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ATP-sensitive potassium channels induced in liver cells after transfection with insulin cDNA and the GLUT2 transporter regulate glucose-stimulated insulin secretion

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Abstract

As part of our research into the liver-directed gene therapy of Type I diabetes, we have engineered a human hepatoma cell line (HEPG2ins/g cells) to store and secrete insulin to a glucose stimulus. The aim of the present study was to determine whether HEPG2ins/g cells respond to glucose via signaling pathways that depend on ATP-sensitive potassium channels (K_{ATP}). Using patch-clamp electrophysiology with symmetrical KCl solutions, the single-channel conductance of K_{ATP} was 61 pS. K_{ATP} was inhibited by ATP (1 mM) or cAMP (50 μ M) applied to the cytosolic side of the membrane. Single K_{ATP} channels and macroscopic whole-cell currents were inhibited by glucose (20 mM) and glibenclamide (20 μ M) and were activated by diazoxide (150 μ M).

Immunoprecipitation and Western blot analysis confirmed the presence of Kir6.2 K_{ATP} channel subunit protein in HEPG2ins/g and HEPG2ins cells. Using radioimmunoassay techniques, we report that exposure of the cells to tolbutamide (100 μ M) resulted in an increase in insulin secretion from 0.3 ± 0.05 to 1.8 ± 0.2 pmol insulin/ 10^6 cells and glibenclamide (20 μ M) from 0.4 ± 0.06 to 2.1 ± 0.3 ($n=4$), similar to what is seen on glucose (20 mM) stimulation. Diazoxide (150 μ M) completely inhibited glucose-stimulated insulin release. Glucose 20 mM and glibenclamide 100 μ M increased intracellular Ca^{2+} level in the HEPG2ins/g cells. However, glucose 20 mM did not stimulate a rise in intracellular Ca^{2+} in the un-transfected parent cell-line HEPG2. We used confocal microscopy to confirm that glucose (20 mM) stimulated the release of insulin from the fluorescently labeled secretion granules in the cells. Furthermore, glibenclamide (20 μ M) also stimulated the release of insulin from fluorescently labeled secretion granules, and diazoxide (150 μ M) blocked that stimulated release of insulin. Our results suggest that HEPG2ins/g cells respond to glucose via signaling pathways that depend on K_{ATP} , similar to a normal pancreatic β cell.

Keywords

HEPG2ins/g; patch-clamp electrophysiology; Western blotting; confocal laser scanning microscopy; radioimmunoassay

Type I diabetes or IDDM is caused by the autoimmune destruction of pancreatic β cells (1). Current treatment of the disease requires daily injections of insulin to control blood glucose levels. Results from The Diabetes Control and Complications Trial Research Group (2) show that the onset of diabetic complications, which greatly reduces the quality and longevity of life in IDDM patients, is reduced by tight glucose control. Glucose control could theoretically be improved by genetically engineering “an artificial β cell” that is capable of synthesizing, storing, and secreting insulin in response to metabolic signals. In pursuit of this goal, hepatocytes have been shown by us and other groups to be a suitable target cell (3-8). Hepatocytes are known to play a crucial role in intermediary metabolism and synthesis of proteins in the liver. Most importantly liver cells express the high-capacity glucose transporter GLUT 2 (9) and the glucose phosphorylation enzyme glucokinase (10), which comprise the key elements of the “glucose sensing system,” which regulates insulin secretion from pancreatic β cells in response to small external nutrient changes. Previous studies of ours have shown that the insertion of insulin cDNA into a human hepatoma cell line (HEPG2) that lacked native GLUT 2 expression to produce the cell-line HEPG2ins, resulted in both the synthesis, storage, and release of insulin to β cell secretagogues, but not to glucose (3). A second insertion of the glucose transporter GLUT 2 resulted in near physiological release of insulin to glucose and other stimuli in the doubly transfected HEPG2ins/g cells (4).

To better understand the mechanisms underlying the transformation of the HEPG2 parent liver cell line into HEPG2ins/g cells that can secrete insulin in response to a glucose stimulus, we investigated the physiology of the glucose-stimulated insulin secretory mechanism in the HEPG2ins/g cells. In a normal pancreatic β cell it is generally accepted that a rise in extracellular glucose initiates the inhibition of ATP-sensitive potassium channels (K_{ATP}), which leads to depolarization, influx of extracellular Ca^{2+} ions, induction of a rise in $[Ca^{2+}]_i$ from intracellular stores, exocytosis, and secretion of insulin (11-14). The K_{ATP} channel has been cloned and found to be a complex of a K^+ channel (Kir 6.2) and an ATP binding cassette protein (SUR1) that functions as a high-affinity receptor for sulphonylureas (15-18).

Although potassium channels have been described in many cell types, apart from pancreatic islets, including skeletal muscle, cardiac, and vascular myocytes; neurons; and renal epithelial cells (19-23), characterization of the role of K^+ channels in hepatocytes has been limited. Henderson et al. (24), described the presence of inwardly rectifying K^+ channels in primary rat hepatocytes that were not affected by voltage or Ca^{2+} stimulation. Capiod and Ogden (25) described the presence of Ca^{2+} -activated and delayed-rectifier K^+ currents, and Lidofsky (26) reported the presence of Ca^{2+} -sensitive and cAMP-dependent K^+ channels in HTC rat hepatoma cells.

