Ca_v1.2 rather than Ca_v1.3 is coupled to glucose-stimulated insulin secretion in INS-1 832/13 cells

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Abstract

In clonal β -cell lines and islets from different species, a variety of calcium channels are coupled to glucose-stimulated insulin secretion. The aim of this study was to identify the voltage-gated calcium channels that control insulin secretion in insulinoma (INS)-1 832/13 cells. The mRNA level of Ca_v1.2 exceeded that of Ca_v1.3 and Ca_v2.3 two-fold. Insulin secretion, which rose tenfold in response to 16.7 mM glucose, was completely abolished by 5 μ M isradipine that blocks Ca_v1.2 and Ca_v1.3. Similarly, the increase in intracellular calcium in response to 15 mM glucose was decreased in the presence of 5 μ M isradipine, and the frequency of calcium spikes was decreased to the level seen at 2.8 mM glucose. By contrast, inhibition of Ca_v2.3 with 100 nM SNX-482 did not significantly affect insulin secretion or intracellular calcium. Using RNA interference, Ca_v1.2 mRNA and protein levels were knocked down by ~65% and ~34% respectively, which reduced insulin secretion in response to 16.7 mM glucose by 50%. Similar reductions in calcium currents and cell capacitance were seen in standard whole-cell patch-clamp experiments. The remaining secretion of insulin could be reduced to the basal level by 5 μ M isradipine. Calcium influx underlying this residual insulin secretion could result from persisting Ca_v1.2 expression in transfected cells since knock-down of Ca_v1.3 did not affect glucose-stimulated insulin secretion. In summary, our results suggest that Ca_v1.2 is critical for insulin secretion in INS-1 832/13 cells.

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Introduction

The β -cells of pancreatic islets secrete insulin in response to an increase in glucose. Glucose enters the cells and is metabolised, provoking a rise in the ATP:ADP ratio, which leads to closure of ATP-sensitive K⁺ (K_{ATP}) channels. This sets off a depolarisation of the plasma membrane, which opens the voltage-gated calcium channels. The subsequent increase in intracellular calcium [Ca²⁺]_i initiates the secretion of insulin granules from the β -cells. The voltage-gated calcium channels are part of a family of multi-subunit proteins, consisting of α_1 -, α_2 -, δ - and β -subunits, and additionally in some cell types a γ -subunit (Jay *et al.* 1990). These have different primary structures and electrophysiological and pharmacological properties (for a review see (Yang & Berggren 2005)). The α_1 -subunits form the ion-conducting pore of the calcium channel. Several pore-forming calcium channel α_1 -subunits of voltage-sensitive calcium channels have been identified in rat pancreatic islets and β -cell lines: Ca_v1.2, Ca_v1.3, Ca_v2.1, Ca_v2.2, Ca_v2.3 and Ca_v3.1 (Plant 1988, Sala & Matteson 1990, Safayhi *et al.* 1997, Ligon *et al.* 1998, Grabsch *et al.* 1999, Ertel *et al.* 2000, Zhuang *et al.* 2000, Scholze *et al.* 2001).

The contribution of the different calcium channels to the increase in [Ca²⁺]_i has been extensively studied. Approximately, 60–70% of the rat whole-cell Ca²⁺ current in β -cells is sensitive to dihydropyridines (Schulla *et al.* 2003, Huang *et al.* 2004, Taylor *et al.* 2005). The major dihydropyridine-sensitive calcium channels in rat β -cells are Ca_v1.2 or Ca_v1.3, with different studies emphasising the role of either one (Iwashima *et al.* 1993, Liu *et al.* 2004, Taylor *et al.* 2005). Ca²⁺ currents that can be inhibited by ω -conotoxin GVIA, which is known to block Ca_v2.2, have been reported in rat β -cells, but their role in insulin secretion is controversial (Satin *et al.* 1995, Sher *et al.* 2003). Ca_v2.3, which is inhibited by SNX-482, accounts for another 18% of the Ca²⁺ current in mouse β -cells (Jing *et al.* 2005). The role of Ca_v2.3 in rat β -cells is not clear since Vajna *et al.* (2001) reported that 25% of insulin secretion in rat insulinoma (INS)-1 β -cells was inhibited by SNX-482, whereas others did not find an effect of SNX-482 in the same cells (MacDonald *et al.* 2005).

