K\textsubscript{ATP} Channels Regulate Mitogenically Induced Proliferation in Primary Rat Hepatocytes and Human Liver Cell Lines

IMPLICATIONS FOR LIVER GROWTH CONTROL AND POTENTIAL THERAPEUTIC TARGETING*

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To determine whether K\textsubscript{ATP} channels control liver growth, we used primary rat hepatocytes and several human cancer cell lines for assays. K\textsubscript{ATP} channel openers (minoxidil, cromakalim, and pinacidil) increased cellular DNA synthesis, whereas K\textsubscript{ATP} channel blockers (quinidine and glibenclamide) attenuated DNA synthesis. The channel inhibitor glibenclamide decreased the clonogenicity of HepG2 cells without inducing cytotoxicity or apoptosis. To demonstrate the specificity of drugs for K\textsuperscript{+} channels, whole-cell patch-clamp recordings were made. Hepatocytes revealed K\textsuperscript{+} currents with K\textsubscript{ATP} channel properties. These K\textsuperscript{+} currents were augmented by minoxidil and pinacidil and attenuated by glibenclamide as well as tetraethylammonium, in agreement with established responses of K\textsubscript{ATP} channels. Reverse transcription of total cellular RNA followed by polymerase chain reaction showed expression of K\textsubscript{ATP} channel-specific subunits in rat hepatocytes and human liver cell lines. Calcium fluxes were unaltered in glibenclamide-treated HepG2 cells and primary rat hepatocytes following induction with ATP and hepatocyte growth factor, respectively, suggesting that the effect of K\textsubscript{ATP} channel activity upon hepatocyte proliferation was not simply due to indirect modulation of intracellular calcium. The regulation of mitogen-related rat hepatocyte proliferation by K\textsubscript{ATP} channels advances our insights into liver growth control. The findings have implications in mechanisms concerning liver development, regeneration, and oncogenesis.

Potassium channels are ubiquitous in eukaryotic cells and play roles in resting membrane potential, frequency of action potential, and membrane potential repolarization rates (1). Membrane currents through ATP-sensitive K\textsuperscript{+} channels, K\textsubscript{ATP}, have been recorded in diverse cell types, including pancreatic \( \beta \) cells; skeletal, cardiac, and vascular myocytes; neurons; and renal epithelial cells (2–7). The K\textsubscript{ATP} channel is a heteromultimer composed of at least two types of subunits, an inwardly rectifying K\textsuperscript{+} channel (Kir6.x) and a sulfonylurea receptor (SUR), that belongs to the ATP-binding cassette superfamily (8). Kir6.x subunits form the pore, and SUR subunits impart regulatory activity (9). The K\textsubscript{ATP} channels are inhibited by sulfonylureas, e.g. glibenclamide and tolbutamide, and activated by diverse substances designated as potassium channel openers (10, 11), which include benzopyrans (cromakalim, levcromakalim, and pinacidil), cyanoguanidines, thiogormamides, pyrimidines (minoxidil), and benzoathiazidines (diizoxide). The activity of K\textsubscript{ATP} channels is dependent upon the energy state of the cell. Various subunits (Kir6.1, Kir6.2, SUR1, and SUR2) form channel subtypes with differences in expression among different organs (10). Kir6.2 and SUR1 form the characteristic K\textsuperscript{+} channel in pancreatic \( \beta \) cells, where an increase in intracellular ATP levels, such as following increased glucose availability, causes channel closure, plasma membrane depolarization, and activation of voltage-sensitive calcium channels, which finally triggers insulin release (10, 12).

It is noteworthy that potassium channels regulate the growth and proliferation of many cell types. For example, receptor-coupled, voltage-sensitive, and calcium-sensitive K\textsuperscript{+} channels regulate proliferation in lymphocytes, adipocytes, and epithelial cells derived from the skin, breast, bladder, and stomach (13–15). This regulation seems to occur at the level of cellular DNA synthesis because inhibition of K\textsuperscript{+} channel activity synchronizes cells in G\textsubscript{0}/G1 (16). Also, in fibroblasts, the Ca\textsuperscript{2\textsuperscript{+}}-sensitive potassium channel has been found to regulate intracellular signal transduction events that concern mitogenic activity (17).