In this paper we report that the HEPG2ins/g cells express functional ATP-sensitive potassium (K_{ATP}) channels. We confirmed the presence of the K^+ channel subunit, Kir6.2, in the cells using immunoprecipitation and Western blotting. The novelty of the engineered HEPG2ins/g cells is that the expression of the insulin and GLUT 2 genes has induced functional K_{ATP} channels in the resting cells. This is compared with the HEPG2 parent cells where the opening of the K_{ATP} channels required pharmacological stimulation (27). We report that these K_{ATP} channels are involved in the control of glucose-stimulated insulin secretion in the HEPG2ins/g cells, using the techniques of patch-clamp electrophysiology, confocal microscopy, fluorescent Ca^{2+} imaging, and biochemistry.

MATERIALS AND METHODS

Cell culture

HEPG2 parent cells were cultured as monolayers in Dulbecco's modification of Eagle's medium (25 mM glucose) containing 10% fetal calf serum (FCS) in 5% CO₂ in air. HEPG2ins cells were cultured as above with the addition of 1 mg/ml G418 antibiotic to maintain selective pressure. HEPG2ins/g cells were cultured as for HEPG2ins cells, plus 500 µg/ml hygromycin B, as were HEPG2 cells transfected with the empty vectors used to deliver insulin and GLUT 2 (4).

Patch-clamp electrophysiology

The cells were plated onto coverslips and superfused with test and control solutions in a specially designed chamber at room temperature (20°C). Patch pipettes with a tip-opening between 0.9 and 1.5 µm were fabricated from special capillary glass tubing (G75-1511). The channel currents were amplified and filtered by using an Axopatch 200B amplifier (Axon Instruments) and sampled on-line by a microcomputer (PC-compatible) by using commercial software and associated A/D hardware (PCLAMP 8.0/Digidata 1200, Axon Instruments Inc.).

Insulin secretion from HEPG2ins/g cells

Insulin secretion from the HEPG2ins/g cells was measured following both (a) static stimulation and (b) perfusion of stimulating agents.

a) Static Stimulation—Before stimulation, tissue culture plates were thoroughly washed with basal medium (PBS containing 1 mM CaCl₂ and were supplemented with 20 mM HEPES and 2 mg/ml BSA, 1.0 mM glucose) to remove culture medium and FCS. Monolayers were incubated in the basal medium at pH 7.4 for two consecutive 1 h periods to stabilize the basal secretion of insulin. Monolayers were then exposed to the inhibitors (tolbutamide: 100 µM, diazoxide: 150 µM) and the activator (glibenclamide: 20 µM) of K_{ATP} channels ± 20 mM glucose for 1 h. Basal medium alone was used as the control. Insulin was measured in the samples by a standard radioimmunoassay, using guinea pig insulin antibody and I¹²⁵ labeled insulin prepared by the chloramine-T method. Data were expressed as means ± SE. Paired sample means were compared with Student's *t*-test.

b) Perfusion—Cells were grown on cytodex beads and were cultured in spinner flasks in normal culture medium for 4 days. Beads with cells (~10⁸) attached were transferred to a column and connected to a peristaltic pump and fraction collector, and basal medium was pumped through the system for 30 min. This was followed by 15 min exposures to 20 mM glucose ± 150 µM diazoxide.

Immunoprecipitation and Western blotting analysis

Sheep anti-peptide antibody that had been raised against synthetic peptides consisted of residue 33 to 47 in the Kir6.2 protein (ARFVSKKGNCNVAHK), and conjugated to a monoclonal anti-goat/sheep IgG were used for immunoprecipitation and Western blotting as described previously (28). Briefly, membrane fractions were obtained from 1 × 10⁸ MIN6 cells (positive control), HEPG2ins/g cells, HepG2 ins cells, and HepG2 cells. Each aliquot of cells was subjected to a freeze-thaw cycle three times with buffer I (Tris 10 mM, NaH₂PO₄ 20 mM, EDTA 1.0 mM, PMSF 0.1 mM, pepstatin 10 µg/ml, leupeptin 10 µl/ml, pH 7.8), then incubated for 20 min at 4°C. Osmolarity was restored with KCl 2.5 M, NaCl 1.2 M, and sucrose 1.25 M, and the preparation was centrifuged at 500 g at 4°C for 30 min. The supernatant was diluted in Buffer II (imidazole 30 mM, KCl 120 mM, NaCl 30 mM, NaH₂PO₄ 20 mM, sucrose 250 mM, pepstatin 10 µg/ml, leupeptin 10 µl/ml at pH=6.8) and centrifuged at 7000 g at 4°C for

30 min. The pellet was removed, and the supernatant centrifuged at 30,000 g at 4°C for 1 h. The pellet contained the membrane fraction and was snap-frozen with liquid nitrogen, and the pellet was ground to powder and diluted with tissue buffer (HEPES 20 mM, NaCl 150 mM, Triton-X100 1%, pH=7.5) and homogenized. Protein concentration was determined the Micro BCA protein Assay Reagent Kit (Pierce, Rockford, IL); 15 µg (0.5mg/ml) of the epitope-specific Kir6.2 antibody was mixed with 45 µg of membrane fraction protein extract at 4°C for 1 h, then 7 µl of protein-G Sepharose beads were added into the mix solution for immunoprecipitation for another 45 min at 4°C. The pellets of this precipitation were run on 10% polyacrylamide (SDS) gels for Western analysis. Western blot probing was performed by using 1/300 dilution of anti-Kir6.2 antibody, and detection was achieved by using monoclonal anti-goat/sheep IgG antibody (1/800 dilution) horse radish peroxidase (Sigma, St. Louis, MO).