Clonal cells derived from the rat insulinoma cell line INS-1, 832/13, have previously been shown to exhibit strong responses to stimulation with glucose (Hohmeier *et al.* 2000). A robust increase in insulin secretion is

demonstrated by 832/13 cells when stimulated with glucose as well as a number of other secretagogues (Hohmeier *et al.* 2000). The identity of voltage-gated calcium channels in these cells is not known and neither is the relative importance of $\text{Ca}_v1.2$ versus $\text{Ca}_v1.3$ for insulin secretion. The distribution pattern of voltage-gated calcium channels could also give rise to differences in insulin secretion between 832/13 and 832/2 cells, the latter being weakly responsive to glucose (Hohmeier *et al.* 2000). However, 832/2 cells do respond to high potassium with an increase in insulin secretion, although not to the same extent as 832/13 cells (Yang *et al.* 2004). In both studies, it was shown that the insulin content of 832/13 and 832/2 cells is similar, and the blunting of insulin secretion can therefore not be attributed to differences in insulin content. Yang *et al.* (2004) showed that increased signalling in the cAMP/protein kinase A system accounts for part of the exaggerated glucose responsiveness of 832/13 cells. However, additional differences must exist. For one, the secretory differences between the two cell types could be explained by the fact that different calcium channels are expressed in the two cell types, resulting in different sensitivities to metabolic coupling signals in the two clones. Also, metabolic differences between the glucose-responsive and -unresponsive lines exist: anaplerosis manifested as pyruvate cycling is much more prominent in 832/13 than 832/2 cells (Lu *et al.* 2002).

To resolve possible differences in Ca^{2+} dynamics between the different 832 lines, we studied the expression levels of three voltage-gated calcium channels ($\text{Ca}_v1.2$, $\text{Ca}_v1.3$ and $\text{Ca}_v2.3$), and the calcium influx in response to changes in glucose concentrations in the presence and absence of calcium channel blockers. The effects of calcium blockers and RNA silencing of calcium channels on insulin secretion, changes in $[\text{Ca}^{2+}]_i$, voltage-gated Ca^{2+} currents and cell capacitance (ΔC_m) were also studied. We found that $\text{Ca}_v1.2$ is the major voltage-gated calcium channel coupled to insulin secretion in 832/13 cells. However, differences in glucose responsiveness of the two clonal β -cell lines could not be attributed to the distribution of voltage-gated calcium channels, since the 832 clones expressed similar levels of calcium channels.

Materials and methods

Materials

All chemicals were obtained from Sigma–Aldrich, unless otherwise indicated.

Cell culture

Insulinoma-derived β -cell clones INS 832/13 and 832/2 were cultured in RPMI 1640 medium containing

11.1 mM glucose supplemented with 10% foetal calf serum, 1 mM sodium pyruvate, 10 mM HEPES, 50 μM β -mercaptoethanol, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2 .

Insulin secretion assays

Cells were cultured in 24-well plates until confluence. After an overnight incubation in full medium containing 5 mM glucose, the cells were first washed and then pre-incubated in HEPES balanced salt solution (HBSS) containing 114 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4 , 1.16 mM MgSO_4 , 20 mM HEPES, 25.5 mM NaHCO_3 , 2.5 mM CaCl_2 at pH 7.2 with 0.575 mM BSA and 2.8 mM glucose for 2 h at 37 °C. The buffer was then replaced by HBSS containing glucose, the calcium channel inhibitors isradipine and SNX-482, and KCl in combination with diazoxide at the indicated concentrations for 1 h at 37 °C. The buffer was carefully removed and the amount of insulin was determined with the Coat-A-Count insulin RIA (DPC, Los Angeles, CA, USA), which recognises human insulin and cross-reacts by $\sim 20\%$ with rat insulin. The cells were washed with PBS and stored at -20 °C for determination of protein content. Protein content was measured with the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL, USA).

Measurements of $[\text{Ca}^{2+}]_i$

Dual-wavelength microfluorimetry was performed to measure changes in $[\text{Ca}^{2+}]_i$ in individual cells. The cells were cultured overnight on 22 mm microscope cover plates. The cells were loaded with 2.5 μM fura-2-acetoxymethyl ester (fura-2 AM) in the presence of 0.0007% w/v pluronic acid (Molecular Probes, Leiden, The Netherlands) for 30–40 min at 37 °C. Experiments were performed in a perfusion chamber; the perfusion solution consisted of 138 mM NaCl, 5.6 mM KCl, 2.6 mM CaCl_2 , 1.2 mM MgCl_2 , 5 mM HEPES at pH 7.4, held at a temperature of 32 °C. Glucose, the calcium channel inhibitors isradipine (which blocks $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$) and SNX-482 (which blocks $\text{Ca}_v2.3$), and KCl were added at this solution at the indicated concentrations. The experiments were conducted on an IonOptix fluorescence imaging system (Milton, MA, USA). Excitation of fura-2 AM at 340 and 380 nm was alternately performed, and the emitted light was collected at 510 nm. The fluorescence (F) ratio F_{340}/F_{380} was determined and equation 5 of (Grynkiewicz *et al.* 1985) with a K_d of 224 nM was then used to estimate $\Delta[\text{Ca}^{2+}]_i$. The maximum ratio (R_{max}) in the equation was established by the addition of 100 μM ionomycin in the presence of 10 mM CaCl_2 at the end of each experiment.

