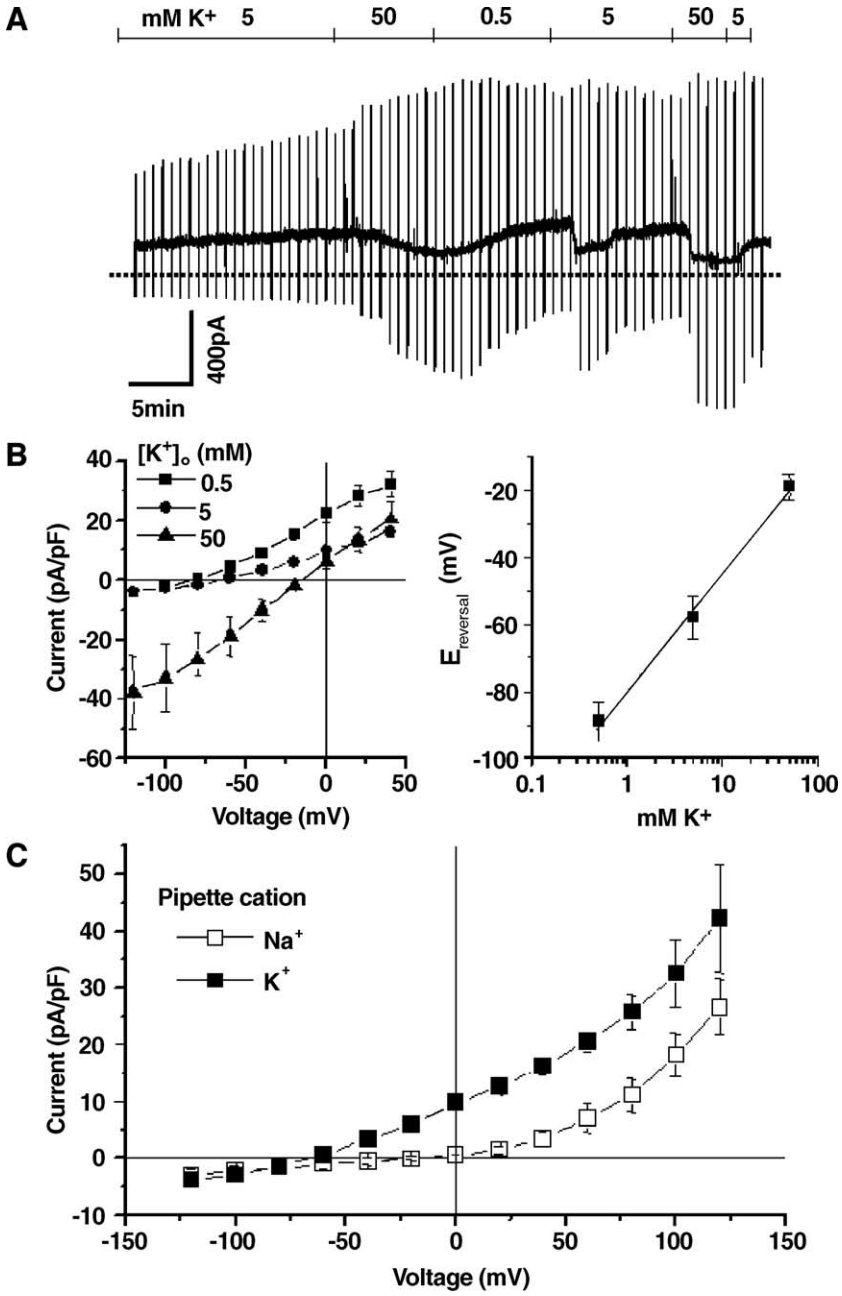

Fig. 2. K^+ selectivity of the hepatocellular swelling-activated current. (A) Current amplitude in a single hepatocyte dialysed with raffinose varied according to the K^+ equilibrium potential. (B) Left panel — Mean (±S.E.M., n=5 for each) current–voltage relationships of swelling-activated current at various $[K^+]_o$. Right panel — The reversal potential of the swelling-induced currents shifted about 30 mV per ten-fold change in $[K^+]_o$. (C) Mean current–voltage relationships from cells dialyzed with 140 mM potassium-gluconate (±S.E.M., n=5) or 140 mM sodium-gluconate (±S.E.M., n=4) in the pipette solution. E_{REV} was −60 and −20 mV, respectively, with potassium-gluconate and sodium-gluconate dialysis solutions.

a smaller component resulting from non-selective cation channels.