Imaging of intracellular Ca^{2+}

Intracellular free Ca^{2+} of HEPG2 and HEPG2 ins/g cells was measured by using fluo3 with an Axiovert 200M inverted deconvolution microscope (Zeiss, Germany). Cells grown on coverslips to 50-70% confluence were incubated in 5% glucose DMEM containing 5 µM fluo3_{AM} (Molecular Probes, Eugene, OR) at 37°C for 60 min. After two washes (5 min/wash), The coverslips were placed in a chamber on a Perspex holder containing 0.3 ml Ca^{2+} -free buffer (in mM: 135 NaCl, 5 KCl, 1 MgCl_2 , 2 D-glucose, 10 HEPES, and pH 7.4). The holder was mounted onto the stage of the microscope, and the fluorescence was recorded at 510 nm. After the intensity of the fluorescence was stable (as in the control), 20 mM glucose or 100 µM glibenclamide was gently added and left in the solution until the end of the experiments. Images were taken every 30 s, and the intensity of fluorescence (reflecting the intracellular free Ca^{2+} level) was analyzed by using the Scion Image software (Beta 4.0.2, Scion Cooperation, Frederick, MD). The intracellular Ca^{2+} concentration is presented as a relative change compared with the average of the control value (= 1.0).

Confocal microscopy of insulin secretory granules

The cell lines were plated onto sterilized no. 1 round coverslips (Marienfeld Superior, 22 mm round, Germany) and grown for 2 days in normal culture medium. Without allowing the attached cells to dry out, the coverslip was sealed, using petroleum jelly, into a cell chamber consisting of a Perspex holder that had been machined to hold the coverslip and 1 mL of medium.

The fluorescent probe for Zn was ethyl [2-methyl-8-*p*-toluenesulphonamido-6-quinolyloxy] acetate (zincquin ester, or zincquin-E: Luminis Pty Ltd., Adelaide, SA), which is an intracellular zinc-specific fluorophore that is concentrated in insulin-storing secretion granules due to their relatively high content of labile zinc. The ester form of zincquin is only weakly fluorescent, but once inside cells it is hydrolyzed by cellular esterases to zincquin acid (zincquin-A). When this binds to zinc it becomes strongly fluorescent and is trapped within cells for several hours due to a permeability change (29). A fresh preparation of stock zincquin-E (5mM) in ethanol was prepared as described by Zalewski et al. (29) before each experiment and diluted to 25 µM zincquin-E in DMEM medium containing 5 mM glucose (5 mM glucose was required to obtain good uptake of zincquin-E). Cells were incubated in zincquin-E for 30 min at 37°C. Excess was washed out with three changes of DMEM medium containing 5 mM glucose before imaging with an inverted microscope by using a 100 × 1.4 NA UV-corrected Planapo oil-immersion objective, UV illumination, BP 490/440, and a Leica TCSNT (Wetzlar, Germany) confocal laser scanning microscope (CLSM).

Test and control [Dulbecco's modification of Eagle's medium with 10% FCS (DMEM medium) and 5 mM glucose] solutions were exchanged gently to avoid removing cells from

the coverslips, and were kept at 37°C. Diazoxide solution: 150 μ M diazoxide in DMEM medium containing 5 mM glucose. Glibenclamide solution: 20 μ M glibenclamide in DMEM medium containing 5 mM glucose; 20 mM glucose solution: DMEM medium containing 20mM glucose.

RESULTS

Patch-clamp electrophysiology

We used patch-clamp electrophysiology to measure the membrane potential of the HEPG2, HEPG2 cells transfected with the empty vectors alone, HEPG2ins, and HEPG2ins/g cells. In those experiments the cells were superfused with a solution containing (pH 7.4, concentration in mM) NaCl (140), KCl (2), CaCl_2 (1), MgCl_2 (1), and HEPES (10). The patch-clamp pipettes contained (pH 7.3, concentration in mM) KCl (140), EGTA (1), and HEPES (10). The results of these experiments are shown in Fig. 1. The HEPG2ins/g cells, in which cDNA for insulin and GLUT2 genes had been inserted, displayed greater membrane potentials (-68.9 ± 7.1 mV, $F=17.933$, $\alpha=0.05$) than either the untransfected HEPG2 cells (-18.2 ± 5.8 mV) or the HEPG2 cells transfected with the empty vectors alone. The HEPG2ins cells had only the insulin gene inserted and had a membrane potential (-45.3 ± 4.4 mV) between that of the HEPG2 and HEPG2ins/g cells.

