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# **Insulin Trafficking in a Glucose Responsive Engineered Human Liver Cell Line is Regulated by the Interaction of ATP-Sensitive Potassium Channels and Voltage-Gated Calcium Channels**

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Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/52839>

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## **1. Introduction**

Type I diabetes is caused by the autoimmune destruction of pancreatic beta ( $\beta$ ) cells [1]. Current treatment requires multiple daily injections of insulin to control blood glucose levels. Tight glucose control lowers, but does not eliminate, the onset of diabetic complications, which greatly reduce the quality and longevity of life for patients. Transplantation of pancreatic tissue as a treatment is restricted by the scarcity of donors and the requirement for lifelong immunosuppression to preserve the graft, which carries adverse side-effects. This is of particular concern as Type 1 diabetes predominantly affects children. Lack of glucose control could be overcome by genetically engineering "an artificial  $\beta$ -cell" that is capable of synthesising, storing and secreting insulin in response to metabolic signals. The donor cell type must be readily accessible and capable of being engineered to synthesise, process, store and secrete insulin under physiological conditions.

The cell type of choice for the gene therapy of diabetes is not the  $\beta$ -cell.  $\beta$ -cells are greatly reduced or absent in people with Type I diabetes because of their autoimmune destruction. This fact will actively work against gene therapists trying to derive surrogate  $\beta$ -cells from

stem cells. There are innumerable theories describing putative mechanisms for preventing a patient's immune system from re-attacking transplanted  $\beta$ -cells, but the fact that the basic processes of islet cell attack have not been fully elucidated makes the search for relevant genes problematic. Thus, the engineering of non-pancreatic  $\beta$ -cells to synthesise, process, store and secrete insulin has several advantages, the most important of which is the ready availability of donor cells. If non  $\beta$ -cells from a diabetic individual can be engineered to produce insulin, then cellular rejection is less likely to occur since donor and recipient are autologous. In pursuit of this goal, hepatocytes have been shown to be suitable target cells for the generation of artificial  $\beta$ -cells [2-9]. Moreover, liver cells that produce insulin may not be prone to autoimmune attack [10]. The suitability of hepatocytes as a  $\beta$ -cell replacement is attributable, in part, to their inherent glucose responsiveness and their embryonic origin from the same endodermal precursor cells as the  $\beta$ -cell. Most importantly, liver cells express the high capacity glucose transporter, GLUT 2 [11], and the high capacity phosphorylation enzyme, glucokinase [12], which constitute the key elements of the "glucose sensing system" that regulates insulin release from pancreatic  $\beta$ -cells in response to small extracellular nutrient changes.

In pancreatic  $\beta$ -cells, a small increase in plasma glucose concentration stimulates significant insulin secretion. Therefore, glucose is the major modulator of  $\beta$ -cell function and this behaviour must be mimicked in insulin-secreting liver cells. In pancreatic  $\beta$ -cells,  $K_{ATP}$  channels, which are composed of four sulphonylurea receptor (SUR) subunits and four inwardly-rectifying potassium channel ( $K_{IR6.2}$ ) subunits [13-18], maintain resting membrane potentials and link plasma glucose concentrations to the insulin secretory machinery. The triggering pathway for insulin release begins with the uptake of glucose via the glucose carrier, GLUT2, and an acceleration of metabolism, such that glucose is used to generate ATP. An increase in the absolute intracellular concentration of ATP, with respect to ADP, stimulates the closure of  $K_{ATP}$  channels [19, 20]. Potassium conductance of the plasma membrane decreases, allowing a background current to shift the membrane potential away from the equilibrium potential for  $K^+$ , thus depolarising the membrane. Consequently, the pancreatic  $\beta$ -cell is able to translate metabolic signals to electrical signals, the latter regulating insulin secretion. Lack of functional  $K_{ATP}$  channels in insulin-secreting NES2Y cells resulted in the unregulated release of insulin, which was restored by expression of both  $K_{IR6.2}$  and SUR1 [21].

When depolarisation of the pancreatic  $\beta$ -cell reaches the threshold for activation of L-type ( $Ca_v1.3$ ), and to a lesser extent P/Q ( $Ca_v1.2$ ) and T-type ( $Ca_v3.x$ ) voltage-gated calcium channels, these open allowing  $Ca^{2+}$  influx down their electrochemical gradient [22]. The opening of  $Ca_v$  channels is intermittent, fluctuating with the membrane potential, therefore generating oscillations in the intracellular (cytosolic) calcium concentration ( $[Ca^{2+}]_i$ ), which, in turn, triggers pulsatile insulin secretion. In  $\beta$ -cells, elevation of  $[Ca^{2+}]_i$  occurs via the release of  $Ca^{2+}$  from intracellular stores (endoplasmic reticulum, mitochondria and secretory granules) and/or influx of extracellular  $Ca^{2+}$  through  $Ca_v$  channels [23, 24]. No functional  $Ca_v$  channels have been previously described in liver cells, however the presence of an  $\alpha 1$ -subunit lacking the voltage sensor has been reported in the rat liver cell line H4IIE [25] and an L-type  $\alpha 1$ -subunit has been detected at low levels in rat liver by RT-PCR [26].

For an insulin-producing liver cell to be of maximum benefit *in vivo* it must be capable of rapid responsive secretion of biologically active insulin. This characteristic demands that artificial  $\beta$ -cells process proinsulin to insulin and store it in granules. Our previous studies have shown that the insertion of genes encoding for insulin and the glucose transporter, GLUT2, into the HEPG2 human hepatoma cell line, resulted in synthesis and storage of (pro)insulin in structures resembling the secretory granules of pancreatic  $\beta$ -cells (HEPG2/ins/g), and the near physiological secretion of (pro)insulin in response to glucose [2, 3]. Similar to pancreatic  $\beta$ -cells, HEPG2ins/g cells responded to glucose via signalling pathways dependent upon  $K_{ATP}$  channels [27]. Therefore, expression of both insulin and GLUT2 in HEPG2 liver cells appeared to be sufficient for the generation of functional  $K_{ATP}$  channels, unlike the parental cell line that required pharmacological stimulation to activate the  $K_{ATP}$  channels [28]. It has previously been shown that stable transfection of the insulin gene into the human liver cell line, Huh7 (which endogenously expresses GLUT2), resulted in synthesis, storage, and regulated release of insulin to the physiological stimulus glucose (Huh7ins cells) [7]. Huh7ins cells are more akin to pancreatic  $\beta$ -cells than HEPG2/ins/g cells. They express a range of  $\beta$ -cell transcription factors [7, 29] and possess storage granules that cleave proinsulin to biologically active diarginyl insulin, due to the expression of the proconvertases PC1 and PC2 [7]. As Huh7ins cells also rapidly secrete insulin in a tightly regulated manner in response to glucose, the Huh7ins cells were able to reverse chemically induced diabetes when transplanted into an animal model [7], which HEPG2ins/g cells [3] failed to achieve [Tuch, unpublished results].

This chapter will detail the use of electrophysiological and biochemical techniques to show that Huh7ins cells respond to a glucose stimulus by closure of  $K_{ATP}$  channels and activation of  $Ca_v$  channels, which is an analogous mechanism to pancreatic  $\beta$ -cells. Patch-clamp electrophysiology of Huh7ins cells yielded current-voltage ( $I$ - $V$ ) curves that indicated the presence of potassium-selective currents; in contrast, currents recorded from Huh7 cells were non-selective. The presence of functional ATP-sensitive potassium ( $K_{ATP}$ ) channels and voltage-gated calcium ( $Ca_v$ ) channels was further validated by measurement of acute insulin secretion by Huh7ins cells in response to pharmacological channel inhibitors and activators and by calcium imaging and patch-clamp electrophysiology experiments. Molecular analyses were used to confirm that the Huh7ins cells express  $Ca_v$  and all the subunits of  $K_{ATP}$  channels. The secretion of insulin from granules in live Huh7ins cells was revealed by confocal microscopy which allowed visualization of secretion of insulin to a zinkin probe or an insulin-enhanced green fluorescent protein (EGFP) fusion protein (EGFP-ins). The glucose responsive mechanism that we observed in the Huh7ins cells was the same as that reported for the pancreatic  $\beta$ -cell line, MIN6 [30]. Prior to this study, the physiological interaction of  $K_{ATP}$  channels and  $Ca_v$  channels had never been shown in liver cells engineered to secrete insulin. As the biochemical properties of Huh7ins cells are akin to those of pancreatic  $\beta$ -cells, engineering hepatocytes in this way opens a promising avenue for the ultimate replacement of the endogenous  $\beta$ -cell function that is lost in Type I diabetes, by modifying a patient's own liver cells to become artificial  $\beta$ -cells. This is the first study that clearly delineates the control of insulin trafficking in a functioning artificial  $\beta$ -cell line that was derived from a human liver cell.