Information concerning the role of K\textsuperscript{+} channels in hepatocytes has been relatively limited. Early studies with primary rat hepatocytes by Sawanobori et al. (18) showed a wide range of resting membrane potentials, along with non-rectifying linear current-voltage relationships and no effect on these currents after norepinephrine or Ca\textsuperscript{2\textsuperscript{+}} administration. In guinea pig hepatocytes, Capiod and Ogden (19) have established the presence of Ca\textsuperscript{2\textsuperscript{+}}-activated, delayed-rectifier K\textsuperscript{+} currents. Similarly, Ca\textsuperscript{2\textsuperscript{+}}-sensitive and cAMP-dependent K\textsuperscript{+} channel currents were reported in HTC rat hepatoma cells by Lidofsky (20). Embryonic chick hepatocytes have been shown to possess large conductance voltage-gated and Ca\textsuperscript{2\textsuperscript{+}}-activated channels (21). Henderson et al. (22) found inwardly rectifying K\textsuperscript{+} channels in primary rat hepatocytes that were unperturbed by voltage or Ca\textsuperscript{2\textsuperscript{+}} stimulation. K\textsuperscript{+} channel activity was also found to regulate additional processes in hepatocytes, such as cell volume (23).

Our own interest in hepatic K\textsuperscript{+} channels concerned potential relationships between K\textsuperscript{+} channel activity and liver growth.
control, which involves growth factor-mediated activation of intracellular signaling cascades and other cellular events (24). The general hypothesis was that modulation of K+ channel activity would affect hepatic DNA synthesis. We studied primary rat hepatocytes as well as several established human cancer cell lines to analyze the effects of KATP channel regulation upon cell proliferation. The results showed that KATP channels play significant roles in regulating hepatocyte proliferation.

**EXPERIMENTAL PROCEDURES**

**Drugs—**Glibenclamide, pinacidil, and minoxidil were from RBI (Natick, MA). Cromakalim, quinidine, and ATOP sodium salt were from Sigma. To obtain stock solutions, glibenclamide and pinacidil were dissolved in dimethyl sulfoxide (10 mM stocks; Sigma); quinidine was dissolved in ethanol (20 mM stock); minoxidil and cromakalim were dissolved in RPMI 1640 medium (1 mM and 1 mM stocks, respectively; Life Technologies, Inc.); and ATP was dissolved in phosphate-buffered saline, pH 7.4 (10 mM stock). Recombinant human hepatocyte growth factor (hHGF)1 was from Genentech Inc. (South San Francisco, CA).

Tetraethyiammonium (Sigma) was dissolved in water (1 mM stock).

Cells—Primary hepatocytes were isolated from F344 rats with a two-step perfusion using 0.03% collagenase as described previously (25). Primary hepatocytes were pelleted in 45% PercollTM in 100% ethanol for 10 min, followed by staining with 4% crystal violet in 1% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; RT-PCR, reverse transcription polymerase chain reaction; Pf, picorafads, bp, base pairs.

**Analysis of Calcium Responses in Cells—**Changes in intracellular calcium levels were demonstrated in HepG2 cells and primary rat hepatocytes plated on glass-bottomed microwells (MatTek Corp., Ashland, MA). Cells were loaded with a 15 μM concentration of the intracellular calcium indicator Fura-2/AM (Molecular Probes, Inc., Eugene, OR) at 37 °C for 45 min. Loaded cells were rinsed with Tyrode’s solution (157 mM NaCl, 2.7 mM KCl, 1.1 mM CaCl2, 1.8 mM NaHCO3, 0.45 mM NaH2PO4, 5.5 mM glucose, and 5 mM HEPES, pH 7.0–7.2) and examined under an epifluorescence microscope. The ratio of Fura-2 fluorescence emitted at 340- and 380-nm excitation wavelengths was obtained using a combined system of optic filter wheel (Sutter Instrument Co., Burlingame, CA) and shutter (Uniblitz, Rochester, NY) driven by an OEI computer (Universal Imaging Corp., West Chester, PA). The images were acquired with an intensified CCD camera and analyzed with Metafluor imaging system software (Universal Imaging Corp.). Fura-2 fluorescence ratio images were continuously acquired at a rate of 0.3 Hz before and after inducing Ca2+ fluxes with ATOP to HepG2 cells at an effective concentration of 50 μM. To establish conditions for demonstrating Ca2+ fluxes in primary rat hepatocytes, we performed pilot studies with hHGF. Rat primary hepatocytes were cultured in 100-mm culture dishes. The drugs were added to cultured cells. Primary rat hepatocytes were cultured with and without 10 ng/ml hHGF and vehicle alone was added to cultured cells. Primary hepatocytes were cultured in RPMI 1640 medium containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA). HepG2, HuH-7, and HFL cells are cancerous liver epithelial cells derived from the human liver (26–28). These cells were cultured in Dulbecco’s minimal essential medium (Life Technologies, Inc.) with antibiotics and fetal bovine serum. Cell morphology was observed under phase-contrast microscopy. All experiments were in triplicate at least and repeated many times.