We recorded macroscopic ionic currents from the HEPG2 and HEPG2ins/g by using patch-clamp electrophysiology in the whole-cell mode. In those experiments the cells were superfused with a solution containing (pH 7.4, concentration in mM) Na-acetate (140), CaCl_2 (1), MgCl_2 (1), and HEPES (10). The patch-clamp pipettes contained (pH 7.3, concentration in mM) K-acetate (136), KCl (4), EGTA (1), and HEPES (10). The currents in the HEPG2ins/g cells were larger than the parent HEPG2 cells. The I-V curve for a group of HEPG2ins/g cells (Fig. 2) indicated that this current was K^+ -selective, since the reversal potential (balance of electrical and concentration potential) ($E_R \approx -50$ mV) for the currents approached the Nernst potential for potassium. The currents in the HEPG2 cells appeared to be non-selective, since the E_R was closer to 0 mV. We found no differences in the macroscopic ionic currents between the HEPG2 and HEPG2 cells transfected with empty vectors alone.

The sensitivity of these potassium channels to ATP was measured by using single-channel recordings from inside-out patches taken from the HEPG2ins/g cells. Both the superfusing and pipette solutions contained (pH 7.3, concentration in mM) K-acetate (136), KCl (4), EGTA (1), and HEPES (10). ATP 1mM inhibited the potassium channels by reducing the frequency of the channel openings, shown by a reduced number of deflection steps in the recordings (Fig. 3a). We also found that cAMP (50 μ M) inhibited potassium channel activity when applied to the cytosolic face of inside-out membrane patches detached from the HEPG2ins/g cell (data not shown).

The sensitivity of the K channels to high glucose concentrations was measured by using whole-cell patch clamping of the HEPG2ins/g cells (Fig. 3b). If the channels had a role in stimulating insulin secretion, extracellular application of glucose (20 mM) would be expected to inhibit the channels. In these experiments the cells were superfused with a solution containing (pH 7.4, concentration in mM) Na-acetate (140), CaCl_2 (1), MgCl_2 (1), and HEPES (10). The patch-clamp pipettes contained (pH 7.3, concentration in mM) K-acetate (136), KCl (4), EGTA (1), and HEPES (10). Glucose (20 mM) added to the superfusing solution inhibited the ATP-sensitive potassium currents.

Using an outside-out patch clamp technique, we showed that the K_{ATP} channel blocker glibenclamide (20 μ M) inhibited the opening probability of the single channel activity while the channel activator diazoxide (150 μ M) increased opening probability (Fig. 4). Both the

superfusing and pipette solutions contained (pH 7.3, concentration in mM) potassium acetate (136), KCl (4), EGTA (1), and HEPES (10).

Western blotting analysis

Western blotting with the specific anti-Kir6.2 antibody revealed signals in MIN 6, HEPG2ins/g, and HEPG2ins cells at the expected size, that is ~35 kDA (Fig. 5). The intensity of the signal is much higher in MIN6 and HEPG2ins/g cells than HEPG2ins cells, with little if any expression in HEPG2 cells.

Acute stimulation of insulin secretion

As can be seen from Fig. 6 static stimulation of the HEPG2ins/g cells with the K_{ATP} channel inhibitor tolbutamide resulted in a significant ($P < 0.001$) increase in insulin secretion, similar to that seen on glucose stimulation. Glibenclamide (20 μ M) had a similar effect (data not shown). Opening the ATP-sensitive potassium channels with diazoxide (150 μ M) completely inhibited glucose-stimulated insulin release, as determined by static incubation (Fig. 6) and perfusion (Fig. 7). We discovered that opening the ATP-sensitive potassium channels with diazoxide reversed the stimulating effects of glucose on insulin secretion (Fig. 7).

Intracellular Ca^{2+} levels in HEPG2 and HEPG2ins/g cells

We compared the effectiveness of glucose 20 mM and glibenclamide 100 μ M in affecting the intracellular Ca^{2+} level in the HEPG2ins/g and HEPG2 cells. Fig. 8 shows that either glucose or glibenclamide were effective only in stimulating an increase in intracellular Ca^{2+} in the transfected HEPG2ins/g cells. In the HEPG2ins/g cells, glucose 20 mM stimulated an increase of 48% in intracellular Ca^{2+} , which peaked 7 min after exposure to glucose and then steadily declined to a 24% rise during the remaining 18 min of exposure (Fig. 8a). Exposure of HEPG2ins/g cells to glibenclamide 100 μ M also stimulated an increase of 30% in intracellular Ca^{2+} , which also peaked ~7 min after exposure and then steadily declined to 24% during the remaining 18 min of exposure to glibenclamide (Fig. 8b). Glucose (20 mM) failed to stimulate an increase in intracellular Ca^{2+} in the un-transfected HEPG2 cells (Fig. 8c).

Confocal microscopy

We visualized secretion granules in live cells as Zinquin-E labeled punctuate fluorescence by using confocal laser scanning microscopy (CLSM) and could follow the effect of glucose and K_{ATP} channel activators and inhibitors on insulin secretion of HEPG2ins/g cells. Both insulin synthesis and secretion were stimulated by glucose in HEPG2ins/g cells (4). Zinquin-E staining was lost (secretion occurred) on addition of either 20 mM glucose alone, or 20 μ M glibenclamide alone (data not shown). Conversely, adding 150 μ M diazoxide blocked secretion and even subsequent washing with 20 mM glucose did not cause insulin-containing granules to be released from HEPG2ins/g cells (Fig. 9). Taken together, these data indicate that secretion of insulin-containing granules was stimulated by depolarizing the HEPG2ins/g cells, with inhibition of the K_{ATP} channels, either by glucose or directly with glibenclamide.