3.3. Sensitivity of I_{Kvol} to cytosolic Ca^{2+}

In order to determine whether the rat hepatocyte I_{Kvol} could be modulated by intracellular Ca^{2+}, we dialyzed cells with solutions containing different Ca^{2+} concentrations ($[Ca^{2+}]_i$) and measured outward current at 0 mV. Fig. 3 shows the mean time course for the development of the I_{Kvol} from 5 cells for each $[Ca^{2+}]_i$. When hepatocytes were dialyzed with solutions containing no added Ca^{2+}, I_{Kvol} at 15 min following attainment of the whole-cell configuration (625±155 pA, n=4) was not significantly different from that in cells dialyzed with 13 nM Ca^{2+} (650±150 pA, n=5). Much higher but still physiological $[Ca^{2+}]_i$ (1 μM) significantly inhibited the development of I_{Kvol}. From these data it is evident that the hepatocellular I_{Kvol} is not dependent on increases in $[Ca^{2+}]_i$ above resting levels but rather these currents are suppressed by high $[Ca^{2+}]_i$.

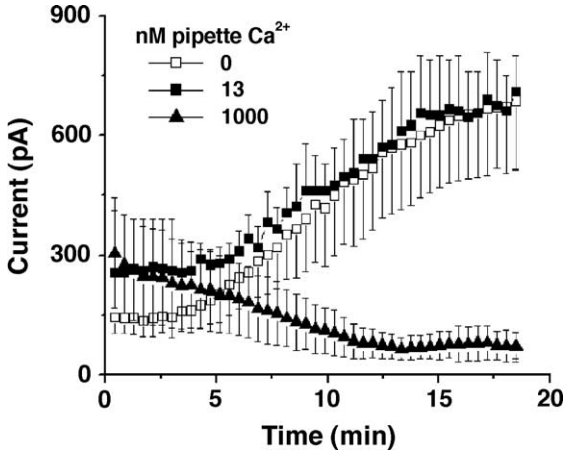

Fig. 3. Sensitivity of the hepatocellular swelling-activated K^+ current to cytosolic Ca^{2+}. Mean time courses of currents at 0 mV in cells dialyzed with solutions containing 80 mM raffinose and either no added Ca^{2+}, 13 nM or 1 μM free Ca^{2+}. There is no significant difference between currents under 0 nM and 13 nM Ca^{2+} whereas 1 μM Ca^{2+} blocked the development of swelling-activated K^+ currents (mean±S.E.M., n=4 cells for each condition).

3.4. Dependence of I_{Kvol} on cytosolic Mg–ATP

Studies of epithelial cell lines suggest a role for cytosolic ATP as a signal that controls K^+ channels [27]. To examine whether cytosolic ATP regulates I_{Kvol} in rat hepatocytes, we determined whether the removal of ATP from the pipette solution affected the currents. The development of I_{Kvol} was evaluated in the presence of Mg^{2+} (1 mM) and raffinose (80 mM), in the absence or presence of 4 mM ATP in the pipette solution. Fig. 4 shows that I_{Kvol} developed only in the presence of ATP. Under the conditions used here, the Mg^{2+} concentration decreased from approximately 4 mM in the absence of ATP to 1 mM when combined with 4 mM ATP suggesting that either Mg–ATP is required for activation or millimolar Mg^{2+} inhibits the development of the hepatocellular I_{Kvol}.