**Cell Viability Assays—**To determine changes in cell viability, utilization of thiazolyl blue (MTT; Sigma) was measured. Cells were plated at a density of 5 × 103/cm2 in 24-well plates. The medium was changed every 48 h. Cells were trypsinized and counted in a modified Neubauer hemocytometer at 1, 3, and 5 days after culture.

To determine DNA synthesis rates, primary hepatocytes were plated at a density of 2 × 104/cm2, and HepG2, HuH-7, and HFL cells were plated at 1 × 104/cm2. Two hours later, drugs affecting potassium channel activity or vehicle alone was added to cultured cells. Primary hepatocytes were isolated from F344 rats with a two-step perfusion using 0.03% collagenase as described previously (25). Primary hepatocytes were pelleted in 45% PercollTM in 100% ethanol for 10 min, followed by staining with 4% crystal violet in 1% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; RT-PCR, reverse transcription polymerase chain reaction; Pf, picorafads, bp, base pairs.

**Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis—**Total RNA was extracted from cells with the guanidium thiocyanate method using Trizol reagent (Life Technologies, Inc.). A commercial kit (Access RT-PCR System; Persema, Madison, WI) was used for one-step reverse transcription-PCR for amplifying rat Kir6.1, Kir6.2, Kir6.3, SUR1, and SUR2 as well as β-actin to serve as an internal control for semi-quantitative comparisons with previously described primers (6). In addition, primers were synthesized in our DNA synthesis facility to amplify human Kir6.1, Kir6.2, SUR1, and SUR2. The effective primers for human sequences were as follows: Kir6.2, 5'-AGC CCA AGT TCA
RESULTS

The general experimental design concerned analysis of proliferation in cultured hepatocytes after the addition of drugs known either to open K\textsubscript{ATP} channels (minoxidil, cromakalim, and pinacidil) or to inhibit K\textsubscript{ATP} channel opening (quinidine and glibenclamide). Since primary rat hepatocytes exhibit limited DNA synthesis in culture, we induced DNA synthesis in these cells by exposure to 10 ng/ml hHGF for 48 h.

K\textsubscript{ATP} Channel Activity and Proliferation of Cultured Hepatocytes—Initial experiments were undertaken to analyze the effects of DNA synthesis in primary rat hepatocytes. Minoxidil, cromakalim, and pinacidil increased DNA synthesis in hHGF-stimulated rat hepatocytes by severalfold (up to ~20-fold; p < 0.05) (Fig. 1A). Stimulation of rat hepatocytes with hHGF itself increased DNA synthesis by 4–10-fold compared with untreated control cells (p < 0.05). Interestingly, DNA synthesis was not increased in rat hepatocytes treated with K\textsuperscript{+} channel openers in the absence of hHGF. The optimal stimulatory drug concentrations were as follows: minoxidil, 500 nM (range tested, 100–1000 nM); cromakalim, 5 \mu M (range tested, 5–25 \mu M); and pinacidil, 100 \mu M (range tested, 5–200 \mu M). In contrast, culture of rat hepatocytes with tetraethylammonium, quinidine, or glibenclamide significantly inhibited hHGF-induced DNA synthesis. More extensive analysis with quinidine (12.5–250 \mu M) and glibenclamide (50–200 \mu M) showed that hHGF-induced DNA synthesis was significantly inhibited. Use of these drugs suppressed DNA synthesis induced by the combination of hHGF and K\textsuperscript{+} channel openers. In a typical experiment, 10 ng/ml hHGF increased DNA synthesis by 15 ± 3-fold and hHGF plus 500 nM minoxidil by 32 ± 6-fold above untreated control cells, whereas in response to 12.5 or 25 \mu M quinidine, DNA synthesis induced by hHGF and minoxidil decreased to 17 ± 1-fold and decreased further to 7 ± 1-fold above untreated control cells with 50 \mu M quinidine (p < 0.05, analysis of variance). Treatment of rat hepatocytes with glibenclamide showed efficient suppression of hHGF-mediated DNA synthesis (Fig. 1B).