DISCUSSION

Our novel results with the HEPG2ins/g cells are very encouraging with regards to the possibility of engineering liver cells to mimic the physiology of pancreatic β cells and their eventual use for treatment of Type I diabetes. The novelty of the HEPG2ins/g cells is that dual expression of the glucose transporter GLUT 2, and the insulin gene has induced functional K_{ATP} channels in the resting cells. Using the techniques of patch-clamp electrophysiology, confocal microscopy, fluorescent Ca^{2+} imaging, and biochemistry, we have defined a role for K_{ATP} channels as part of a Ca^{2+} -dependent mechanism that regulates the secretion of insulin

in the genetically engineered HEPGins/g liver cell-line. Transfection of the parent HEPG2 cell-line with both the GLUT2 transporter and insulin cDNA resulted in cells (HEPG2ins/g) that stored insulin in secretory granules and secreted insulin in response to glucose stimulation, at near physiological levels (4). Although ATP-sensitive potassium channel subunits are found in the parent cell line, HEPG2, the opening of functional channels in resting HEPG2 cells requires pharmacological stimulation (27). Our results from immunoprecipitation and Western blotting indicated that there was little, if any, expression of Kir6.2 in the HEPG2 cells. That observation confirmed the lack of functional K_{ATP} channel activity in the HEPG2 cells in our patch-clamp experiments, and supports the previous observation of Mahli et al. (27), whereby K_{ATP} currents could only be recorded in HEPG2 cells after stimulation by pharmacological activators.

The expression of functional K_{ATP} channels in the resting HEPG2ins/g cells provides a mechanism to explain the rapid triggering of insulin secretion that we recorded in response to stimulation of the cells by glucose. The results we report in this paper suggest that the HEPG2ins/g cells follow the classical triggering pathway whereby glucose enters the cell and there is a rise in the ATP-to-ADP ratio leading to closure of K_{ATP} channels, which causes membrane depolarization and subsequent activation of insulin secretion by exocytosis of secretory granules (30). We report that the HEPG2ins/g cells have a resting membrane potential similar to that of pancreatic β cells, which is in contrast to the relatively depolarized potential of the parent HEPG2 liver cell-line. Moreover, compared with the parent HEPG2 cells, the HEPG2ins/g cells expressed a macroscopic K^+ -selective current that was inhibited by glucose. The single channels underlying the macroscopic K^+ -current had the characteristics of ATP-sensitive channels reported previously in pancreatic β cells (11-14). These functional recordings of K_{ATP} channel activity are supported by immunoprecipitation and Western blotting, which suggested that the Kir6.2 subunits were most strongly expressed in the HEPG2ins/g cells and extremely weakly, if at all, in the HEPG2 cells.

The subunits comprising the K_{ATP} channel in the parent HEPG2 cells were reported to be SUR2 and Kir6.2, whose mRNA expression was unaltered by glibenclamide (27). The results of Mahli et al. (27) indicate that the K_{ATP} channels present in the parent HEPG2 cells do not have the same subunits as native pancreatic β cells, as K_{ATP} channels in native pancreatic β cell comprise the subunits SUR1 and Kir6.2. In native pancreatic β cells, the primary site at which ATP acts to mediate K_{ATP} channel inhibition is located on Kir6.2, and SUR1 is required for sensitivity to sulphonylureas and diazoxide and for activation by Mg-ADP (31).

Our combined transfection of the GLUT2 and insulin genes (4) induces the activation of functional K_{ATP} channels that do not require pharmacological stimulation to generate currents. Indeed, the pinacidil-activated K_{ATP} current reported by Mahli et al. (27) has an identical current-voltage relation to the currents we recorded in the unstimulated HEPG2ins/g cells. Taken together with the Western blotting reported in this paper, we can draw the hypothesis that our transfection may have induced a modification of the SUR2 subunit present in the HEPG2 cells to allow functional K_{ATP} channels to be expressed in the HEPG2ins/g cells. Evidence for this hypothesis can be found in the following literature. It is known that co-expression of Kir6.2 with SUR1 generates K_{ATP} channels that are blocked by tolbutamide and activated by diazoxide, whereas co-expression of Kir6.2 with SUR2A results in K_{ATP} channels that are blocked by glibenclamide, activated by pinacidil, and insensitive to diazoxide (16). There is 71% identity between the genes SUR1 and SUR2, which encodes the subunits that provide the complete K_{ATP} channel with sensitivity to Mg^{2+} nucleotides and therapeutic drugs, such as sulphonylureas and K_{ATP} openers (15). Alternative splicing of SUR2 produces SUR2A and SUR2B, which differ only in their COOH-terminal 42 amino acids (16). Also, it has been recently reported that the sensitivity of the K_{ATP} channel to nicorandil (channel opener) and

sulfonylureas can depend on sequence variations in the COOH-terminal group of certain trans-membrane loops of SUR2 and the last 42 amino acids of SUR2 (32).