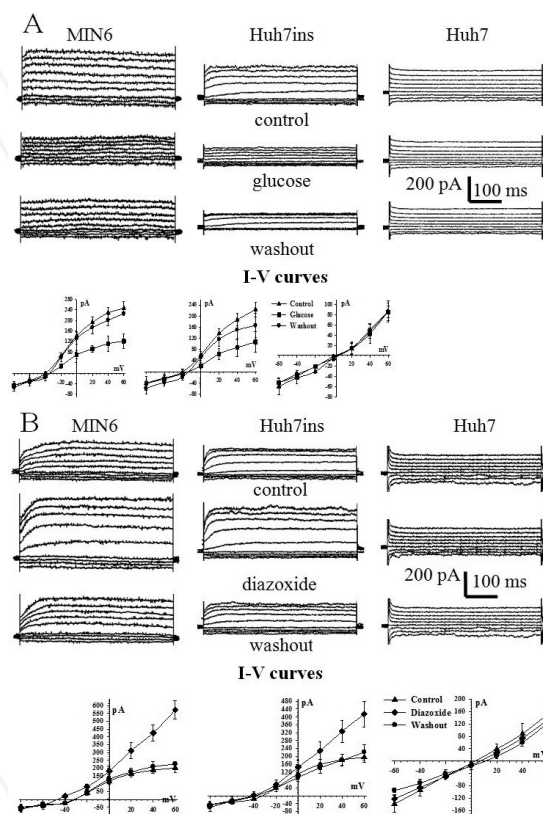
## 2. Understanding the mechanism by which liver-derived artificial beta cells respond to glucose and pharmacological stimulators and inhibitors of insulin secretion:

The mechanisms by which liver-derived artificial  $\beta$ -cells respond to glucose are poorly understood. Indeed, the majority of engineered insulin-secreting liver cells lack a truly regulated pathway of insulin release [31]. As pancreas and liver are derived from the same endodermal origin, the capacity of liver cells to differentiate into cells bearing pancreatic characteristics is well documented. A number of studies have shown that the expression of  $\beta$ -cell transcription factors in liver cells leads to pancreatic transdifferentiation, glucose-regulated insulin secretion and reversal of diabetes [4-7, 9, 32, 33]. Spontaneous pancreatic transdifferentiation and glucose-regulated insulin secretion have also been shown in dedifferentiated liver cells that express  $\beta$ -cell transcription factors such as the HEPG2ins/g and Huh7ins liver cell lines [3, 7, 9], as well as liver cells that have experienced a metabolic insult such as hepatic oval cells cultured in high glucose [34]. Consistent with this, our laboratory has shown spontaneous pancreatic transdifferentiation in hyperglycaemic rat livers and reversal of diabetes following the delivery of the insulin gene using a lentiviral vector [8]. Other recent studies in our laboratory have employed the H4IIE liver cell line, which does not express  $\beta$ -cell transcription factors and lacks a regulated pathway of insulin release [31]. When engineered to express the  $\beta$ -cell transcription factor *Neurod1* and rat insulin (H4IIEins/ND), H4IIE cells underwent pancreatic transdifferentiation and glucose-regulated insulin secretion from secretory granules. However, when *Neurod1* alone was expressed, an array of  $\beta$ -cell transcription factors and pancreatic hormones were expressed, but glucose-regulated insulin-secretion was not observed [9]. The Huh7 parent cell line, from which the insulin-secreting Huh7ins cells were derived, represents an ideal candidate for the engineering of an artificial  $\beta$ -cell. These cells possess several characteristics inherent to  $\beta$ -cells but not intrinsic to primary hepatocytes, such as the expression of  $\beta$ -cell transcription factors *Neurod1* [7], *Pdx1*, *Nkx2-2*, *Nkx6-1*, *Neurog3* and *Pax 6* [29]. Importantly, however, the process of transfection with insulin resulted in the formation of insulin secretory granules and the development of a regulated insulin secretory pathway [7] as was observed in the rodent H4IIEins/ND cells. Results of a mechanistic microarray analysis comparing Huh and Huh7ins cells following insulin transfection indicated that the formation of secretory granules and the development of a regulated secretory pathway was likely related to a protein interaction or posttranslational effect in combination with increased gene expression of secretory granule proteins such as chromogranin A [29].

### 2.1. Huh7ins cells possess potassium-selective plasma membrane channels

Huh7 (parental human liver cell line), Huh7ins (parental human liver cell line transfected with human insulin cDNA) [7] were maintained in Dulbecco's Modified Eagle's Medium

(DMEM) supplemented with 10% v/v fetal calf serum (FCS) (Trace Biosciences, Australia) in 5% CO<sub>2</sub> at 37°C. Although of murine origin, MIN6 cells are one of the few  $\beta$ -cell lines that are responsive to glucose in the physiological range, and, accordingly provide an established  $\beta$ -cell-like cell line for comparative purposes [30]. MIN6 cells were grown in DMEM supplemented with 15% v/v FCS (37°C, 5% CO<sub>2</sub>). For the Huh7ins cell line, the selective antibiotic G418 (0.55 mg/ml) was added to maintain stable transfectants.

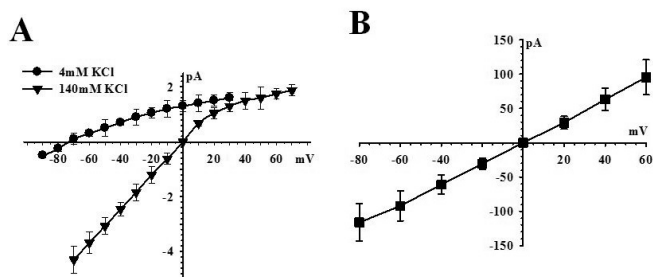


**Figure 1.** Sensitivity of potassium channels to glucose and diazoxide in Huh7ins cells. The upper three sets of current traces in panels A and B show superimposed families of whole-cell K<sup>+</sup> currents elicited by 450 ms test pulses from -80 to +80 mV in 10-mV steps. Lower graphs show the I-V relationship of the late current measured at the end of the test pulse and shows the mean  $\pm$  SEM at each potential. (A) Glucose (20 mM) reversibly inhibited the potassium currents of MIN6 and Huh7ins cells (left and centre columns;  $n = 4$ ), however glucose did not affect the non-selective currents of Huh7 cells (right column;  $n = 3$ ). (B) The channel opener diazoxide (100  $\mu$ M) reversibly increased the potassium currents of MIN6 and Huh7ins cells (left and centre columns;  $n = 9$ ), but did not effect the non-selective currents of Huh7 cells (right column;  $n = 3$ ).

To determine if functional K<sub>ATP</sub> channels were present in Huh7ins or Huh7 cells, K<sub>ATP</sub> channel currents were recorded using whole-cell patch-clamp electrophysiology, with MIN6 cells

being included as the positive control. Whole-cell patch-clamp recordings from potassium channels were made as previously described [27]. Cells grown on coverslips were transferred to a recording chamber and were perfused with a bath solution of the following composition (in mM): 140 Na acetate, 1  $\text{CaCl}_2$ , 1  $\text{MgCl}_2$ , 10 HEPES (pH 7.4). Patch pipettes were filled with an internal solution containing (in mM): 136 K acetate, 5 CsF, 5 KCl, 1 EGTA, 10 HEPES (pH 7.3). For inside-out patch-clamp recordings, the patch pipette was filled with (in mM): 135 NaCl, 5 KCl, 5  $\text{CaCl}_2$ , 2  $\text{MgSO}_4$ , 5 HEPES or a high  $\text{K}^+$  extracellular solution in which KCl replaced NaCl. The bath solution contained (in mM): 107 KCl, 11 EGTA, 2  $\text{MgSO}_4$ , 1  $\text{CaCl}_2$ , 11 HEPES (pH 7.2). For  $\text{Ca}_v$  channel analyses the bath solution contained (in mM): 115 NaCl, 5 KCl, 10  $\text{CaCl}_2$ , 10 HEPES, 2 D-glucose and 100  $\mu\text{M}$  tetrodotoxin (pH 7.4) and the internal solution contained (in mM): 10 CsCl, 115 Cs aspartate, 2.5 EGTA, 10 HEPES (pH 7.2). Channel currents were amplified and filtered using a MultiClamp amplifier (Molecular Devices, MDS Analytical Technologies, Toronto, Canada) and sampled on-line using a Digidata 1322 (A/D converter) and pClamp 8.2 software program (Molecular Devices).

The electrophysiological properties of the  $\text{K}_{\text{ATP}}$  channel in the Huh7ins cells closely resemble those reported for normal pancreatic  $\beta$ -cells [19]. The outward potassium currents of MIN6 and Huh7ins cells were sensitive to glucose and inhibited by perfusing 20 mM glucose for 5 min, with partial recovery of current amplitude after the washout of glucose for 10 min. In contrast, the non-selective outward and inward currents of Huh7 cells were not altered by the addition of 20 mM glucose (Figure 1A). The outward potassium currents of MIN6 and Huh7ins cells were also reversibly increased by perfusing with the  $\text{K}_{\text{ATP}}$  channel opener, diazoxide, 100  $\mu\text{M}$  (Figure 1B), whereas the non-selective currents of Huh7 cells were unaffected by diazoxide.