To exclude whether inhibition of DNA synthesis could have been due to drug toxicity, utilization of MTT by cells was analyzed. It was noteworthy that neither quinidine nor glibenclamide was cytotoxic to our primary hepatocytes or HepG2, HuH-7, and HFL cell lines across the range of drug concentrations tested. However, changes in the morphology of primary hepatocytes were observed following treatment with glibenclamide (Fig. 2). The most characteristic feature was that cells appeared more rounded in culture dishes. When DNA fragmentation was analyzed by staining HepG2 cells or primary rat hepatocytes with the DNA-binding Hoechst 33322 dye, there was no evidence of increased apoptosis (Fig. 2, D and F). TUNEL staining showed the presence of some apoptosis in untreated control primary rat hepatocytes at 48 h of cell culture (~10%), which was in agreement with the generally limited survival of primary hepatocytes under these conditions. In contrast, only occasional apoptotic nuclei were detected in hepatocytes following culture with hHGF. TUNEL positivity in glibenclamide-treated hepatocytes did not increase, and apoptosis rates remained similar to those in untreated control cells or hHGF-treated cells (Fig. 2, G and H). In control liver tissue from rats subjected to ischemia-reperfusion injury, TUNEL staining showed extensive apoptosis.

To determine whether inhibition of DNA synthesis was associated with sustained impairment in cell replication, we cultured HepG2 cells with 100 \mu M glibenclamide and measured cell numbers in culture dishes for up to 5 days. The number of untreated control cells increased by 468 ± 94% between 1 and
5 days of culture. In contrast, the number of glibenclamide-treated cells increased by 350\% during this period (p, 0.05, analysis of variance). Additional studies were undertaken to demonstrate whether clonogenic capacity of HepG2 cells was altered by glibenclamide treatment. In our colony-forming assay, at 18 days of culture, 1735 colonies formed in dishes containing control cells, which were treated by vehicle alone, whereas 934 colonies (56\%) formed in dishes containing 100 \(\mu\)M glibenclamide (p < 0.001, t test). This suggested that inhibition of \(K^+\) channel activity affected DNA synthesis and cell proliferation, including perturbation of clonogenic capacity.

**Molecular Expression of \(K_{\text{ATP}}\) Channels in Hepatocytes**—The intact rat liver contained Kir6.1 and SUR1 mRNAs as well as SUR2 mRNA, although the abundances of Kir6.1 and SUR2 were greater in the rat heart compared with the rat liver (Fig. 3A). The expression of Kir6.2 mRNA in the intact rat liver was uncertain in our hands; and therefore, analysis of Kir6.2 expression in rat hepatocytes was not pursued further. When primary rat hepatocytes were cultured for 48 h, it was still possible to detect Kir6.1, SUR1, and SUR2 mRNAs, although SUR2 mRNA was detected only at low levels (Fig. 3B). Neither hHGF nor glibenclamide altered the overall abundance of Kir6.1 and SUR2 mRNA in primary rat hepatocytes. In contrast, SUR1 mRNA expression was perturbed. In rat hepatocytes treated with glibenclamide alone compared with no treatment, the relative SUR1 mRNA expression, after normalization to \(\beta\)-actin expression, was consistently decreased (Fig. 3B). Densitometric scanning showed that this decrease ranged from 0.25 to 0.5 in three independent experiments performed.
of these three experiments, the addition of hHGF prevented glibenclamide-induced decreases in SUR1 mRNA expression. These data suggested that glibenclamide inhibited SUR1 expression, whereas hHGF could potentially prevent glibenclamide-induced inhibition of SUR1 expression. Our findings concerning Kir6.1 and SUR expression in primary rat hepatocytes were in agreement with the observations of Kir6.1 protein expression by Suzuki et al. (32), who studied plasma and mitochondrial membranes from primary rat hepatocytes with immunofluorescence. Also, Inoue et al. (33) showed binding of radiolabeled glibenclamide to hepatocyte membranes, which is in agreement with the presence of SUR in hepatocytes. Further verification of our findings concerning modulation of SUR expression will be facilitated by the availability of reliable antibodies, e.g. Western blot analysis, but this is lacking at present.