As described previously in β cells (33), we found that the specific K_{ATP} -activating drug diazoxide inhibited insulin secretion in the HEPG2ins/g cells that had been stimulated previously by glucose. Application of diazoxide also inhibited the glucose-stimulated secretion of insulin-containing granules that had been labeled with a fluorescent marker, visualized by CLSM. The mechanism for this effect would be for diazoxide to hyperpolarize the HEPG2ins/g cells and inhibit any depolarization-mediated entry of extracellular Ca^{2+} or release of intracellular Ca^{2+} from intracellular stores. This lack of Ca^{2+} would inhibit the release of insulin-containing secretory granules from the HEPG2ins/g cells. Furthermore, static stimulation of the HEPG2ins/g cells with the K_{ATP} channel inhibitor tolbutamide resulted in a significant increase in insulin secretion, similar to what is seen on glucose stimulation. Glibenclamide, another K_{ATP} channel inhibitor, also caused insulin secretion from HEPG2ins/g cells, as shown by static stimulation and by visualization with CLSM of insulin-containing granules labeled with a fluorescent marker. Both glibenclamide and glucose stimulated a rise in intracellular Ca^{2+} levels in the HEPG2ins/g cells, where the mechanism of action is for the K_{ATP} channel inhibitor to depolarize the cell, allowing entry of extracellular calcium or release of calcium from intracellular stores, resulting in Ca^{2+} -mediated release of insulin from secretion granules. Glucose is known to stimulate a rise in intracellular Ca^{2+} in β cells (34).

In conclusion, we report that there are functional K_{ATP} channels expressed in the transfected liver cell-line HEPG2ins/g. These channels are activated only in the parent HEPG2 cell line by pharmacological stimulation. We have demonstrated, by using patch-clamp electrophysiology, confocal laser scanning microscopy with labeling of insulin-containing secretory granules, measurement of intracellular Ca^{2+} levels, and biochemical studies of glucose-stimulated insulin secretion, that the HEPG2ins/g cells secrete insulin in response to glucose in a manner consistent with the classical triggering pathway present in pancreatic β cells. Our results further support the potential value of the HEPG2ins/g cells as a genetically engineering “an artificial β cell” in the treatment of Type I diabetes.

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REFERENCES

1. Eisenbarth GS. Type I diabetes mellitus: a chronic autoimmune disease. *N. Engl. J. Med* 1986;14:1360–1368. [PubMed: 3517648]
2. The Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N. Engl. J. Med* 1993;329:977–986. [PubMed: 8366922]
3. Simpson AM, Tuch BE, Swan MA, Tu J, Marshall GM. Functional expression of the human insulin gene in a human hepatoma cell line (HEPG2). *Gene Ther* 1995;2:223–231. [PubMed: 7614254]
4. Simpson AM, Marshall GM, Tuch BE, Maxwell L, Szymanska B, Tu J, Beynon S, Swan MA, Camacho M. Gene therapy of diabetes: glucose stimulated insulin secretion in a human hepatoma cell line (HEPG2ins/g). *Gene Ther* 1997;4:1202–1215. [PubMed: 9425444]
5. Simpson AM, Szymanska B, Tuch BE, Marshall GM. Secretion and storage of insulin from a human hepatoma cell line: Huh7-ins. *Transplant. Proc* 1999;31:812. [PubMed: 10083351]
6. Ferber S, Halkin A, Cohen H, Ber I, Einav Y, Goldberg I, Barshack I, Seijffers R, Kopolovic J, Kaiser N, Karasik A. Pancreatic and duodenal homeobox gene I induces expression of insulin genes in liver and ameliorates streptozotocin-induced hyperglycaemia. *Nat. Med* 2000;6:568–572. [PubMed: 10802714]