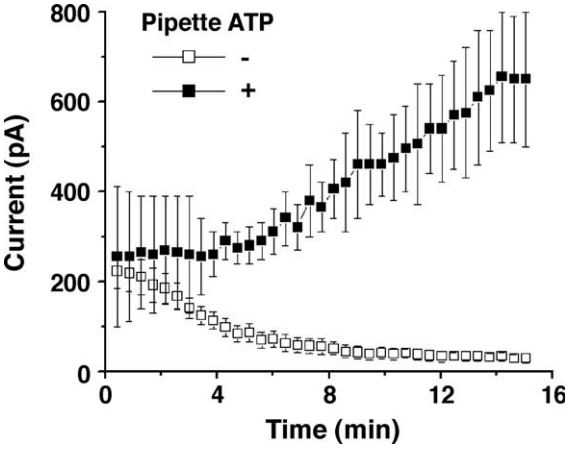

Fig. 4. Effect of cytosolic ATP on the hepatocellular swelling-activated K^+ current. Mean time courses of currents at 0 mV in cells dialyzed with 80 mM raffinose and 1 mM Mg^{2+} in the absence (±S.E.M., n=4) or presence of 4 mM ATP (±S.E.M., n=5). Currents decayed in the absence of ATP.

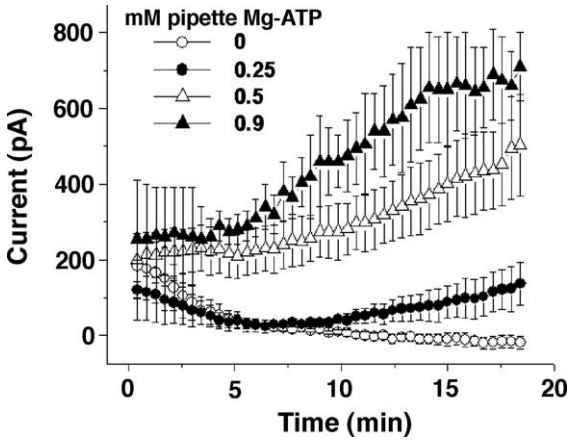

Fig. 5. Effect of cytosolic Mg–ATP on the hepatocellular swelling-activated K^+ current. Mean time courses of currents at 0 mV in cells dialyzed with 80 mM raffinose and 1 mM Mg^{2+} in the absence or presence of varying concentrations of ATP to give the indicated Mg–ATP concentration; ±S.E.M., n=4 cells for each condition. Mg–ATP stimulated development of the swelling-activated current.