In HepG2, HuH-7, and HFL cells, we were able to detect Kir6.2 as well as SUR2 mRNAs by RT-PCR. We were unable to obtain PCR products for Kir6.1 and SUR1 in our cell lines. There was no change in Kir6.2 and SUR2 mRNA expression after treatment with glibenclamide alone, which was similar to primary rat hepatocytes.

Electrophysiological Studies of K^+ Channel Activity—To determine the functional expression of K_{ATP} channels in hepatocytes, we performed whole-cell current recordings with the patch-clamp technique. ATP, an inhibitor of K_{ATP} channel activity, was excluded from the internal pipette solution, and 5 mM UDP, a channel activator, was included. In primary hepatocytes cultured for 24 h, the reversal potential shifted toward the equilibrium potential for K^+ upon the addition of minoxidil (n = 6) and pinacidil (n = 3) (Fig. 4A and Table I). The current-voltage plot in Fig. 4A shows a minoxidil-activated current with some inward rectification in isolated hepatocytes. This current was inhibited by glibenclamide (Table I), which was in agreement with K_{ATP} channel activity. The subtraction of current recorded in the presence of glibenclamide from K^+ channel openers yielded an inward current of
16 ± 19 pA/pF and an outward current of 48 ± 70 pA/pF with a reversal potential of −64 ± 2 mV in primary rat hepatocytes.

In HepG2 cells, we recorded a pinacidil-activated, outwardly rectifying current that reversed at −55 ± 16 mV (n = 8) (Fig. 4B). The reversal potential in these cells shifted toward the equilibrium potential for K+ from −48 ± 26 to −55 ± 16 mV upon the addition of pinacidil (n = 3). This current was partially inhibited by both glibenclamide and tetrathylenammonium. K+ channel openers and antagonists affected these currents at drug concentrations that also altered cellular DNA synthesis.

**Analysis of Intracellular Calcium following Stimulation of Glibenclamide-treated HepG2 Cells with ATP**—These studies showed that mean basal intracellular calcium levels in HepG2 cells were 141 ± 12 ng/ml hHGF, intracellular Ca2+ levels increased significantly (by 4-fold) (Fig. 5). The intracellular calcium levels increased rapidly, followed by a sustained plateau during observations lasting ~90 s. When cells were treated with 100 µM glibenclamide (n = 50), mean resting calcium levels were 155 ± 2 ng/ml (p = 0.98), similar to untreated control cells (Table I). Cells treated with glibenclamide immediately before analysis responded to ATP stimulation with a 3-± 1-fold increase in intracellular calcium. Similarly, HepG2 cells cultured with 100 µM glibenclamide for 24 h prior to analysis showed basal intracellular calcium levels as well as responses to ATP stimulation that were not significantly different from those of untreated control cells (Fig. 5 and Table I). There was a delay in the response of cells to ATP stimulation, nor was a difference elicited in the magnitude of fluxes. It was noteworthy that intracellular Ca2+ fluxes were not perturbed calcium fluxes.

In primary hepatocytes for <3 h, hHGF stimulation was effective in activating Ca2+ fluxes. On the other hand, when primary hepatocytes had been in culture for 24 h, hHGF stimulation (up to 600 ng/ml) was completely ineffective. Therefore, we restricted our analysis to primary hepatocytes that were cultured for <3 h, which was adequate for cell attachment to dishes. In untreated primary hepatocytes, mean basal intracellular calcium levels were 292 ± 14 ng/ml (n = 20). When hHGF was added to the hepatocytes, most cells (60–80%), but not all, responded with increased Ca2+ fluxes. It was noteworthy that intracellular Ca2+ levels did not change during the hHGF pulse itself. However, 5 min after removing hHGF from the solution, Ca2+ levels increased by 1.6 ± 1-fold (p < 0.001, t test), which was sustained for 15–20 min, although intracellular Ca2+ levels oscillated in some hepatocytes. After incubation with 100 µM glibenclamide for 5 min, intracellular Ca2+ levels declined slightly in primary hepatocytes (n = 36) from 344 ± 19 to 242 ± 16 ng/ml (p < 0.05, t test). However, when glibenclamide-treated cells were pulsed with 12 ng/ml hHGF, intracellular Ca2+ levels increased by 1.55 ± 0.1-fold at 5 min (p < 0.001, t test). This induction of intracellular Ca2+ flux was identical to control primary hepatocytes that had not been treated with glibenclamide (p = not significant).