7. Soria B, Roche E, Berna G, Leon-Quinto T, Reig JA, Martin F. Insulin-secreting cells derived from embryonic stem cells normalize glycaemia in streptozotocin-induced diabetic mice. *Diabetes* 2000;49:157–162. [PubMed: 10868930]
8. Tuch BE, Szymanska B, Yao M, Tabiin MT, Gross DJ, Holman S, Swan MA, Humphrey RKB, Marshall GM, Simpson AM. Function of a genetically modified human liver cell line that stores, processes and secretes insulin. *Gene Ther* 2003;10:490–503. [PubMed: 12621453]
9. Permutt MA, Koranyi L, Keller K, Lacy PE, Scharp DM, Mueckler M. Cloning and functional expression of a human pancreatic islet glucose-transporter cDNA. *Proc. Natl. Acad. Sci. USA* 1989;86:8688–8692. [PubMed: 2479026]
10. Weinhouse, S. Regulation of glucokinase in liver. In: Horecker, BL.; Stadtman, ER., editors. *Current Topics in Cellular Regulation*. New York, San Francisco, London: Academic Press; 1976. p. 1-50.
11. Cook DL, Hales CN. Intracellular ATP directly blocks K^+ channels in pancreatic β cells. *Nature* 1984;311:271–273. [PubMed: 6090930]
12. Ashcroft FM, Harrison DE, Ashcroft SJH. Glucose induces closure of single potassium channels in isolated rat pancreatic β cells. *Nature* 1984;312:446–468. [PubMed: 6095103]
13. Pralong WK, Bartley C, Wollheim CB. Single islet β cell stimulation by nutrients: relationship between pyridine nucleotides, cytosolic Ca^{2+} and secretion. *EMBO J* 1990;9:53–60. [PubMed: 2403930]
14. Sakurada M, Kanasuka, Saitoh T, Makino H, Yamamura K, Miyazaki J-I, Kikuchi M, Yoshida S. Relation between glucose-stimulated insulin secretion and intracellular calcium accumulation studies with a super fusion system of a glucose-responsive pancreatic β cell line MIN6. *Endocrinology* 1993;132:2659–2665. [PubMed: 8504766]
15. Aguilar-Bryan L, Nichols CG, Wechsler SW, Clement JP, Boyd AE, Gonzalez G, Herrera-Sosa H, Nguy K, Bryan J, Nelson DA. Cloning of the β cell high affinity sulfonylurea receptor: a regulator of insulin secretion. *Science* 1995;268:423–426. [PubMed: 7716547]
16. Inagaki N, Gonoi T, Clement JP IV, Namba N, Inazawa J, Gonzalez G, Aguilar-Bryan L, Seino S, Bryan J. Reconstitution of IKATP: an inward rectifier subunit plus the sulfonylurea receptor. *Science* 1995;270:1166–1170. [PubMed: 7502040]
17. Sakura H, Ammala C, Smith PA, Gribble FM, Ashcroft FM. Cloning and functional expression of the cDNA encoding a novel ATP-sensitive potassium channel expressed in pancreatic β cells, brain, heart and skeletal muscle. *FEBS Lett* 1995;377:338–344. [PubMed: 8549751]
18. Babenko AP, Aguilar-Bryan L, Bryan J. A view of sur/KIR6.X K_{ATP} channels. *Annu. Rev. Physiol* 1998;60:308–315. [PubMed: 9558481]
19. Spruce AE, Standen NB, Stanfield PR. Voltage-dependent ATP-sensitive potassium channels of skeletal muscle membrane. *Nature* 1985;316:736–738. [PubMed: 2412127]
20. Noma A. ATP-regulated K^+ channels in cardiac muscle. *Nature* 1983;305:147–148. [PubMed: 6310409]
21. Wellman GC, Barrett-Jolley R, Koppel H, Everitt D, Quayle JM. Inhibition of vascular $K(ATP)$ channels by U-37883A: a comparison with cardiac and skeletal muscle. *Br. J. Pharmacol* 1999;128:909–916. [PubMed: 10556925]
22. Lee K, Dixon AK, Freeman TC, Richardson PJ. Identification of an ATP-sensitive potassium channel current in rat striatal cholinergic interneurons. *J. Physiol* 1998;510:441–453. [PubMed: 9705995]
23. Russ U, Rauch U, Quast U. Pharmacological evidence for a K_{ATP} channel in renin-secreting cells from rat kidney. *J. Physiol* 1999;517:781–790. [PubMed: 10358118]
24. Henderson RM, Graf J, Boyer JL. Inward-rectifying potassium channels in rat hepatocytes. *Am. J. Physiol* 1989;171:G1028–G1035. [PubMed: 2735409]
25. Capiod T, Ogden DC. The properties of calcium-activated potassium ion channels in guinea-pig isolated hepatocytes. *J. Physiol* 1989;409:285–295. [PubMed: 2511294]
26. Lidofsky SD. Convergent and parallel activation of low-conductance potassium channels by calcium and cAMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* 1995;92:7115–7119. [PubMed: 7624380]
27. Malhi H, Irani AN, Rajvanshi P, Suadcani SO, Spray DC, McDonald TV, Gupta S. K_{ATP} channels regulate mitogenically induced proliferation in primary rat hepatocytes and human liver cell lines. *J. Biol. Chem* 2000;275:26050–26057. [PubMed: 10862612]

28. Ranki HJ, Budas GR, Crawford RM, Jovanovic A. Gender-specific difference in cardiac ATP-sensitive K^+ Channels. *J. Am. Coll. Cardiol* 2001;38:906–915. [PubMed: 11527652]
29. Zalewski PD, Millard SH, Forbes IJ, Kapaniris AS, Betts WH, Ward DA, Lincoln SF, Mahadevan I. Video image analysis of labile zinc in viable pancreatic islet cells using a specific fluorescent probe for zinc. *J. Histochem. Cytochem* 1994;42:877–884. [PubMed: 8014471]
30. Henquin J-C. Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes* 2000;49:1751–1760. [PubMed: 11078440]
31. Tucker SJ, Gribble FM, Zhao C, Trapp S, Ashcroft FM. Truncation of Kir6.2 produces ATP-sensitive K channels in absence of the sulphonylurea receptor. *Nature* 1997;387:179–185. [PubMed: 9144288]
32. Reimann F, Ashcroft FM, Gribble FM. Structural basis for the interference between nicorandil and sulphonylurea action. *Diabetes* 2001;50:2253–2259. [PubMed: 11574406]
33. Straub SG, James RFL, Dunne MJ, Sharp GWG. Glucose activates both K_{ATP} channel-dependent and K_{ATP} Channel-independent signalling pathways in human islets. *Diabetes* 1998;47:758–763. [PubMed: 9588447]
34. Yamada K, Nakata M, Horimoto N, Saito M, Matsuoka H, Inagaki N. Measurement of glucose uptake and intracellular calcium concentration in single, living pancreatic beta-cells. *J. Biol. Chem* 2000;275:22278–22283. [PubMed: 10748091]

Figure 1. Membrane potential recorded for samples of HEPG2 ($n=6$), HEPG2ins ($n=6$), and HEPG2ins/g ($n=7$) cells

The vertical bars indicate SD. One-way ANOVA and Tukey posthoc testing showed a significant difference in the membrane potentials between the three cell types ($F=17.933$, $\alpha=0.05$). Dashed lines indicate published membrane potentials for isolated hepatocytes and pancreatic islet cells.