Real-time PCR

Total RNA was isolated from 832/13 and 832/2 cells grown to confluence with the RNeasy Plus Mini kit (Qiagen GmbH), which removes genomic DNA from the sample. One microgram of RNA was transcribed into single-stranded cDNA with 100 U M-MuLV Reverse Transcriptase RNase H⁻ (Finnzymes Oy, Espoo, Finland). TaqMan gene expression assays were purchased from Applied Biosystems (ABI; Stockholm, Sweden) for the various target genes: Rn00709287, directed against rat calcium channel *Ca_v1.2*; Rn00568820, directed against rat calcium channel *Ca_v1.3*; Rn00494444, directed against rat calcium channel *Ca_v2.3* and Rn0138745, directed against hypoxanthine-guanine phosphoribosyl transferase, which served as endogenous control gene. The reaction consisted of 50 ng cDNA, 10 µl TaqMan Universal PCR mix (ABI), primers at a final concentration of 300 nM, a final concentration of the probe of 100 nM and RNase-free water for a volume of 20 µl per well. The PCR was performed in an ABI 7900HT Fast Real-Time PCR system. The reactions were performed in duplicate. The relative quantity of calcium channel *Ca_v1.2*, *Ca_v1.3* and *Ca_v2.3* mRNA was calculated using the comparative *C_T* method after initial experiments showed similar quantitative PCR efficiency rates for all assays.

RNA interference

Two different siRNA sequences for *Ca_v1.2* were purchased with siRNA identification numbers 199812, with an antisense sequence of 5'-AUUCAAUGGUGAAAAUGG-3' and a sense sequence of 5'-CCAUUUU-CACCAUUGAAAU-3', and 47874, with an antisense sequence of 5'-AAAUACAGACAUACAUCCC-3' and a sense sequence of 5'-GGGAUGUUAGUCUGUAUUU-3'. For *Ca_v1.3*, one siRNA sequence was obtained with identification number 198492 and with an antisense sequence of 5'-UCCUUGCAUAGUUUGCUG-3' and a sense sequence of 5'-CGAGGCAAACUAUGCAAGA-3' (Ambion, Huntingdon, UK). Negative control siRNA coupled to the fluorophore Alexa488 were used (1027284; Qiagen). Cells were reverse transfected with 30 nM siRNA, using HiPerFect transfection medium for siRNA (Qiagen). The transfection efficiency amounted to 70%. The medium was changed after 24 h and the cells were assayed for knock-down at the mRNA level after yet another 24 h. Alternatively, the cells were cultured for 48 h post-transfection and then insulin secretion was subsequently performed. For patch-clamp measurements, the cells were co-transfected with *Ca_v1.2* siRNA and negative control siRNA or negative control siRNA; the presence of control siRNA allowed us to identify cells transfected with siRNA.

Western blot

Cells transfected with siRNA for *Ca_v1.2* or *Ca_v1.3* were cultured for 48 h and homogenised in a homogenisation buffer containing 50 mM Tris, 5 mM MgCl₂, 1 mM CaCl₂, 1 mM EDTA, 0.1 × 10⁻³ mM EGTA and 1:100 protease inhibitor cocktail. Protein content was determined by the BCA method and 20 and 35 µg protein was run onto a 7.5% SDS-PAGE gel for *Ca_v1.2* and *Ca_v1.3* respectively, and subsequently blotted onto polyvinylidene difluoride (PVDF) membranes. Protein for *Ca_v1.2* was detected with primary antibody rabbit anti-Ca²⁺ channel (*Ca_v1.2* (α-1C)) polyclonal antibody in a dilution of 1:1000 (BD Biosciences, Stockholm, Sweden) and for *Ca_v1.3* with primary rabbit anti-*Ca_v1.3* polyclonal antibody in a dilution of 1:350 (Alomone Labs, Jerusalem, Israel). Horseradish peroxidase-coupled anti-rabbit IgG antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as a secondary antibody (1:5000). Enhanced chemiluminescence was used to detect protein on the blots. The protein levels were determined by densitometry, and normalised to the levels of α-tubulin (Santa Cruz Biotechnology), which corrected for variations in protein loading. The results are expressed as proportion of the density for samples treated with negative control siRNA.