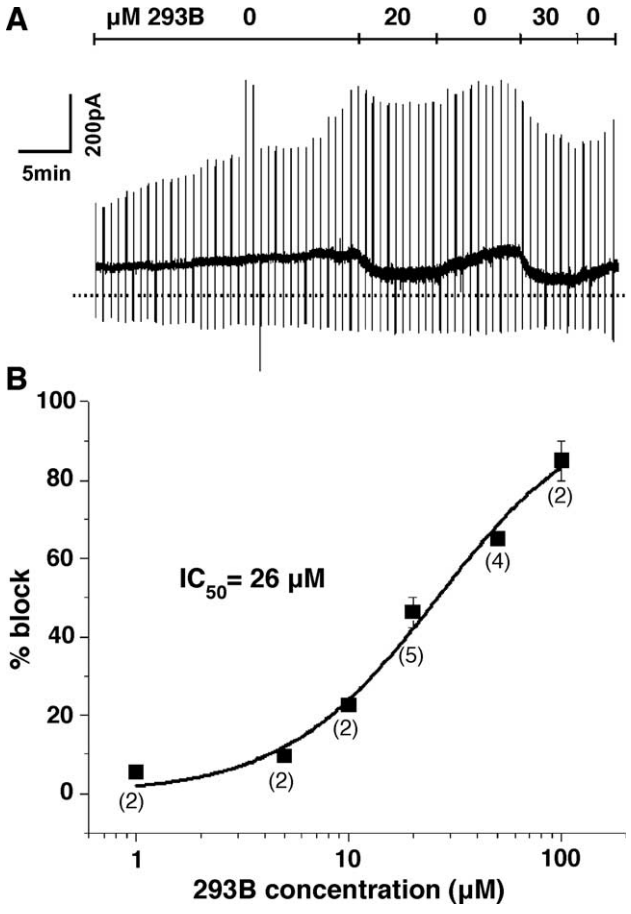

Fig. 6. Effect of chromanol 293B on the hepatocellular swelling-activated K^+ current. (A) Representative current trace from a rat hepatocyte dialyzed with 80 mM raffinose and perfused with 0, 20 or 30 μM chromanol 293B as indicated during the application of voltage steps (from −80 through 0 to +80mV). Exposure to chromanol 293B reversibly inhibited outward currents at 0 and +80 mV. (B) The concentration–response relationship for chromanol 293B inhibition of I_{Kvol}; mean±S.E.M., n=number of cells for each condition.

To determine whether I_{Kvol} is dependent on ATP alone or the Mg–ATP complex, we first examined the effect of removing Mg^{2+} from the pipette solution containing 4 mM ATP. As shown in Fig. 5, I_{Kvol} did not activate in the absence of Mg^{2+} in the pipette solution (○) indicating that the presence of the Mg–ATP complex was required for current activation. To examine the concentration dependence of I_{Kvol} activation on Mg–ATP we added increasing amounts of ATP to pipette solutions containing 1 mM Mg^{2+}. Increasing the Mg–ATP from 0.25 through 0.5 to 0.9 mM activated I_{Kvol} faster and to a larger extent. At 15 min following the attainment of the whole-cell configuration the mean currents at 0 mV increased from 90±40 pA (n=4) through 400±100 pA (n=4) to 650±150 pA (n=5) when the cells were dialyzed with 0.25, 0.5 and 0.9 mM Mg–ATP respectively. These results indicate that the hepatocellular I_{Kvol} is dependent on Mg–ATP.

3.5. Sensitivity of I_{Kvol} to chromanol 293B

Since I_{Kvol} requires Mg–ATP, it is possible that this activation is mediated by a kinase-dependent phosphorylation. The voltage-gated K^+ channel KCNQ1 is an appropriate candidate for the hepatocellular I_{Kvol} since it is sensitive to tonicity [13,14,28], has both protein kinase A (PKA) and protein kinase C (PKC) phosphorylation sites and is activated by PKA [29]. We used chromanol 293B (293B), a KCNQ1 channel blocker [30,31], to pharmacologically identify whether KCNQ1 could mediate some of the hepatocellular I_{Kvol}. Fig. 6A is a recording from a hepatocyte perfused with 20 μM and 30 μM chromanol 293B with washout periods between each. Both of these concentrations reversibly depressed currents at 0 mV, in other words, I_K. The concentration–response relationship for chromanol 293B is plotted in Fig. 6B. The percentage block of I_{Kvol}, calculated as $(I_0-I_{293B}/I_0)\times 100$ in which I_0 is the current at 0 mV prior to 293B exposure and I_{293B} is the current at 0 mV after 5 min exposed to 293B, is plotted as a function of blocker concentration. The mean data were reasonably well fitted with the Hill equation, $I=I_{max}/(1+(IC_{50}/[293B])^{nH})$, where I_{max} is the maximum degree of block expressed as a percentage, IC_{50} is the concentration of 293B ([293B]) causing half-maximal block, and nH is the Hill coefficient. Fig. 6B shows that the value for IC_{50} is 26±1.2 μM.