**DISCUSSION**

These studies provide new knowledge concerning KATP channels in hepatocytes and their biological significance with respect to liver growth control. Our work showed functional KATP channels in freshly isolated primary hepatocytes as well as in hepatocyte-derived cell lines, which was previously unknown. The currents recorded from primary rat hepatocytes and HepG2 cells were consistent with KATP channels because these currents were observed in the presence of UDP in internal solution, without ATP, and with K+ as the major permeant cation. Moreover, the addition of K+ channel openers to the external solution shifted the reversal potential toward the equilibrium potential for K+.

Previously recorded hepatic K+ currents have been either linear or Ca2+-dependent inward rectifiers (18, 19). Our electrophysiological data are in agreement with additional demonstrations of K+ channel mRNA expression in liver cells. Kir6.1 and SUR1 mRNAs were well expressed in the intact rat liver as well as in isolated primary rat hepatocytes, whereas SUR2 was expressed weakly. In our cell lines, we detected Kir6.2 and SUR1 proteins and Kir6.5 transcripts, which are consistent with our electrophysiological data.
K+ Channels and Hepatocyte Proliferation

Changes in intracellular calcium after stimulation of HepG2 cells with ATP

| Condition                        | Base-line [Ca\(^{2+}\)], median (range) | [Ca\(^{2+}\)] after ATP stimulation, median (range) | \(\Delta\)Fold in [Ca\(^{2+}\)] (mean ± S.D.) | \(p\) values (\(t\) tests) |
|---------------------------------|----------------------------------------|---------------------------------------------------|------------------------------------------|------------------------------|
| Untreated cells                 | na                                     | na                                                | 4 ± 1                                    | <0.001                      |
| Immediately after glibenclamide treatment | 117 (23–435)\(^a\)                   | 441 (113–1845)\(^b\)                              | 3 ± 1                                    | <0.001                      |
| Glibenclamide pretreatment for 24 h | 121 (24–647)\(^b\)                   | 318 (96–1306)\(^b\)                              | 5 ± 2                                    | <0.001                      |

\(^a\)\(^b\) Comparisons between footnotes \(a\), \(b\), \(c\), as well as between footnotes \(d\), \(e\), and \(f\), were not statistically significant.

SUR2 mRNAs. These findings concerning regulation of SUR1 in rat hepatocytes are in agreement with previous studies in chick cardiomyocytes showing that K+ channel openers and glibenclamide regulated K\(_{ATP}\) activity (34). The K+ currents in primary rat hepatocytes and HepG2 cells were different, as indicated above. The difference in the properties of currents in primary rat hepatocytes and HepG2 cells might be due to the expression of different K\(_{ATP}\) channel subunits.

K+ channels are known to regulate proliferation in many cell types, although the type of K+ channel involved in this varies, as indicated by the identification of small conductance Ca\(^{2+}\)-activated K+ channels in fibroblasts (17), voltage-activated and calcium-gated K+ channels in lymphocytes (16), and K\(_{ATP}\) channels in bladder cells (14). Interestingly, activation of K+ channels occurs early during mitogenic stimulation, occurring prior to DNA synthesis in hematopoietic cells as well as in neural cells (16, 35, 36). In fibroblasts, small conductance Ca\(^{2+}\)-activated K+ channels are involved in activating intracellular signaling, such as Ras signaling (17). Similarly, over-expression of ras or raf proto-oncogenes, which are downstream effectors of the intracellular signaling pathways, increases K+ channel current density in cultured fibroblasts (37, 38).

Our work shows that in primary rat hepatocytes as well as in human liver cell lines, quinidine and glibenclamide dose-dependently inhibit cell proliferation. Although quinidine is a relatively nonspecific potassium channel blocker, glibenclamide has specificity for K\(_{ATP}\) channels. In cultured primary hepatocytes, it was necessary to add a growth factor (hHGF) to elicit DNA synthesis because these cells show very limited proliferative activity. Various cancer cell lines were presumably capable of producing autocrine factors to sustain higher levels of proliferation. The efficacy of glibenclamide in suppressing cell proliferation in primary hepatocytes without the addition of hHGF (Fig. 1B) may suggest interference with growth factor-independent pathways, although suppression of growth factor-dependent increases in DNA synthesis was more obvious. Whether other activities of glibenclamide in cells, such as regulation of the cystic fibrosis transmembrane conductance regulator chloride channel, GLUT2 or GLUT4 glucose transporters, and glucose synthesis or storage (39), could contribute to regulating cell proliferation will require further analysis. It is noteworthy that although the pancreatic K\(_{ATP}\) channel responds to nanomolar concentrations of glibenclamide, such responses can vary widely in cell- and tissue-specific fashions, ranging from 10^-7 to 10^-5 M (40, 41). Also, glibenclamide is largely bound to serum proteins, and the total effective concentrations in cultured cells may not be reflective of free glibenclamide concentrations achieved in vivo (42).