Figure 2. Summary of whole-cell I-V curves for the samples of HEPG2ins/g ($n=3$) and HEPG2 cells ($n=5$)

Vertical bars indicate SEM . The position of the reversal potential ($E_R \approx -50$ mV) for the HEPG2ins/g cells indicates a potassium-selective current, whereas the current for the HEPG2 cells is non-selective ($E_R \approx 0$ mV).

Figure 3. Sensitivity of potassium channels to ATP and high-glucose concentration

a) Recordings of K_{ATP} activity in an inside-out membrane patches of HEPG2ins/g cell ($n=8$). Closed channel level is shown by the horizontal arrows, and open channels are upward deflections. ATP (1 mM) reduced the frequency of open channel activity. Transmembrane potential = +40 mV. The vertical scale-bar represents 10 pA, and the horizontal scale-bar represents 200 ms. **b)** Whole-cell patch clamping of the HEPG2ins/g cells ($n=4$). Glucose (20 mM) was added to the superfusing solution and reversibly inhibited the ATP-sensitive potassium currents. The vertical scale-bar represents 200 pA, and the horizontal scale-bar represents 200 ms.

Figure 4. Activity of K_{ATP} recorded in an outside-out patch was increased by the channel opener diazoxide (150 μ M) and inhibited by the channel blocker glibenclamide (20 μ M)

Closed channel level is indicated by the horizontal arrows, and open levels are downward deflections. Transmembrane potential = +40mV. The vertical scale-bar represents 5 pA, and the horizontal scale-bar represents 100 ms.

Figure 5. Qualitative Western blot analysis for Kir6.2 in HEPG2ins/g (lane 1), MIN 6 (lane 2), HEPG2ins (lane 3), and HEPG2 cells (lane 4)

The molecular weight of Kir6.2 is 35 kDa. The MIN 6 cells (positive control) had the strongest staining for Kir6.2. There was a strong presence of Kir6.2 in the HEPG2ins/g cells. There was weaker staining for Kir6.2 in the HEPG2ins cells and almost no staining for Kir6.2 in the HEPG2 cells. This pattern of Kir6.2 expression follows the electrophysiology data, which indicated that the functional presence of the KATP channels was greatest in the HEPG2ins/g cells and absent in the HEPG2 cells.

Figure 6. Static stimulation of insulin secretion in cultures of HEPG2ins/g cells

Secretion of insulin from HEPG2ins/g cells in response to (a) 20 mM glucose, (b) 100 μ M tolbutamide, and (c) 20 mM glucose + 150 μ M diazoxide. Cells were incubated in basal medium for two consecutive 1-h periods before being exposed to the stimulus for 1 h (solid bars); cells in the control group were treated throughout with basal medium (unfilled bars). n, Number of experiments; B1, first basal period; B2: second basal period; B3: basal period following stimulation; S: stimulation period. Values are expressed as means $\pm$ SE.

Figure 7. Static stimulation of insulin secretion in cultures of HEPG2ins/g cells

Data have been normalized to the average insulin secretion measured in samples between 0 and 15 min (1.00 ± 0.001 pmoles insulin/ 10^8 cells), during which cells were perfused with basal medium without any stimuli. The y-axis shows the increase in insulin secretion as a multiple of the normalized basal secretion ($= 1.0$). Data are representative of three independent experiments.

Figure 8. Intracellular free Ca^{2+} changes in HEPG2 and HEPG2ins/g cells induced by glucose and the K_{ATP} channel antagonist glibenclamide

The vertical bars on each data point indicate SE where this is larger than the symbol. **A)** Glucose 20 mM stimulated an increase in the intracellular free Ca^{2+} level in HEPG2ins/g cells ($n=12$). **B)** Glibenclamide 100 μM also stimulated an increase in the intracellular free Ca^{2+} level in HEPG2ins/g cells ($n=9$). **C)** Glucose 20 mM failed to stimulate an increase in intracellular Ca^{2+} level in the HEPG2 cells ($n=10$). The horizontal bars indicate the time of application of glucose or glibenclamide.

Figure 9. Fluorescent labeling of secretory granules in HEPG2ins/g cells

a) Zinquin staining of secretory granules in HEP Gins/g cells superfused with 5 mM glucose in DMEM medium (control). **b)** Same HEP Gins/g cells as in (a), after changing the superfusing solution to one containing 150 μ M diazoxide in DMEM medium followed by the addition of 20 mM glucose in DMEM medium 15 min later. The image was taken after 45 mins' exposure to 20 mM glucose medium. The greater punctate staining indicates that the K_{ATP} -channel activator (diazoxide) has inhibited the glucose-stimulated secretion of insulin-containing granules in the HEP Gins/g cells despite prolonged exposure to 20 mM glucose.