Electrophysiology

Whole-cell currents and exocytosis were recorded on single 832/13 cells 48 h after transfection using an EPC-9 patch-clamp recorder and the software Pulse (Heka Elektronik, Lambrecht/Pfalz, Germany). Measurements were only performed on transfected cells that were identified by Alexa488. All measurements were conducted at 32–34 °C. Patch electrodes were pulled from borosilicate glass (tip resistance 3–6 MΩ when filled with intracellular solution). Experiments were conducted using the standard whole-cell configuration, which results in electrical contact with the whole cell and allows the pipette solution to diffuse into the cell and exchange the cytosol. The pipette solution contained 125 mM Cs-Glut, 10 mM NaCl, 10 mM CsCl, 1 mM MgCl₂, 0.05 mM EGTA, 3 mM Mg-ATP, 10 mM HEPES (pH 7.15 using CsOH) and 0.1 mM cAMP. The standard extracellular solution consisted of 118 mM NaCl, 20 mM tetraethylammonium chloride (to block voltage-gated K⁺-currents), 5.6 mM KCl, 2.6 mM CaCl₂, 1.2 mM MgCl₂, 5 mM glucose and 5 mM HEPES (pH 7.4 using NaOH). Exocytosis was detected as changes in membrane capacitance using the software-based lock-in application (which add a sine wave with a frequency of 500 Hz to the holding potential) of the amplifier. Exocytosis was elicited by a train of 500 ms

voltage-clamp depolarisations from -70 to 0 mV applied at 1 Hz. The steady-state inactivation of the current was investigated using a conventional two-pulse protocol in which a 5 ms test depolarisation to zero was preceded by a 50 ms conditioning pulse to voltages between -150 and 0 mV. The current(I)–voltage(V) relationship was studied using a protocol in which a series of 50 ms depolarisations from -70 mV to increasing voltages between -50 mV to 20 mV is applied to the cell. Both the inactivation properties and the I–V relationship were analysed using different features of the PulseFit function of the Heka software.

Statistical analysis

Values are expressed as mean $\pm$ S.E.M. Differences between the two clones or the groups were performed by Student's *t*-test for unpaired data, unless otherwise indicated, and $P < 0.05$ was considered significant.

Results

Insulin secretion is reduced in the presence of isradipine in 832/13 but not 832/2 cells

The two clones of the INS-1 cell line differed in their basal secretion of insulin in the presence of 2.8 mM glucose. 832/2 cells displayed basal insulin secretion, which was three- to fourfold higher than basal insulin secretion in 832/13 cells (Fig. 1A). However, whereas 832/13 cells responded to stimulation by 16.7 mM glucose with a tenfold increase in insulin secretion, 832/2 cells did not significantly increase insulin secretion from the basal level. The K_{ATP} -independent pathway of glucose-stimulated insulin secretion was tested by incubating the cells with 2.8 or 16.7 mM glucose in the presence of 35 mM KCl and 250 μ M diazoxide. In 832/13 cells, this resulted in increased insulin secretion both at basal (by 10-fold) and high glucose concentrations (by 25-fold over basal glucose concentrations and by 2.6-fold over 16.7 mM glucose).

Figure 1 Insulin secretion in 832 INS-1 cells. (A) Insulin secretion in response to stimulation with 2.8 and 16.7 mM glucose with and without 35 mM KCl and 250 μ M diazoxide; 832/13 cells (black bars), 832/2 cells (white bars). (B) Insulin secretion in 832/13 cells after stimulation with 2.8 or 16.7 mM glucose in combination with 5 μ M isradipine or 100 nM SNX-482. (C) Effect of 5 μ M isradipine on insulin secretion elicited by 35 mM KCl and 250 μ M diazoxide and 16.7 mM glucose. Number 2 denotes 832/2 cells. $n=3$ for each condition (* $P < 0.05$, ** $P < 0.01$).

In 832/2 cells, an increase in insulin secretion could also be observed, although not to the same extent as in 832/13 cells (a 4.1-fold increase over basal glucose concentrations). There was, however, no stimulatory effect of 16.7 mM glucose in 832/2 cells under K_{ATP} -independent conditions.