**Figure 2.** I-V curves of Huh7ins and Huh7 cells. (A) Mean current-voltage relations for inside-out patches of Huh7ins cells exposed to an external  $\text{K}^+$  concentration of either 140 mM or 5 mM  $\text{K}^+$  ( $n = 6$ ). (B) Using an internal and external  $\text{K}^+$  concentration of 5 mM and 140 mM respectively, the reversal potential ( $E_{\text{rev}}$ ) of Huh7 whole-cell currents ( $n = 6$ ), was approximately 0 mV, indicating a non-selective current. Values represent means  $\pm$  SEM.

Further support for the presence of functional  $\text{K}_{\text{ATP}}$  channels in Huh7ins cells was obtained by analysis of current-voltage ( $I$ - $V$ ) relationships of single channel currents, which had similar kinetics to that of pancreatic  $\beta$ -cells. Recordings were made from inside-out patches exposed to 140 mM  $[\text{K}^+]_{\text{i}}$  and either 140 mM  $\text{K}^+$   $[\text{K}^+]_{\text{o}}$  or 5 mM  $\text{K}^+$   $[\text{K}^+]_{\text{o}}$ . As would be expected



for a K<sup>+</sup>-selective channel, the single channel currents recorded with symmetrical [K<sup>+</sup>] reversed close to 0 mV, with a mean slope conductance of 48.5 pS (−80 to −10 mV). In comparison, the slope conductance was reduced to 12.4 pS (0 to +60 mV) when the [K<sup>+</sup>]<sub>o</sub> was reduced to 5 mM, indicating that the channel was K<sup>+</sup>-selective (Figure 2A). In contrast the *I*-*V* curve for K<sub>ATP</sub> channels in Huh7 cells indicated that currents from these cells were non-selective as the reversal potential was closer to 0 mV (Figure 2B). As secretory granules require K<sub>ATP</sub> channels for the appropriate release of insulin [35, 36], it is likely that Huh7ins cells also contained K<sub>ATP</sub> channels located intracellularly at the secretory granule membrane.

## 2.2. Secretion of insulin observed in real time in response to glucose and K<sub>ATP</sub> channel blockers

In order to observe, in real time, the secretion of insulin from granules in response to stimulators and inhibitors of insulin secretion by confocal microscopy, Huh7 and MIN 6 cells were engineered to express insulin fused to EGFP. To accomplish this, human insulin cDNA pC<sub>2</sub> (a gift from Dr. M. Walker, Weizmann Institute, Israel) [7] was cloned into the multicloning site of the pEGFP-N1 vector (Clontech, CA, USA). As there were no intervening stop codons, EGFP/insulin (EGFPins) was expressed as a fusion protein, which allowed visualization and localization of the fusion protein in cells. The construct (20 µg) or vector alone was introduced into Huh7 and MIN6 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), following the instructions of the manufacturer. To obtain stable transfectants, containing the construct (EGFPins) or empty vector (EGFP), G418 antibiotic (0.55 mg/ml) (Gibco Laboratories, Grand Island, NY) was added to the culture medium after 48 h. Media and G418 were changed every 2–3 days. After 3–4 weeks of selection, 25 colonies were chosen and screened for production of insulin by radioimmunoassay (RIA) [7] and EGFP by fluorescence microscopy. Human c-peptide was measured as previously described [8]. Clones were expanded into mass cultures and maintained in G418 selection media (37°C, 5% CO<sub>2</sub>). Huh7-EGFP (parental human liver cell line expressing EGFP) and Huh7-EGFPins (parental human liver cell line expressing EGFPins) cells were maintained in DMEM supplemented with 10% v/v fetal calf serum (FCS) (Trace Biosciences, Australia) in 5% CO<sub>2</sub> at 37°C. MIN6-EGFP (EGFP-expressing MIN6 cells) and MIN6-EGFPins (EGFPins-expressing MIN6 cells) cells were grown in DMEM supplemented with 15% v/v FCS (37°C, 5% CO<sub>2</sub>). For these transfected cell lines, the selective antibiotic G418 (0.55 mg/ml) was added.

To compare the function of Huh7-EGFPins and Huh7ins cells, chronic insulin secretion, insulin storage, and glucose-responsiveness were assessed. Acute insulin secretion was measured by static stimulation in basal medium consisting of PBS supplemented with (in mM): 1 CaCl<sub>2</sub>, 20 HEPES, 2 mg/ml BSA, 1.0 D-glucose; pH 7.4, as previously described [7]. Insulin was measured by RIA using human or rodent standards as previously described [7]. To assay insulin content, insulin was extracted from cells using 0.18 N HCl in 70% ethanol for 18 h at 4°C, as previously described [7]. To assess the quantity of human as compared to rodent insulin secreted by MIN6-EGFPins cells, a commercial RIA for human insulin (Linco Re-

search, MO, USA), was used. This has less than 1% and 6% cross-reactivity with rodent insulin and human proinsulin, respectively.

Of the 25 clones of Huh7-EGFPins isolated for analysis, insulin secretion differed 3-fold ( $0.11 \pm 0.2$  vs.  $0.32 \pm 0.2$  pmol insulin/ $10^6$  cells/24 h;  $n = 6$ ) and insulin storage varied 2-fold ( $3.4 \pm 1.2$  vs.  $7.1 \pm 0.3$  pmol insulin/ $10^6$  cells;  $n = 6$ ). Subsequently, six clones which secreted and stored the highest levels of insulin and exhibited consistently bright EGFP fluorescence, were examined for glucose responsiveness. Whilst all clones were glucose responsive, one clone (clone 16) was most comparable to Huh7ins cells [7] as it secreted equal amounts of insulin over a 24 h period ( $0.32 \pm 0.2$  vs.  $0.30 \pm 0.1$  pmol insulin/ $10^6$  cells for Huh7ins cells;  $n = 6$ ). Insulin storage was also comparable between the two cell lines with Huh7-EGFPins (clone 16) and Huh7ins cells storing  $7.1 \pm 0.3$  and  $7.0 \pm 0.2$  pmol/ $10^6$  cells ( $n = 6$ ), respectively. Glucose concentration-response curves for the Huh7-EGFPins (clone 16) and Huh7ins cell lines were also determined and revealed that there was no significant difference to previously published values [7] (data not shown). Levels of human proinsulin (not insulin) were  $11.4 \pm 1.2\%$  of total insulin ( $n = 6$ ). Human c-peptide levels were  $1.0 \pm 0.4\%$  of total insulin activity ( $n = 6$ ). Therefore clone 16 was used for all subsequent analyses, and is referred to as Huh7-EGFPins hereafter. As expected, Huh7-EGFP cells did not synthesize, store nor secrete insulin. Examination of the insulin secreted chronically by MIN6-EGFPins cells revealed that  $20.5 \pm 2.3\%$  ( $n = 6$ ) was of human origin, the remainder being rodent insulin. Of the insulin stored by MIN6-EGFPins cells,  $17.9 \pm 2.4\%$  ( $n = 6$ ) was human insulin. As expected, all of the insulin stored and secreted by MIN6-EGFP cells was of rodent origin. These data suggest that MIN6 cells handled EGFPins in a similar fashion to native rodent insulin.