3.6. KCNQ/KCNE expression in the rat hepatocyte and human liver

KCNQ1 has been identified in some gastrointestinal epithelia cells [30–32]. We measured, using RT-PCR, KCNQ and KCNE family expression in mRNA from

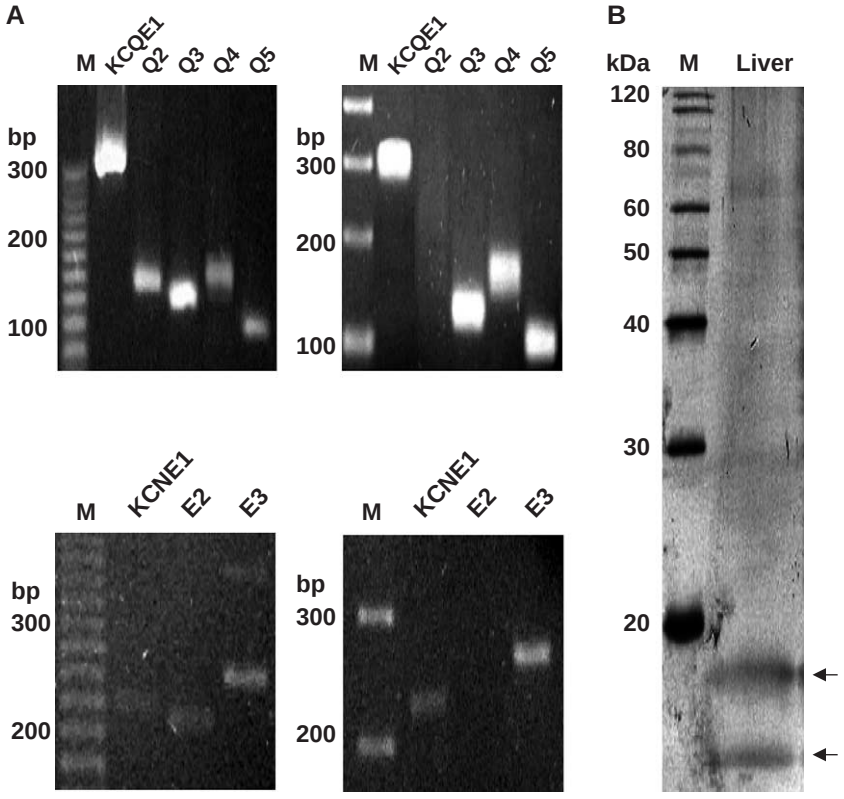

Fig. 7. Expression of KCNQ/KCNE in rat hepatocytes and human liver. (A) PCR of cDNA synthesized from rat hepatocyte (left panels) or human liver (right panels) mRNA using oligonucleotide primers specific for each of the indicated KCNQ (upper panels) and KCNE (lower panels) subfamily subunits; M=DNA mass markers. (B) Western blot analysis of rat liver plasma membrane fraction probed with affinity-purified antibody against KCNE3. 50 μg protein was dialyzed and loaded on SDS–10% polyacrylamide; M=protein mass markers.

freshly isolated rat hepatocytes and from commercially prepared human liver cDNA (Fig. 7A). The amplification products of the expected lengths (see Table 1) were detected using rat hepatocyte or human liver cDNA as template. The cloned fragments were identical to the sequences from rat brain (GenBank Human KCNQ1 accession # HSU89364). Whereas KCNQ1 through KCNQ5 were expressed in the rat hepatocytes (left panel), all but KCNQ2 were found in human liver (right panel). No fragments were amplified with the rat template without reverse transcriptase treatment.

KCNQ subunits generally associate with a member of the KCNE gene family to form a functional channel complex. The five-member KCNE family are single transmembrane domain proteins that can significantly affect the gating of KCNQ1 [33]. Since KCNE proteins have an influential role to play in KCNQ function, we studied the expression of three members of the KCNE gene family in rat hepatocytes and human liver (Fig. 7A, lower panels). We did not look at KCNE4 and KCNE5 because their effects on KCNQ1 in heterologous expression systems indicate that they are not suitable candidates for the hepatocellular I_{Kvol}. Specifically, although KCNE4 transcript has been detected in human liver [34], it slows or completely inhibits KCNQ1 activation [34,35]. KCNE5 also decreases the activation rate, in addition to shifting the threshold of activation of KCNQ1 by +140 mV [36]. Oligonucleotide primers for KCNE1, KCNE2 and KCNE3 resulted in amplified products of the expected size of KCNE1, KCNE2 and KCNE3 in the rat hepatocytes (left panel), and KCNE1 and KCNE3 but not KCNE2 in the human liver (right panel). The nucleotide sequences of these amplified DNA fragments were confirmed by sequencing.