Since we hypothesised that the disparity in glucose responsiveness could be attributed to differences in the expression of the various calcium channels, we incubated the cells with inhibitors of three different calcium channels: $Ca_v1.2$, $Ca_v1.3$ and $Ca_v2.3$. Isradipine, which blocks $Ca_v1.2$ and $Ca_v1.3$, was added at 5 μ M to basal and high glucose concentrations. In 832/13 cells, isradipine did not significantly affect insulin secretion at 2.8 mM glucose. However, insulin secretion in response to 16.7 mM glucose was reduced by 90% in the presence of isradipine (Fig. 1B). In 832/2 cells, no effect of isradipine could be detected irrespective of the glucose concentration (results not shown). When the inhibitor of $Ca_v2.3$, SNX-482 (100 nM), was used, there was no significant effect on insulin secretion in either 832/13 or 832/2 cells. Under K_{ATP} channel-independent conditions (in the presence of KCl and diazoxide), addition of isradipine resulted in a significant decrease in insulin secretion at 2.8 and 16.7 mM glucose in both 832/13 and 832/2 cells (Fig. 1C).

Dynamics of $[Ca^{2+}]_i$ in 832/13 cells

The differences in insulin secretion between the two clones were also reflected in the measurements of $\Delta[Ca^{2+}]_i$. Figure 2A shows the pattern of changes in $[Ca^{2+}]_i$ in 832/13 cells. Here, an increase in glucose concentration of the perfusion medium from 3 to 15 mM resulted in a rise in $[Ca^{2+}]_i$. This rise in calcium was not constant but rather of a more oscillatory nature. When expressed as number of peaks per minute, it increased from 0.44 ± 0.04 at 3 mM glucose to 0.74 ± 0.06 ($P < 0.001$) at 15 mM glucose. In 832/2 cells (Fig. 2B), there was no significant increase in the number of $[Ca^{2+}]_i$ peaks per minute when the glucose concentration was raised (0.46 ± 0.07 vs 0.37 ± 0.06 peaks per minute at basal and high glucose respectively). Comparison of the two clones revealed that 832/13 cells displayed significantly more calcium peaks per minute than 832/2 cells at 15 mM glucose (0.74 ± 0.06 vs 0.37 ± 0.06 , $P < 0.001$), while there was no difference in the frequency of peaks at 3 mM glucose. The change in $[Ca^{2+}]_i$ after application of 15 mM glucose was also significantly different between 832/13 and 832/2 cells. In 832/13 cells, the mean increase in $[Ca^{2+}]_i$ was 61 ± 15 nM, whereas in 832/2 cells, it amounted to 7.4 ± 3.4 nM ($P < 0.001$).

Both clones displayed a rise in $[Ca^{2+}]_i$ after the addition of 75 mM KCl to the perfusion solution

(Fig. 2C and D). In fact, the increase in $[Ca^{2+}]_i$ was similar in 832/2 and 832/13 cells, averaging ~ 1 μ M. This increase in 832/2 cells shows that the cells do contain voltage-gated calcium channels that permit the entry of Ca^{2+} into the cell in response to depolarisation of the cell membrane. These results are consistent with the results from the insulin secretion experiments performed with KCl (Fig. 1).

Next, measurements of $[Ca^{2+}]_i$ in both cell types were performed in the presence of inhibitors of the voltage-gated calcium channel. The $Ca_v1.2$ and $Ca_v1.3$ channels were blocked by the addition of isradipine (5 μ M) to the perfusion medium containing 15 mM glucose. In 832/13 cells, this led to a decrease in the frequency of $[Ca^{2+}]_i$ peaks to that seen at 3 mM glucose (0.40 ± 0.05 , $P < 0.001$; Fig. 2E). The effect of isradipine was also observed as a decrease in $[Ca^{2+}]_i$ by 21.23 ± 11.53 nM ($P < 0.01$). In 832/2 cells, no effect of isradipine on the number of peaks per minute, nor on the $[Ca^{2+}]_i$, could be seen. In separate experiments, SNX-482, which is a blocker of $Ca_v2.3$, was co-perfused with 15 mM glucose. Here, no effect on the number of peaks per minute was noted; only a slight decrease in $[Ca^{2+}]_i$ was seen in 832/13 cells (Fig. 2F), whereas no effect on 832/2 cells was observed (results not shown).

Real-time PCR reveals the presence of several voltage-dependent Ca^{2+} channel subtypes

The relative levels of RNA for $Ca_v1.2$, $Ca_v1.3$ and $Ca_v2.3$ were determined by real-time PCR. The expression of all three channels was readily detected; however, no differences between the 832 lines with respect to the mRNA expression levels were found (Fig. 3). However, the expression levels of the individual calcium channels varied widely. $Ca_v1.2$ mRNA expression levels were two orders of magnitude higher than those for $Ca_v1.3$ and $Ca_v2.3$. The expression levels for $Ca_v1.3$ and $Ca_v2.3$ were low but did not differ from each other.