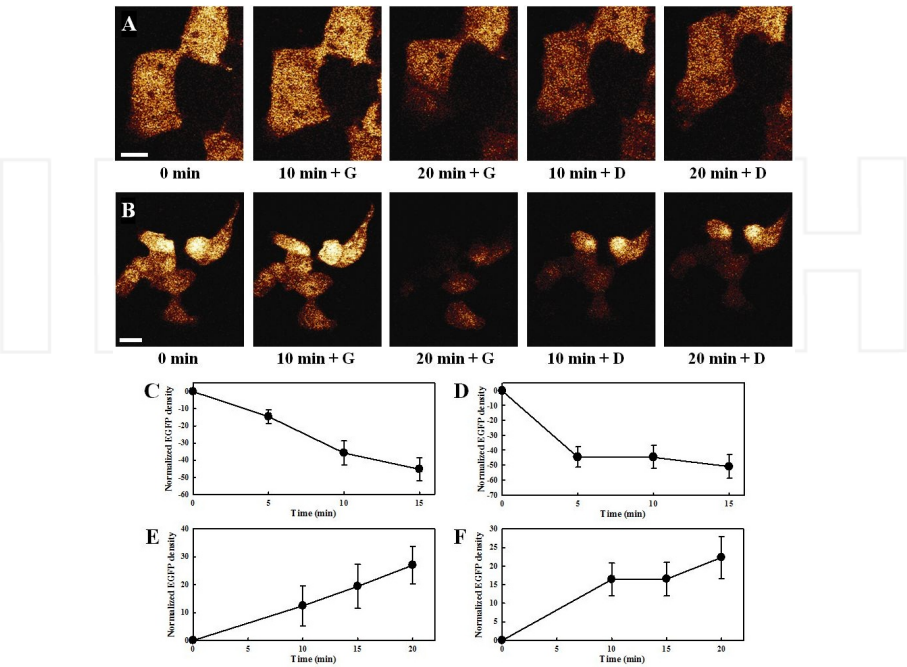
In order to perform confocal microscopy, cells were plated on coverslips (Marienfeld superior 22 mm diameter) and grown for 2–4 days. Each coverslip was inserted into a Perspex cell chamber, sealed with silicone grease, and overlaid with 1 ml DMEM containing 5 mM glucose (confocal scanning laser microscope [CSLM] medium). For Zinquin-E (zinquin ester, ethyl[2-methyl-8-*p*-toluenesulphonamido-6-quinolyloxy]acetate) staining, cells were incubated at 37°C for 30 min with CSLM medium containing 25  $\mu$ M zinquin E (Luminis Pty Ltd, Australia), as previously described [27]. After incubation, cells were rinsed with CSLM medium before recording confocal images with a Leica TCSNT (Wetzlar, Germany) with an inverted microscope (Leica DMRBE). Cells were imaged with a UV laser, oil 100x (N.A.1.4 UV-corrected Planapo) or oil 63x (N.A.1.32 UV-corrected Planapo). Emissions were collected with a BP490/440 filter. For analyses of stable transfectants expressing EGFP, incubation with a fluorescent probe was not required. These cells were imaged with an Ar/Kr laser and DP488/568 dichroic and emissions were collected with a BP525/550 filter.

CSLM medium or test solutions containing glibenclamide (20  $\mu$ M), or diazoxide (150  $\mu$ M) in CSLM medium, or DMEM containing 20 mM glucose, were exchanged at 37°C. Density measurements on images were performed using the public domain NIH Image program [37].

Defined regions of interest (ROI) for individual cells (10–30 cells per experiment) were followed through a time series before, and after, addition of test solutions. All values were normalized by subtracting the initial density, before addition of the test solution, from all the measurements in the series for each individual ROI to give a value of zero density for the



initial time point. Confocal microscopy detected intracellular EGFP-ins or Zinquin-E as punctuate fluorescence, which was indicative of insulin stored within secretion granules.



**Figure 3.** Confocal microscopic visualization of Huh7-EGFPins cells after exposure to glucose and diazoxide. (A) Huh7-EGFPins and (B) MIN6-EGFPins cells were incubated in DMEM containing 5 mM glucose (CLSM medium), then stimulated with glucose (20 mM, G) and diazoxide (150  $\mu$ M, D). Images were recorded in CLSM medium at 0, 10 and 20 min after glucose addition. At 20 min, cells were placed in CLSM medium containing diazoxide and images were recorded at 10 and 20 min after diazoxide exposure (bars = 10  $\mu$ m). Normalized EGFP density indicated that (C) Huh7-EGFPins ( $n = 60$ ) and (D) MIN6-EGFPins cells ( $n = 42$ ) showed decreasing EGFP density after addition of glucose, whereas (E) Huh7-EGFP ( $n = 19$ ) and (F) MIN6-EGFP cells ( $n = 12$ ) showed increasing EGFP density after addition of glucose. Values represent the mean  $\pm$  SEM.

For statistical analysis of all the confocal measurements described below SPSS version 11.5 (SPSS Inc) was used to determine a one-way analysis of variance after testing for homogeneity of variance using the Levene statistic. Huh7-EGFPins and MIN6-EGFPins cells responded in the same way to 20 mM glucose after 10 and 15 min, with loss of fluorescence from the ROI indicative of insulin secretion (Figure 3A-D). There was no significant difference between the response of Huh7-EGFPins and MIN6-EGFPins cells at 10 min ( $p > 0.5$ ,  $n = 60$ ) and 15 min ( $p \geq 0.7$ ,  $n = 42$ ). The MIN6-EGFPins cells responded more rapidly to the glucose stimulus than the Huh7ins-EGFP cells, with close to maximum loss of fluorescence achieved at 5 min, but after 10 min the two cell lines had achieved the same response level (Figure 3C-D). When diazoxide (150  $\mu$ M) was added to cells that had been stimulated by 20 mM glucose for 20 min, cytoplasmic fluorescence accumulated, as the release of insulin from

secretory granules was blocked (Figure 3A-B). The response of the control cell lines, Huh7-EGFP and Min6-EGFP to 20 mM glucose was significantly different ( $p < 0.00001$ ) at all time points from that of the engineered cell lines. In the control cell lines fluorescence increased over time (Figure 3E-F) since cells accumulated considerable amounts of EGFP within their cytoplasm and they were unresponsive to 20 mM glucose. Presumably this phenomenon is attributable to an inability of the parental cell lines to direct EGFP to secretory granules, whereas the cells engineered to synthesize insulin responded by releasing insulin from secretory granules so that their fluorescence decreased (Figure 3C-D).

Huh7ins and Min6 cells stained with the zinquin-E probe responded to 20 mM glucose with decreasing fluorescence. There was no significant difference between Huh7ins cells labelled with zinquin-E and Huh7-EGFPins cells in their response to 20 mM glucose after 5 min ( $p > 0.8$ ,  $n = 41$ ) and 15 min ( $P > 0.1$ ,  $n = 59$ ). After incubation with the  $K_{ATP}$  channel blocking sulphonylurea, glibenclamide (20  $\mu$ M), the two cell lines responded as they did in the presence of glucose (i.e. decreasing fluorescence was observed). Conversely, treatment with 150  $\mu$ M diazoxide, which inhibits glucose-activated  $\beta$ -cell depolarisation by suppressing closure of  $K_{ATP}$  channels, caused increased fluorescence, showing that secretion of insulin was blocked in Huh7ins-EGFP cells (Figure 3A) and Huh7ins cells (with zinquin-E probe).

Huh7ins-EGFP and MIN6ins-EGFP cells responded to either glucose or glibenclamide with a decrease in fluorescence (indicative of insulin secretion). The same secretory response to glucose or glibenclamide was seen in MIN6 and Huh7ins cells using the zinquin probe. Through its high affinity for the sulphonylurea subunit of the  $K_{ATP}$  channel, glibenclamide renders the  $K_{ATP}$  channel inactive and calcium influx through  $Ca_v$  channels ensues due to depolarisation of the cell membrane. The release of insulin from intracellular storage granules is the net result of these processes. As this response to glibenclamide was observed for Huh7ins, Huh7-EGFPins, MIN6 and MIN6-EGFPins cells, insulin secretion likely occurred via the classic insulin triggering pathway utilized by pancreatic  $\beta$ -cells. In contrast, the negative controls (Huh7-EGFP and MIN6-EGFP cells) or Huh7 cells in the case of Zinquin-E labelling, were unresponsive to glucose or glibenclamide. The increased fluorescence of the negative control cells MIN6-EGFP and Huh7-EGFP after glucose stimulation showed that there was no trafficking of EGFP to secretory granules. In an earlier publication, Arvan and Halban [38] questioned the specificity of the trans Golgi network sorting process, but the fact that in our cell lines the secretion of EGFP-ins was regulated while EGFP was not, shows that the sorting of EGFP was specific, with only EGFP-insulin being trafficked to secretory granules.

### 2.3. Huh7ins cells express the $K_{ATP}$ channel subunits, $K_{IR}6.2$ , SUR2A and SUR2B, and the $\alpha 1$ -subunit of the $Ca_v1.3$ channel

Primers were designed to the cDNA sequences encoding the human  $K_{ATP}$  subunits,  $K_{IR}6.2$  (F: AGCCCAAGTTCAGCATCTCTCC, R: CCAGAAATAGCATAGTGACAAGTGCC), SUR1 (F: TCAGGGTTGTGAACCGCA, R: GTTCTGCGAAGCATAGGC), SUR2A (F:

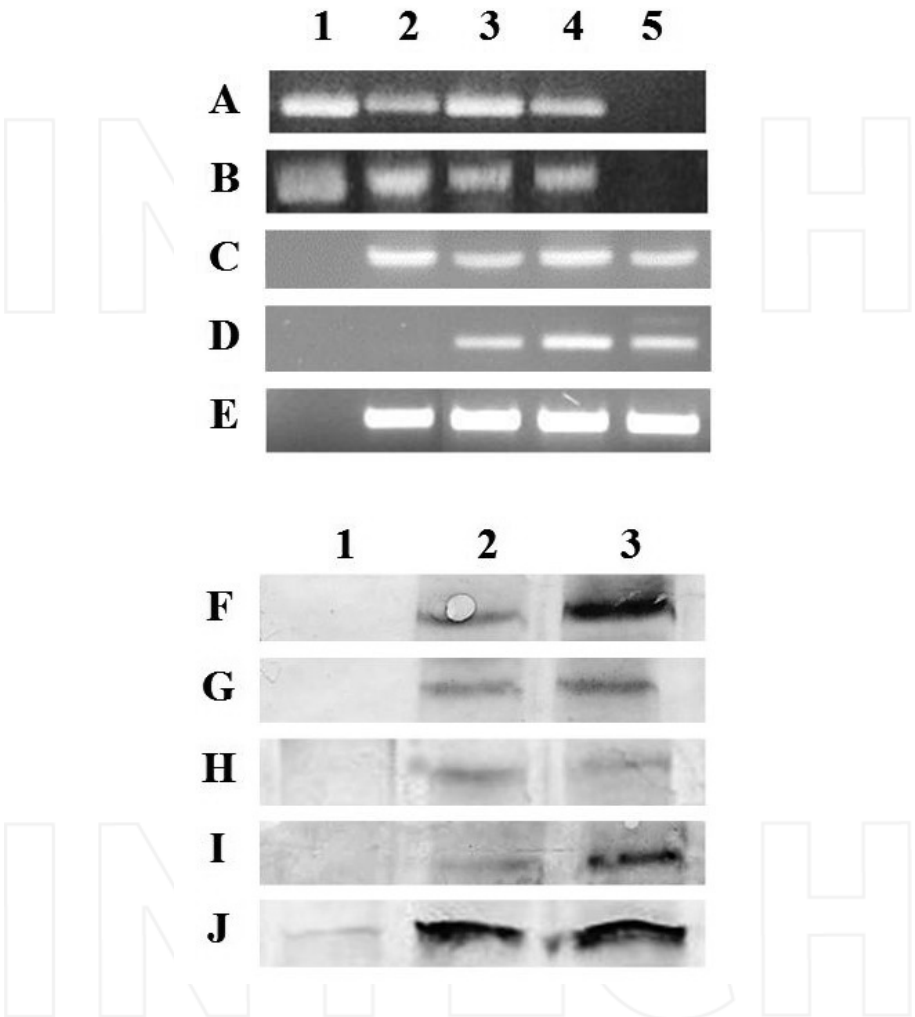
GGCAGGTGGGAAATCATCGTTA, R: TCCCCACCTTCAGTGACAA') and SUR2B (F: GATGCGGTTGTCAGTAA, R: ACTCCTTCACATGTCTGC). Primers were also designed to amplify the  $\alpha$ 1-subunit of the  $\text{Ca}_v1.3$  channel of pancreatic  $\beta$ -cells (F: TGGCAGGAGATCATGCTGG, R: CTAATCTCTTGCTCGCTACC). RT-PCR analyses were performed using the cDNA synthesised from RNA isolated from Huh7 and Huh7ins cells using TRIzol® Reagent (Invitrogen). Positive controls were HEPG2 cells that express the human  $\text{K}_{\text{IR}}6.2$  and SUR2A subunits [27], or human pancreatic islets.

Immunoblot analyses were performed using protein extracted from Huh7 and Huh7ins cells and human pancreatic islets to detect the human  $\text{K}_{\text{ATP}}$  subunits, SUR1, SUR2A and SUR2B, and the  $\alpha$ 1-subunit of the  $\text{Ca}_v1.3$  channel. Detection of the  $\text{K}_{\text{IR}}6.2$  subunit was determined as previously described [27]. For detection of  $\text{K}_{\text{IR}}6.2$ , SUR1, SUR2A, SUR2B and the  $\alpha$ 1-subunit of the  $\text{Ca}_v1.3$  channel, cell supernatants were suspended in buffer I containing (in mM): 10 Tris, 20  $\text{NaH}_2\text{PO}_4$ , 1 EDTA, 0.1 PMSF, 10  $\mu\text{g/ml}$  pepstatin, 10  $\mu\text{l/ml}$  leupeptin (pH 7.8), subjected to three freeze-thaw cycles, and then incubated for 20 min at 4°C. The protein concentration of the supernatant was determined using a Micro Bicinchoninic Protein Assay Reagent Kit (PIERCE, Thermo Fisher Scientific, Rockford, IL, USA). Protein samples (15  $\mu\text{g}$ ) were electrophoresed in 10% polyacrylamide gels (100 V) and then transferred to nitrocellulose membranes (Millipore Corporation, USA) for immunoblot analyses. Nitrocellulose membranes were blocked in PBS with 5% w/v skim milk overnight at 4°C. Immunoblotting was performed using a 1:1000 dilution of goat anti-human  $\text{K}_{\text{IR}}6.2$ , SUR1, SUR2A, SUR2B and the  $\alpha$ 1-subunit of the  $\text{Ca}_v1.3$  channel polyclonal IgGs (Santa Cruz Biotech. USA) and detection was achieved using monoclonal (mouse) anti-goat/sheep horseradish peroxidase IgG conjugate (1:800 dilution) (Sigma).

RT-PCR analysis revealed that the Huh7 and Huh7ins cells expressed the human  $\text{K}_{\text{ATP}}$  channel subunit,  $\text{K}_{\text{IR}}6.2$ , and the  $\beta$ -cell sulfonylurea receptor subunits, SUR2A and SUR2B (Figure 4A-C), together with the human  $\alpha$ 1-subunit of the  $\text{Ca}_v1.3$  channel (Figure 4E). SUR1 was only detected in the Huh7ins liver cell line (Figure 4D). Immunoblot analysis for the presence of  $\text{K}_{\text{IR}}6.2$ , SUR1, SUR2A and SUR2B, revealed strong expression in Huh7ins cells and human pancreatic islets, with no detectable expression in Huh7 cells (Figure 4F-I). The presence of protein product for the  $\alpha$ 1-subunit of the  $\text{Ca}_v1.3$  channel was confirmed by immunoblot analysis of protein extracted from Huh7ins cells, with only low expression in Huh7 cells (Figure 4J).

Thus, unlike the glucose-responsive insulin-secreting cell line, HEPG2ins/g [26], the Huh7ins cells expressed the SUR1 receptor as do pancreatic  $\beta$ -cells. The functional recording of  $\text{K}_{\text{ATP}}$  activity in Huh7ins cells are supported by the immunoblot analyses, which suggests that  $\text{K}_{\text{IR}}6.2$ , SUR1, SUR2A and SUR2B are strongly expressed in Huh7ins cells. There was no detectable expression of  $\text{K}_{\text{IR}}6.2$ , SUR1, SUR2A and SUR2B in Huh7 cells, which is supported by the absence of  $\text{K}_{\text{ATP}}$  currents in the patch-clamp recordings (Figure 2B). Expression of  $\text{K}_{\text{IR}}6.2$  and SUR1, the two relevant subunits of the pancreatic  $\beta$ -cell  $\text{K}_{\text{ATP}}$  channel, is commonly seen in primary hepatocytes, although dedifferentiated cell lines such as HEPG2 [28] and Huh7 cells appear to have lost expression of SUR1 at the mRNA level. It is apparent that the process of pancreatic transdifferentiation, which has caused the formation

of secretory granules, has resulted in expression of  $K_{IR}6.2$  protein and SUR1 at the mRNA level and protein expression in Huh7ins cells.



**Figure 4.** RT-PCR and immunoblot analysis for  $K_{ATP}$  and  $Ca_v$  channel subunits. RT-PCR analysis of liver cell lines for (A) human  $K_{IR}6.2$ : HuH7 (lane 1), Huh7ins (lane 2), Huh7-EGFPins (lane 3), HEPG2 (lane 4, positive control), and no cDNA control (lane 5); (B) human SUR2A: Huh7 (lane 1), Huh7ins (lane 2), Huh7-EGFPins (lane 3), HEPG2 (lane 4, positive control), and no cDNA control (lane 5); (C) human SUR2B: no cDNA (lane 1), human pancreas (lane 2, positive control), Huh7 (lane 3), Huh7ins (lane 4), Huh7-EGFPins (lane 5), (D) Human SUR1: no cDNA (lane 1), Huh7 (lane 2), Huh7ins (lane 3), Huh7-EGFPins (lane 4), human pancreas (lane 5, positive control), (E) human  $\alpha 1$ -subunit of the  $Ca_v1.3$  channel: no cDNA (lane 1), human pancreas (lane 2, positive control), Huh7 (lane 3), Huh7ins (lane 4), Huh7-EGFPins (lane 5). Immunoblot analysis for (F) human  $K_{IR}6.2$ , (G) human SUR2A, (H) SUR2B, (I) SUR1 and (J) the  $\alpha 1$ -subunit of the  $Ca_v1.3$  channel in Huh7 (lane 1), Huh7ins (lane 2) and human islet (lane 3).