We also performed Western blots of rat hepatocyte plasma membranes with a focus on KCNE3 since this subunit renders KCNQ1 currents time independent and weakly rectifying [31]. KCNE2 has a similar effect on KCNQ1 currents [37] but because it was not expressed in human liver (Fig. 7A), and it imparts a much higher sensitivity of KCNQ1 to 293B [38] than we observed here (Fig. 6B), we did not look for KCNE2 protein by immunoblot. Fig. 7B shows two distinct bands with a molecular weight of 12 and 18 kDa. The 12 kDa band corresponds with the theoretical mass calculated for KCNE3. The 18 kDa band could represent a glycosylated form of KCNE3 as was noted for KCNE1 in equine and guinea pig heart in which this small protein appears as three bands between 18 and 25 kDa [39].

4. Discussion

Volume regulation in the face of the changing nutrient-containing environment of the portal vein is a vital function of hepatocytes. Although it is well established that RVD involves K^+ efflux from hepatocytes and other cells, the molecular identity of the channels involved has not been clearly demonstrated. Our results show that short-term cultured rat hepatocytes possess K^+ channels that are activated as the cells swell. This conclusion is based on two groups of observations. First, the dialysis of hepatocytes with 80 mM raffinose caused the development of significant outward current at E_{Cl} (0 mV) whereas currents at E_K (−80 mV) were not affected. Second, the E_{rev} of the swelling-activated current was close to E_K and shifted in the expected direction when extracellular K^+ concentration was altered. Further characterization of this swelling-activated K^+ current, I_{Kvol}, indicated that a significant fraction is mediated by KCNQ1, probably with its accessory protein KCNE3.

4.1. I_{Kvol} and cytosolic Ca^{2+}

I_{Kvol} in many cell types appears to require increases in cytosolic Ca^{2+} [40]. However, there are examples of I_{Kvol} that are not activated by Ca^{2+}, including KCNQ1 [41]. How an increase in volume mediates channel stimulation is not well understood although it is thought that signal pathways are involved in the liver [3]. Hepatocytes from different species express different arrays of K^+ channels, particularly Ca^{2+}-sensitive K^+ channels (IK_{Ca}). The SK2 and SK3 channels found in the mammalian hepatocyte and hepatoma as well as in other epithelial cells, sense cell regulatory volume decreases [16,17]. Our studies demonstrate that I_{Kvol} still developed in the absence of any added Ca^{2+} in the rat hepatocytes. This suggests that I_{Kvol} are Ca^{2+} independent in these cells, in keeping with earlier observations [42]. IK_{Ca}, such as SK2 and SK3 activity, attain their maximal activity at about 1 μM intracellular Ca^{2+} [17,21]. The present study shows that I_{Kvol} was blocked at this Ca^{2+} concentration in the rat hepatocyte. Furthermore, we have identified BK channels in embryonic chick hepatocytes [43] but we have never observed similar channels in rat hepatocytes. Therefore, I_{Kvol} in the rat hepatocytes do not seem to be SK2, SK3 or BK.

4.2. I_{Kvol} and cytosolic Mg–ATP

Our results show that hepatocellular I_{Kvol} is dependent on Mg–ATP. The requirement of Mg–ATP for the activation of I_{Kvol} raises the possibility that this channel may be regulated physiologically by phosphorylation. A plausible mechanism underlying the Mg–ATP dependence of I_{Kvol} could be lipid phosphorylation. In the plasma membrane phosphatidylinositol-4,5-bisphosphate (PIP_2) is produced by the consecutive phosphorylation of phosphatidylinositol (PI) by PI kinase and phosphatidylinositol monophosphate (PIP) by PIP kinase. PIP_2 can regulate several ion channels and transporters in the presence of Mg–ATP [27]. In particular, PIP_2 has been reported to stabilize whole-cell or excised patch currents and modulate the voltage dependency of KCNQ1 [44]. In rat hepatocytes, phosphoinositide 3-kinase is activated by cell swelling [45]. Therefore, PIP_2

synthesis might be the event underlying Mg–ATP requirements of the hepatocellular I_{Kvol}.