RNA interference of $Ca_v1.2$ reduces insulin secretion and exocytosis in 832/13 cells

Since isradipine is a pharmacological inhibitor of both $Ca_v1.2$ and $Ca_v1.3$, our results could not discern the importance of $Ca_v1.2$ versus $Ca_v1.3$ in glucose-stimulated insulin secretion in 832/13 cells. Therefore, expression of $Ca_v1.2$ was knocked down, using RNA interference. Two different siRNAs were employed and found to be equally efficient; a knock-down by 65% at the mRNA level was achieved (Fig. 4A, results of one siRNA shown). Knock-down of $Ca_v1.2$ did not influence mRNA expression levels of $Ca_v1.3$ (Fig. 4A), which concurs with the fact that the siRNA sequences were

Figure 2 Changes in $[Ca^{2+}]_i$ in 832 INS-1 cells. Response to 3 mM (3G) and 15 mM glucose (15G) on $\Delta[Ca^{2+}]_i$ in an (A) 832/13 cell and (B) 832/2 cell. (C) Same cell as in (A) but the response on $\Delta[Ca^{2+}]_i$ to 70 mM KCl (KCl). (D) The same cell as in (B) stimulated as in (C). (E) Changes in $\Delta[Ca^{2+}]_i$ in response to 15 mM glucose and 5 μ M isradipine in 832/13 (black traces) and 832/2 cells (grey traces). (F) The response on $\Delta[Ca^{2+}]_i$ in an 832/13 cell stimulated with 15 mM glucose and after the addition of 100 nM SNX-482 in the continued presence of glucose. The traces are representative examples of respective traces.

found to be specific for $Ca_v1.2$ upon blasting of the sequences in the NCBI database. The signal of the protein level on western blot for $Ca_v1.2$ was found to be reduced (Fig. 4C) by 34% upon RNA interference, when the density of the protein band of siRNA-treated

cells was compared with the density of the protein band of negative control-treated cells (Fig. 4D). As a consequence of $Ca_v1.2$ knock-down, insulin secretion in response to 16.7 mM glucose was decreased by 50% (Fig. 5A). Co-incubation of 5 μ M isradipine and

Figure 3 mRNA expression levels for $\text{Ca}_v1.2$, $\text{Ca}_v1.3$ and $\text{Ca}_v2.3$ in 832/13 and 832/2 cells. Expression levels in 832/13 cells (black bars; $n=4$ for $\text{Ca}_v2.3$ or $n=5$ for $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$), and 832/2 cells (white bars; $n=3$). The results were evaluated with a one-way ANOVA and the *post hoc* Bonferroni test (* $P<0.05$, *** $P<0.001$).

16.7 mM glucose further reduced insulin secretion by 55 and 75% respectively, of that provoked by 16.7 mM glucose alone in $\text{Ca}_v1.2$ siRNA-treated and negative control-treated cells. Expression of $\text{Ca}_v1.3$ was knocked

down to a similar extent as $\text{Ca}_v1.2$ (by 70%), whereas the expression levels of $\text{Ca}_v1.2$ were not affected (Fig. 4B) and $\text{Ca}_v1.3$ protein signals were decreased by 31% (Fig. 4C and D). The siRNA sequences were specific for $\text{Ca}_v1.3$ in the analysis of the sequence in the NCBI database. Nevertheless, the knock-down of $\text{Ca}_v1.3$ did not impact on glucose-stimulated insulin secretion (Fig. 5B).

To investigate whether the 50% reduction in insulin secretion was due to effects on the exocytotic machinery, standard whole-cell patch-clamp recordings on single INS 832/13 cells transfected with negative control siRNA or siRNA against $\text{Ca}_v1.2$ were performed. First, we investigated the voltage-dependent currents in 832/13 cells by application of 50 ms depolarisations from -70 mV to increasing voltages between -50 mV to $+20$ mV. The voltage-dependent current contained both a transient Na^+ -dependent component and a Ca^{2+} -dependent component (Fig. 6A), as has been observed in primary Sprague-Dawley rat β -cells (Hiriart & Matteson 1988). The peak of the Na^+ component ($I_{p, \text{Na}}$) was maximal when the membrane potential was depolarised to 0 mV and was not significantly different between