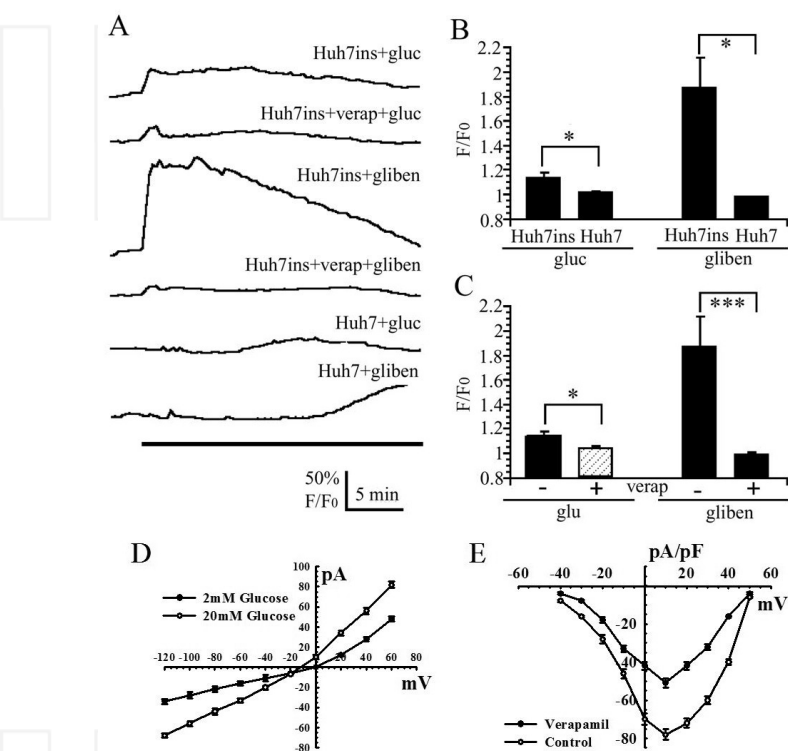
## 2.4. Huh7ins cells possess $\text{Ca}_v$ channels

The level of intracellular free  $\text{Ca}^{2+}$  was measured using Fluo4-AM and pluronic F-127 with a Zeiss microscope (Axiovert 200M; Zeiss, Germany). Cells were grown on coverslips until 50–70% confluent and were then incubated in culture medium containing 8  $\mu\text{M}$  Fluo4-AM (Invitrogen, Carlsbad, CA) and 0.1% pluronic F-127 (Invitrogen) at 37°C for 60 min. To remove excess Fluo4-AM and F-127, the cells were incubated with HEPES buffer containing (in mM): 140 NaCl, 5 KCl, 2  $\text{CaCl}_2$ , 1  $\text{MgCl}_2$ , 5 D-glucose, 10 HEPES (pH 7.4), for 30 min images were captured. The coverslips were then placed in a chamber containing HEPES buffer. After control images were taken (before addition of glucose or glibenclamide), the cells were exposed to 20 mM glucose or 20  $\mu\text{M}$  glibenclamide until the completion of experiments. For the experiments in the presence of  $\text{Ca}_v$  channel blocker, the cells were incubated with 10  $\mu\text{M}$  verapamil for 30 min before the addition of glucose or glibenclamide. Fluorescence intensity was observed under a Zeiss microscope and images were captured with a digital camera (Axio-Cam, Zeiss) and the Axiovision program (Zeiss). Images were taken every 20 s and analyzed using ImageJ software [39]. Results were presented as relative fluorescence values ( $F/F_0$ ), where  $F_0$  represents the fluorescence of controls (before addition of glucose or glibenclamide).

While the expression of  $\text{Ca}_v$  channels in pancreatic  $\beta$ -cells has been well documented [23, 40], their precise role in hepatocytes is yet to be elucidated. It has been reported that  $\text{Ca}_v1$  channels are found in endocrine (pancreatic), cardiac and neural cells [41], but no physiologically-active  $\text{Ca}_v1$  channels have been identified in hepatocytes prior to this study. Calcium imaging revealed that an increase in the extracellular glucose concentration from 5 to 20 mM immediately stimulated an elevated level of free  $[\text{Ca}^{2+}]_i$  in Huh7ins cells, which peaked within 2 min and then gradually recovered to the level observed prior to application of 20 mM glucose (Figure 5A). The  $F/F_0$  value at 2 min after the application of 20 mM glucose in Huh7ins cells was  $1.14 \pm 0.038$  ( $n = 33$ , Figure 5B). However, 20 mM glucose did not significantly increase the level of free  $[\text{Ca}^{2+}]_i$  in Huh7 cells ( $F/F_0 = 1.02 \pm 0.01$ ,  $n = 19$ ), which was significantly lower than that of Huh7ins cells (Fig 5A-B). To examine if blockade of  $\text{K}_{\text{ATP}}$  channels mimicked the effect of 20 mM glucose, glibenclamide (20  $\mu\text{M}$ ) was applied in the bath solution containing 5 mM glucose. Glibenclamide dramatically increased the level of intracellular free  $\text{Ca}^{2+}$  ( $F/F_0 = 1.87 \pm 0.24$ ,  $n = 25$ ), which had a similar time course to that observed in the presence of 20 mM glucose, but with a greater peak amplitude. Similar to the effects of 20 mM glucose on Huh7 cells, glibenclamide did not alter calcium flux in Huh7 cells (Figure 5A-B). It should be noted that both 20 mM glucose and 20  $\mu\text{M}$  glibenclamide produced a more delayed increase in the  $[\text{Ca}^{2+}]_i$  in Huh7 cells in comparison with data recorded in Huh7ins cells (Figure 5A).

Verapamil (10  $\mu\text{M}$ ), a phenylalkylamine  $\text{Ca}_v1.x$  channel blocker, inhibited the increase in  $[\text{Ca}^{2+}]_i$  in Huh7ins cells produced by 20 mM glucose ( $1.04 \pm 0.02$ ,  $n = 31$ ) and glibenclamide ( $0.99 \pm 0.02$ ,  $n = 31$ ; Figure 5A and C). This indicated that the observed glucose-induced block and diazoxide-induced increase in free  $[\text{Ca}^{2+}]_i$  was mediated by  $\text{Ca}_v$  channels. To further validate this interpretation, we used the whole-cell patch-clamp technique to measure the effect of increased glucose on membrane currents in Huh7ins and Huh7 cells. The resultant  $I$ - $V$  curve indicated that increasing the concentration of glucose from 2 to 20 mM resulted in ac-

tivation of an inwardly-rectifying current in Huh7ins cells (Figure 5D). This current was blocked by the addition of CsCl thereby lending further support to the premise that it was mediated via  $K^+$  channels. No activation was seen when Huh7 cells were used in these experiments (results not shown).  $Ca_v$  channel currents recorded from Huh7ins cells were inhibited by verapamil (10  $\mu$ M), indicating that  $Ca_v1.x$  channels were involved in the response (Figure 5E). This further corroborates the calcium imaging data described above.



**Figure 5.** Calcium imaging and patch-clamp electrophysiology of Huh7ins and Huh7 cells. High glucose and blockade of  $K_{ATP}$  channels elevated levels of intracellular free  $Ca^{2+}$  in Huh7ins cells. (A) Averaged time courses of relative fluorescence intensity ( $F/F_0$ ) induced by 20 mM glucose (gluc) and 20  $\mu$ M glibenclamide (gliben) in the presence, and absence, of 10  $\mu$ M verapamil (verap,  $Ca_v1.x$  channel blocker) in Huh7ins and Huh7 cells. The black bar at the base of panel A represents the time of application of glucose or glibenclamide. Each trace represents an average  $F/F_0$  value of the cells investigated. (B) Glucose and glibenclamide increased the level of free  $[Ca^{2+}]_i$  in Huh7ins cells, but not in Huh7 cells. (C) Glucose- and glibenclamide-induced increases in intracellular free  $Ca^{2+}$  in Huh7ins cells were significantly inhibited by 10  $\mu$ M verapamil. The values shown in B and C were taken 2 min after application of glucose or glibenclamide. \*  $p < 0.05$  and \*\*\*  $p < 0.001$ . (D) Mean I-V relationship in Huh7ins cells under low (2 mM) and high (20 mM) glucose conditions ( $n = 4$ ). (E) I-V curves for  $Ca_v$  channel currents in Huh7ins cells in the presence of 20 mM glucose and following the addition of 10  $\mu$ M verapamil ( $n = 6$ ). Values are expressed as means  $\pm$  SEM

The  $Ca_v1.3$   $\alpha 1$  subunit (Figure 4J), expressed in pancreatic  $\beta$ -cells [42], was detected in both Huh7ins cells and the parental Huh7 cells, at both the mRNA and the protein level, suggest-