K\(_{ATP}\) channels play significant roles in liver growth control as indicated by stimulation of DNA synthesis as well as by elicitation of characteristic currents following exposure to K+ channel openers specific for the K\(_{ATP}\) channel. On the other hand, we found that quinidine and glibenclamide, which block the K\(_{ATP}\) channel, inhibited DNA synthesis both with and without growth factor stimulation in hepatocytes. These findings indicate that K\(_{ATP}\) channels play critical roles in hepatocytes. Also, K\(_{ATP}\) channel activity was preserved despite oncogenic transformation in cancer cell lines. Persistent inhibition of proliferation in glibenclamide-treated HepG2 cells was in agreement with the lack of redundancy in regulation of cell proliferation by K\(_{ATP}\) channels. Previous studies with bladder cancer cells showed that K+ channel blockers inhibited cell proliferation without cytotoxicity (14), similar to our findings in hepatocytes.

Hepatocyte growth factor acts on hepatocytes via the c-Met receptor, which is a receptor tyrosine kinase, with activation of intracellular signaling pathways, including calcium-mediated signals. Also, hepatocyte growth factor exhibits morphogenic and motogenic activities in cells (24). It was noteworthy that our glibenclamide-treated cells underwent morphological alterations in culture. Several mechanisms could have been responsible for this change. Some K+ channels and other ion channels regulate cell volume, which may have secondary effects upon mitogenesis as well (23). Also, membrane depolarization associated with inhibition of K+ channels could modulate calcium channels with perturbation of signal transduction mechanisms. Fluxes in intracellular calcium ions as well as in other inorganic ions participate in mediating mitogenic stimuli, including hHGF (24, 43, 44). Hepatocyte and epidermal growth factors have previously been shown to elicit Ca\(^{2+}\) fluxes in freshly isolated primary rat hepatocytes (45). It is noteworthy that glibenclamide did not alter hHGF-stimulated increases in intracellular Ca\(^{2+}\) fluxes. Furthermore, stimulation of glibenclamide-treated HepG2 cells with ATP elicited intracellular calcium fluxes, which was similar to previous studies of Ca\(^{2+}\) fluxes in HepG2 cells (46). These findings indicate that K\(_{ATP}\) channels regulated cell proliferation independently of intracellular Ca\(^{2+}\) response.

Further analysis of hepatic K+ channel activity will offer insights into liver growth control. An understanding of the hepatic activity of K+ channel openers and blockers may offer novel drug targets for regulating cell proliferation events. A working model would suggest that modulation of K\(_{ATP}\) channels on the cell membrane alters intracellular processes involved in DNA synthesis. Potential interactions among intracellular regulators in the context of K\(_{ATP}\) channel activity can be examined in our systems described here. For instance, we are currently defining alterations in cellular gene expression following inhibition of K\(_{ATP}\) channel activity at a genome-wide level. Initial findings indicate perturbations in specific genes involved in growth factor activity and cell cycle regulation. Inhibitors of cell signaling mechanisms have recently been found to have potent anticancer effects in intact animals (47), which suggests that insights into K\(_{ATP}\) channel activity in liver cancer could potentially offer novel therapeutic targets.

Insights into the regulation of hepatic K\(_{ATP}\) channel activity...
will help define potential hepatotoxic manifestations of sulfonylureas and related drugs (48), which are poorly understood at present, but could involve perturbation of cell cycling or cell survival. Finally, amplification of hepatic growth factor responses with effective drugs capable of modulating K\textsubscript{ATP} activity might also find applications. For instance, this might facilitate hepatic gene transfer for somatic gene therapy with specific vectors requiring DNA synthesis (49) as well as liver repopulation with transplanted hepatocytes for cell therapy.

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K<sub>ATP</sub> Channels Regulate Mitogenically Induced Proliferation in Primary Rat Hepatocytes and Human Liver Cell Lines: IMPLICATIONS FOR LIVER GROWTH CONTROL AND POTENTIAL THERAPEUTIC TARGETING
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