4.3. KCNQ/KCNE as candidates for the hepatocellular I_{Kvol}

KCNQ1 (with KCNE2 or KCNE3) appears to be responsible for I_{Kvol} in the rat hepatocyte since both currents are weakly outwardly rectifying, insensitive to cytosolic Ca^{2+}, inhibited by chromanol 293B and stimulated by hypotonic solutions [13,14,28,31,41,46]. Although we show that KCNQ2 through KCNQ5 are also expressed in the rat hepatocyte (and KCNQ3 through KCNQ5 in human liver), all of these channels form voltage-gated K^+ currents [33], making them poor candidates for the hepatocellular I_{Kvol}. KCNQ1 also forms voltage-gated channels as a homotetramer, but becomes essentially time-independent and voltage-insensitive when expressed as a heterotetramer with KCNE2 and KCNE3, but not KCNE1 [31,37,47,48]. Since we found KCNE1, KCNE2 and KCNE3 transcripts in rat hepatocytes and KCNE1 and KCNE3 in human liver, I_{Kvol} in these cells may result from a heteromeric association between KCNQ1 and KCNE3 in the human, or KCNQ1 and either KCNE2 or KCNE3, in the rat. Based on the different sensitivity of KCNQ1 to chromanol 293B, which is ten times higher (4.3 vs. 0.4 μM) when coexpressed with KCNE2 as compared with KCNE3 in mammalian cells [38], we would predict that KCNE3 is the major β-subunit supporting KCNQ1 function in hepatocellular volume regulation. Conclusive identification of the KCNQ1 partner in the rat hepatocyte awaits co-localization or co-immunoprecipitation studies.

There is conflicting evidence whether KCNQ sensitivity to cell volume changes may be modified by association with KCNE subunits. When co-expressed with KCNQ1 in *Xenopus* oocytes, KCNE1 inhibits whereas KCNE2 or KCNE3 does not effect the swelling-induced K^+ current [13]. Conversely, proximal convoluted tubule epithelial cells from KCNE1 knock-out mice do not respond to cell swelling in the same way as do cells from wild-type animals [49]. In addition to KCNQ1, KCNQ4 is also sensitive to cell volume changes, although again, this response seems to be dependent on the expression system [13,50]. As stated earlier, the activation kinetics and voltage-sensitivity of KCNQ4 exclude this channel as a major contributor to hepatocellular I_{Kvol}.

4.4. Functional implications of a hepatocellular KCNQ/KCNE current

KCNQ1 in combination with either KCNE2 or KCNE3 is thought to conduct the basolateral K^+ current that hyperpolarizes secretory epithelial cells thereby increasing apical Cl^- secretion [31,51–53]. In the apical membrane of gastric cells KCNQ1, as a multimer with either KCNE2 or KCNE3, is proposed to be involved in acid secretion [32,54]. KCNE1 is not required for KCNQ1 to control ion secretion in the small and large intestine, the exocrine pancreas [30] and parietal cells [32]. We suggest that KCNQ1/KCNE2 and KCNQ1/KCNE3 are suitable candidates to regulate ion fluxes in response to cell swelling in rat hepatocytes, whereas KCNQ1/KCNE3 is the likely candidate in human hepatocytes since KCNE2 is not expressed in human liver.

In summary, we have presented functional evidence for an I_{Kvol} in rat hepatocytes that is Mg–ATP dependent and insensitive to cytosolic Ca^{2+}. Pharmacological and molecular evidence supports the view that KCNQ1, probably with KCNE2 or KCNE3 as its accessory subunit, underlies this current in the rat hepatocyte and potentially human hepatocytes. We speculate that K^+ efflux through sinusoidal KCNQ1 heteromeric channels could mediate recovery from cell volume increases in response to nutrient, bile acid and xenobiotic uptake in the liver.

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