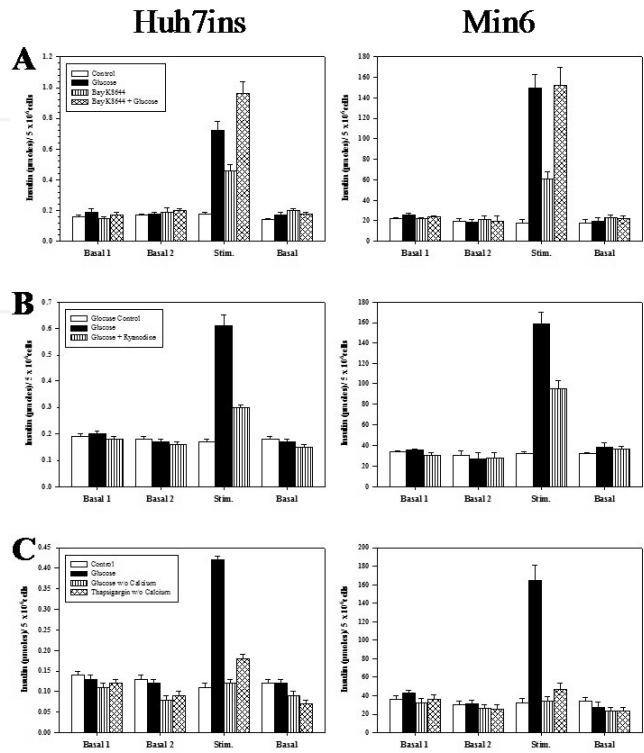
ing that Huh7ins and Huh7 cells possess  $\text{Ca}_v1.3$  channels that are similar to those found in pancreatic  $\beta$ -cells.  $\text{Ca}^{2+}$  imaging and patch-clamp electrophysiology experiments further detected a  $\text{Ca}_v$  channel current in Huh7ins cells, which was stimulated by glucose and inhibited by verapamil. The expression of functional  $\text{Ca}_v$  channels in Huh7ins cells may explain, in part, the acute secretion of insulin in response to glucose stimulation. The mechanism of insulin secretion depends upon the activities of ion channels in the plasma membrane, and, more critically, upon the activation of  $\text{Ca}_v$  channels, caused indirectly by increased glucose metabolism. Influx of  $\text{Ca}^{2+}$ , through open  $\text{Ca}_v$  channels, is responsible for the exocytosis of insulin storage granules, emphasising the importance of  $\text{Ca}_v$  channels in glucose-stimulated insulin secretion [41]. The lack of functional  $\text{Ca}_v$  channels in Huh7 cells is likely related to the low level of expression of the  $\text{Ca}_v1.3$   $\alpha 1$ -subunit. Once it was determined that Huh7ins cells possessed functional  $\text{Ca}_v$  channels, static stimulation experiments using the inhibitor verapamil, and the activator BayK8644, established that  $\text{Ca}_v$  channels in Huh7ins cells function in a similar manner to  $\text{Ca}_v$  channels in pancreatic  $\beta$ -cells.

## 2.5. Huh7ins cells appear to be glucose-responsive through the presence of functional $\text{K}_{\text{ATP}}$ channels and $\text{Ca}_v$ channels

To measure insulin secretion, monolayers of cells were incubated with  $\text{K}_{\text{ATP}}$  channel modulators, using concentrations determined from concentration-response curves in the corresponding cell lines. These included the  $\text{K}_{\text{ATP}}$  channel activators tolbutamide (100  $\mu\text{M}$ ) or diazoxide (150  $\mu\text{M}$ ) and the  $\text{K}_{\text{ATP}}$  channel blocker glibenclamide (20  $\mu\text{M}$ ) with or without 20 mM glucose for 1 h. The effects of the  $\text{Ca}_v$  channel blocker verapamil (10  $\mu\text{M}$ ), the  $\text{Ca}_v$  channel activator Bay K8644 (1  $\mu\text{M}$ ), the sarcoplasmic and endoplasmic reticulum family of  $\text{Ca}^{2+}$ -ATPases (SERCA) blocker ryanodine (20  $\mu\text{M}$ ), the SERCA stimulator thapsigargin (1  $\mu\text{M}$ ), and the hemi-channel blocker oleic acid (20  $\mu\text{M}$ ) were also assessed. Inhibitors and activators were purchased from Sigma, Sydney, Australia. Results were expressed as means  $\pm$  standard error of the mean (SEM). The statistical analysis of insulin RIA results was by univariate repeated measures analysis of variance using Systat<sup>TM</sup> version 9. Post-hoc comparisons were made using Tukey's HSD test (Minitab<sup>TM</sup> version 13, Minitab Inc).

Stimulation with 20 mM glucose resulted in a 3.6- and 5.2-fold increase in insulin secretion by Huh7ins and MIN6 cells, respectively (Figures 6 and 7). Incubation of Huh7-EGFPins cells with the  $\text{K}_{\text{ATP}}$  channel blocker, glibenclamide, significantly increased insulin secretion by Huh7-EGFPins from  $0.06 \pm 0.01$  to  $0.26 \pm 0.03$  pmol/ $10^6$  cells ( $p < 0.001$ ,  $n = 6$ ). The  $\text{K}_{\text{ATP}}$  activator, diazoxide, completely inhibited glucose-stimulated insulin release from Huh7-EGFPins ( $0.05 \pm 0.02$  pmol/ $10^6$  cells,  $n = 6$ ) and MIN6-EGFPins cells (data not shown). It was also noted that, diazoxide treatment prevented glucose-induced insulin secretion in Huh7ins and Huh7-EGFPins cells. Diazoxide causes sustained opening of  $\text{K}_{\text{ATP}}$  channels causing hyperpolarisation of the cell membrane, thereby preventing the voltage-dependant calcium response and inhibiting insulin exocytosis [43]. Static glucose stimulation experiments demonstrated that the insulin secretory response of Huh7ins and Huh7-EGFPins cells functioned via the channel-dependant pathway of insulin secretion. The responses of Huh7ins and MIN6 cells to diazoxide and

glibenclamide treatment were identical to that observed in each of the cell lines in which insulin was fused to EGFP (data not shown). Therefore, fusion of EGFP to insulin did not alter the physiological mechanism of insulin secretion.



**Figure 6.** Secretion of insulin from Huh7ins cells and MIN6 cells. Insulin secretion was activated in response to 20 mM glucose alone or (A) 1  $\mu$ M BayK8644  $\pm$  20 mM glucose, (B) 20  $\mu$ M ryanodine  $\pm$  20 mM glucose; and (C) 1  $\mu$ M thapsigargin in the absence of extracellular calcium. Cells were incubated in basal medium for two consecutive 1 h periods before being exposed to the stimulus for 1 h, followed by a third period of basal incubation. Cells in the control group were treated throughout with basal medium. Values are expressed as means  $\pm$  SEM ( $n = 6$ ).

The application of the  $\text{Ca}_v1.x$  channel activator, BayK8644 to Huh7ins and MIN6 cells significantly increased insulin secretion above basal levels ( $p < 0.01$ ,  $n = 6$ ; Figure 6A). In the presence of 20 mM glucose, BayK8644 further amplified glucose-stimulated insulin secretion in Huh7ins cells ( $p < 0.05$ ,  $n = 6$ , Fig. 6A). Application of the SERCA blocker, ryanodine, which prevents increases in  $[\text{Ca}^{2+}]_i$ , caused a decrease in glucose-stimulated insulin secretion from Huh7ins and MIN6 cells ( $p < 0.05$ ,  $n = 6$ ; Figure 6B).

The dihydropyridine, BayK8644, functions as a  $\text{Ca}_v1$  channel agonist, which interacts with the  $\alpha_1$  subunit of  $\text{Ca}_v$  channels to stabilise the channel in the open state, thereby enhancing  $\text{Ca}^{2+}$  influx to cause the exocytosis of insulin [41]. BayK8644 does not change the membrane

potential of resting  $\beta$ -cells [43]. Rather, it acts on the  $\text{Ca}_v$  channel in the open state, failing to affect basal insulin secretion at non-stimulatory glucose concentrations [43], but exaggerating glucose-stimulated insulin secretion [44, 45]. The addition of BayK8644 increased insulin secretion by both the Huh7ins and MIN6 cells. However, the amount of insulin secreted in the presence of BayK8644 was lower than that released in response to 20 mM glucose alone. Putatively, this concentration of glucose may have stimulated the influx of extracellular  $\text{Ca}^{2+}$ , the release of  $[\text{Ca}^{2+}]_i$  from intracellular stores and increased  $\text{Ca}^{2+}$  via other  $\text{Ca}^{2+}$ -related pathways to such an extent that the total increase of  $\text{Ca}^{2+}$  in the cell was higher in the presence of 20 mM glucose as compared to BayK8644 alone.