Figure 4 $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ downregulated by RNA interference in 832/13 cells. (A) $\text{Ca}_v1.2$ or $\text{Ca}_v1.3$ mRNA levels in cells treated with $\text{Ca}_v1.2$ siRNA (black bars) or negative control siRNA (white bars) respectively ($n=3$, * $P<0.05$). (B) $\text{Ca}_v1.3$ or $\text{Ca}_v1.2$ mRNA levels in cells treated with $\text{Ca}_v1.3$ siRNA (black bars) or negative control siRNA (white bars) respectively. Negative control expression levels for each voltage-gated calcium channel were set to 1 ($n=4$). Protein expression signals for $\text{Ca}_v1.2$ after siRNA treatment (C, upper panel) or for $\text{Ca}_v1.3$ (C, lower panel). (D) Protein expression was normalised to expression levels of α -tubulin and expressed as proportion of negative control protein signal. The results were evaluated with a paired *t*-test: $\text{Ca}_v1.2$ ($n=4$, $P=0.0135$), $\text{Ca}_v1.3$ ($n=3$, $P=0.0536$).

Figure 5 Influence of Ca_v RNA knock-down on insulin secretion. (A) Insulin secretion after RNA interference for $Ca_v1.2$ (black bars). Cells were stimulated with 2.8 and 16.7 mM glucose or 16.7 mM glucose with 5 μ M isradipine. Results were evaluated with a paired t -test due to variation in absolute values of secreted insulin between different experiments ($n=3$, * $P<0.05$). (B) Insulin secretion after siRNA for $Ca_v1.3$ (black bars). Cells were stimulated with 2.8 mM glucose or 16.7 mM glucose ($n=3$).

control cells and cells treated with $Ca_v1.2$ siRNA (data not shown). Thus, the RNA silencing had no effect on the Na^+ current. The steady-state inactivation properties of the Na^+ current measured using a two-pulse protocol with a 5 ms test pulse preceded by a 50 ms conditioning pulse was also the same in control and siRNA-treated cells; the half-maximal inactivation

(V_{half}) was -55 ± 4 mV ($n=8$) in the control cells and -58 ± 3 ($n=11$) in the $Ca_v1.2$ siRNA-treated cells. This is slightly higher than that previously reported in primary rat β -cells, where the half-maximal inactivation of Na^+ currents was -70 mV (Hiriart & Matteson 1988), but closer to that reported in human islets (Barnett *et al.* 1995).

Figure 6 Ca^{2+} current and exocytosis upon RNA silencing of $Ca_v1.2$ in 832/13 cells. (A) Voltage-dependent inward current initiated by a depolarisation from -70 to 0 mV in a control cell and $Ca_v1.2$ siRNA-treated cell as indicated. Note that the rapid Na^+ peak inactivated within a few milliseconds is followed by the more sustained Ca^{2+} current. (B) Current (I)–voltage (V) relationship of the peak Ca^{2+} current ($I_{p, Ca}$) in control cells (black) and siRNA $Ca_v1.2$ cells (grey). (C) Charge (Q)–voltage (V) relationship of the voltage-dependent current under same conditions as in (B). (D) Increase in membrane capacitance (ΔC_m , bottom) evoked by a train of membrane depolarisations (V , top) in a control cell. (E) The same as in (D) but the experiment was performed on a cell transfected with siRNA against $Ca_v1.2$. (F) Histogram summarising the total increase in membrane capacitance in the experiments performed in (D)–(E) ($n=7$ – 10 , * $P<0.05$ and ** $P<0.01$).

To analyse the voltage dependence of the Ca^{2+} current, the peak current ($I_{p,\text{Ca}}$) was measured 2.5 ms after the onset of the depolarization when the transient Na^+ current was inactivated (Hiriart & Matteson 1988). The peak Ca^{2+} current was maximal at 0 mV and amounted to -163 ± 36 pA ($n=8$) in the control cells (Fig. 6B). This maximal peak current was significantly smaller in the siRNA-treated cells, demonstrating that the knock-down of $\text{Ca}_v1.2$ by RNA silencing indeed reduced $I_{p,\text{Ca}}$ by $\sim 60\%$ ($P<0.05$; $n=8-10$). This was also confirmed by analysis of the voltage dependence of the charge (Q ; Fig. 6C), which mainly represents the amount of Ca^{2+} that is transferred into the cell through the voltage-dependent Ca^{2+} channels. The charge at 0 mV was estimated to be -6.1 ± 1.8 ($n=8$) and -2.7 ± 0.7 ($n=10$; $P<0.05$) in control and $\text{Ca}_v1.2$ siRNA-treated cells respectively.