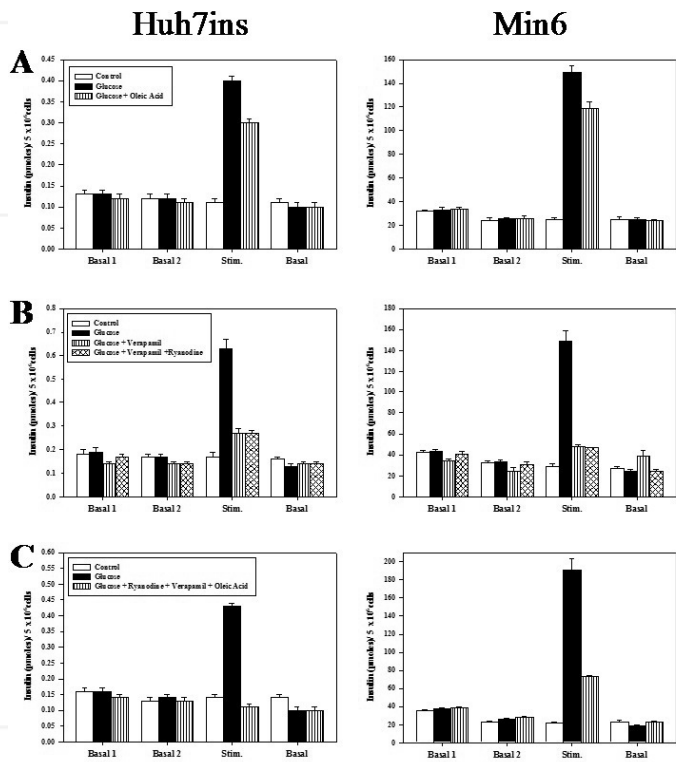
Consistent with reports that BayK8644 is known to stimulate the opening of  $\text{Ca}_v$  channels in pancreatic  $\beta$ -cells without altering the membrane potential [44], static stimulation of Huh7ins cells with 1  $\mu\text{M}$  BayK8644 plus 20 mM glucose amplified glucose-stimulated insulin release. However, BayK8644 failed to amplify glucose-stimulated insulin secretion in MIN6 cells. This finding may be attributable to the ability of 20 mM glucose alone to cause the maximum threshold in the activation of insulin release in MIN6 cells, such that the addition of BayK8644 was unable to exert any additional stimulatory effects. Nevertheless, these results demonstrate that the insulin secretory response of the Huh7ins cells is dependent upon the activation of  $\text{Ca}_v$  channels, as is the case for pancreatic  $\beta$ -cells.

Static stimulation of Huh7ins cells with the highly specific SERCA blocker thapsigargin, which induces the release of  $\text{Ca}^{2+}$  from intracellular stores resulted in a significant increase (two-fold increase over basal levels), in insulin secretion in the absence of extracellular  $\text{Ca}^{2+}$  ( $p < 0.05$ ,  $n = 6$ ; Figure 6C). Consistent with results from Tuch *et al.* [7], the response of the Huh7ins cells to glucose was abolished when  $\text{Ca}^{2+}$  was removed from the basal medium before 20 mM glucose was added ( $p > 0.05$  vs. control,  $n = 6$ ; Figure 6C). MIN6 cells showed a similar response; namely, in the absence of extracellular  $\text{Ca}^{2+}$  the glucose-responsiveness was abolished ( $p > 0.05$  vs. control,  $n = 6$ ; Figure 6C), and the presence of 1  $\mu\text{M}$  thapsigargin significantly increased insulin secretion 1.8-fold over basal levels ( $P < 0.05$ ,  $n = 6$ ; Figure 6C).

The connexon (hemi-channel blocker), oleic acid, significantly reduced acute insulin secretion by 1.4-fold ( $p < 0.05$ ,  $n = 6$ ; Figure 7A), while verapamil (10  $\mu\text{M}$ ) resulted in a significant decrease in insulin secretion to glucose in both cell lines ( $p < 0.05$ ,  $n = 6$ ; Figure 7B). However, the combination of verapamil and ryanodine did not exert an additive effect on insulin secretion, compared to treatment with verapamil alone ( $p < 0.05$ ,  $n = 6$ ; Fig. 7B). Nevertheless, a greater decrease in insulin secretion was observed after the addition of verapamil, ryanodine and oleic acid in both Huh7ins ( $p < 0.05$ ,  $n = 6$ ) and MIN6 cells ( $p < 0.05$ ,  $n = 6$ ; Figure 7C).

SERCA operate to restore diminished intracellular endoplasmic and sarcoplasmic reticulum  $\text{Ca}^{2+}$  stores, thereby decreasing cytoplasmic  $\text{Ca}^{2+}$  levels [46-50]. Thapsigargin is a highly selective inhibitor of SERCA. Stimulation of  $\beta$ -cells with glucose causes an initial, thapsigargin-inhibitable, drop in  $[\text{Ca}^{2+}]_i$  that precedes the increase in  $[\text{Ca}^{2+}]_i$  due to the pumping of  $\text{Ca}^{2+}$  into the endoplasmic reticulum [51, 52]. Blocking of SERCA by thapsigargin augments the glucose-induced  $[\text{Ca}^{2+}]_i$  increase by activating a depolarising store-operated current, which then facilitates the opening of  $\text{Ca}_v$  channels [51, 53, 54]. Consistent with the results reported by Tuch *et al.* [7], in the absence of extracellular  $\text{Ca}^{2+}$ , the glucose responsiveness of

both Huh7ins and MIN6 cells in the absence of extracellular  $\text{Ca}^{2+}$ , was lost, while normal glucose responsiveness was seen when  $\text{Ca}^{2+}$  was present in the medium. However, thapsigargin, which raises cytosolic  $\text{Ca}^{2+}$ , stimulated insulin secretion by both Huh7ins and MIN6 cells in the absence of extracellular  $\text{Ca}^{2+}$ . This finding further supports the role of intracellular  $\text{Ca}^{2+}$  storage in insulin secretion in both pancreatic  $\beta$ -cells and in the insulin-secreting liver cell line, Huh7ins.



**Figure 7.** Secretion of insulin from Huh7ins cells and MIN6 cells. Insulin secretion was activated in response to 20 mM glucose alone or in the presence of (A) 20  $\mu\text{M}$  oleic acid; (B) 10  $\mu\text{M}$  verapamil  $\pm$  10  $\mu\text{M}$  ryanodine and (C) 10  $\mu\text{M}$  verapamil, 20  $\mu\text{M}$  ryanodine and 20  $\mu\text{M}$  oleic acid. Cells were incubated in basal medium for two consecutive 1 h periods before being exposed to the stimulus for 1 h, followed by a third period of basal incubation. Cells in the control group were treated throughout with basal medium. Values are expressed as means  $\pm$  SEM ( $n = 6$ ).

The presence of 20  $\mu\text{M}$  ryanodine, which blocks  $\text{Ca}_v$  channels at concentrations  $\geq 10 \mu\text{M}$  [55] and prevents the release of  $\text{Ca}^{2+}$  from the endoplasmic reticulum, reduced the glucose-responsiveness of both Huh7ins cells and MIN6 cells, although to a lesser extent than was observed in the presence of 10  $\mu\text{M}$  verapamil. This finding is consistent with previous reports that intracellular  $\text{Ca}^{2+}$  stores (and therefore SERCA) contribute to the intracellular  $\text{Ca}^{2+}$  response during insulin secretion. The application of verapamil to Huh7ins cells caused a

complete abrogation of glucose-responsiveness upon extracellular  $\text{Ca}^{2+}$  levels has been previously reported for pancreatic  $\beta$ -cells [40, 43, 56]. As expected the addition of oleic acid to Huh7ins and MIN6 cells resulted in reduced glucose responsiveness, due to the blockage of hemi-channels, similar to what has been reported in pancreatic  $\beta$ -cells [57].

### 3. Conclusion

The results described in this chapter indicate that insulin secretion in engineered hepatocytes (Huh7ins cells) was controlled, as precisely as in the pancreatic  $\beta$ -cell, by a fully functional  $\text{K}_{\text{ATP}}$  and  $\text{Ca}_v$  channel system. The results clearly document that Huh7ins cells respond to glucose via insulin secretion from secretory granules by the same mechanism observed in pancreatic  $\beta$ -cells. This is the first study to demonstrate a clear physiological and biochemical interaction of  $\text{K}_{\text{ATP}}$  channels and  $\text{Ca}_v$  channels in liver cells, and as such reveals that hepatocytes are ideal candidates for the engineering of artificial  $\beta$ -cells. Testament to this, we have successfully engineered a liver cell line to synthesize, store and secrete insulin. Regardless of whether this hepatoma cell line will be a viable  $\beta$ -cell alternative for transplantation into patients, the present study provides valuable information with regards to the future engineering of glucose-responsive insulin-secreting liver cells. Elucidation of the minimal molecular modifications required for the creation of an artificial  $\beta$ -cell from a hepatocyte may one day provide therapeutic avenues to engineer a patient's own liver cells to synthesize, store and secrete insulin in response to metabolic stimuli.

### Acknowledgements

This work was supported by grants from Diabetes Australia Research Trust, Rebecca L. Cooper Medical Research Foundation and the University of Technology Sydney. We would like to thank Wayne Hawthorne and Philip O'Connell from the Westmead Millennium Institute for human pancreatic islets and Richard Limburg for IT support.

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