Next, we investigated the effects of RNA silencing of the $\text{Ca}_v1.2$ channel on exocytosis in the 832/13 cells, using capacitance measurement in combination with standard whole-cell patch-clamp recordings. Exocytosis was evoked by a train of ten 500 ms depolarisations from -70 to 0 mV (Fig. 6D and E). The total increase in membrane capacitance under control conditions was 182 ± 38 fF ($n=7$). When 832/13 cells had been transfected with siRNA against $\text{Ca}_v1.2$, the increase in membrane capacitance was significantly reduced and amounted to 81 ± 15 fF ($n=9$; $P<0.01$). The decrease in membrane capacitance was equal throughout the train and not linked to the first depolarisations, as in primary mouse β -cells (Schulla *et al.* 2003, Jing *et al.* 2005). The difference between control cells and siRNA-treated cells was sustained also during the application of a second train, 2 min after the first. Here, the increase in membrane capacitance was 209 ± 38 ($n=7$) during control conditions and 121 ± 13 ($n=9$; $P<0.05$) in the $\text{Ca}_v1.2$ siRNA-treated cells.

Discussion

We found that $\text{Ca}_v1.2$ mRNA is expressed at a substantially higher level in 832 cells than either $\text{Ca}_v1.3$ or $\text{Ca}_v2.3$ mRNA. The expression levels for $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ have previously been reported for different types of rat β -cells (Safayhi *et al.* 1997, Taylor *et al.* 2005) and rat islets (Iwashima *et al.* 1993, Taylor *et al.* 2005). Whereas minor differences in expression of the two channels have been found before, differences of the extent that we found here have not been reported. However, the mRNA expression levels of the three calcium channels studied did not differ between the two INS-1 832 clones, indicating that the number of calcium channels is not the causal factor for the discrepant insulin secretion responses. By contrast, we have previously reported that pyruvate cycling (Lu *et al.*

2002) and signalling in the cAMP/protein kinase A system (Yang *et al.* 2004) are exaggerated in the highly glucose-responsive 832/13 cells compared with unresponsive 832 clones.

The 832/13 cells are a model for β -cells with a strong insulin secretory response to increases in glucose concentration (Hohmeier *et al.* 2000). This increase in insulin secretion is preceded by a rise in Ca^{2+} influx into the cytoplasm. This increase and its oscillatory nature was recently described in 832/13 cells (Baroukh *et al.* 2007). Ca^{2+} enters the cells via dihydropyridine-sensitive voltage-gated calcium channels. We found that isradipine blocked calcium influx and virtually abolished glucose-induced insulin secretion in the 832/13 cells. These findings are in accordance with the findings of others, who noted significant inhibition of insulin secretion with dihydropyridines (Satin *et al.* 1995, MacDonald *et al.* 2005, Taylor *et al.* 2005). This emphasises the importance of dihydropyridine-sensitive calcium channels in rat β -cells. Next, we silenced $\text{Ca}_v1.2$ mRNA levels in order to distinguish between the role of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$, since both these channels are inhibited by dihydropyridines. The cells treated with $\text{Ca}_v1.2$ siRNA exhibited a significant decrease in glucose-stimulated insulin secretion, a similar decrease in calcium currents and a decrease in capacitance upon a train of depolarisations. Knock-down of $\text{Ca}_v1.3$ with siRNA did not affect glucose-stimulated insulin secretion. Given the high expression level of $\text{Ca}_v1.2$, the decrease in insulin secretion after RNA interference, the effect on capacitance increases and calcium currents after RNA interference, and the lack of effect on insulin secretion of $\text{Ca}_v1.3$ downregulation, we conclude that $\text{Ca}_v1.2$ is the critical voltage-gated calcium channel in 832/13 cells. The residual insulin secretion that was inhibited by isradipine could be attributed to remaining $\text{Ca}_v1.2$ expression considering the relatively modest decreases in protein signal and/or persistent expression of $\text{Ca}_v1.3$. $\text{Ca}_v1.3$ mRNA expression levels were not significantly altered by $\text{Ca}_v1.2$ downregulation, in contrast to the reported compensatory upregulation of $\text{Ca}_v1.2$ in $\text{Ca}_v1.3$ knockout mice (Namkung *et al.* 2001). Liu *et al.* have previously performed studies on the role of $\text{Ca}_v1.2$ versus $\text{Ca}_v1.3$ in INS-1 cells. They found that $\text{Ca}_v1.3$ was responsible for insulin secretion and calcium influx into the cells (Liu *et al.* 2003, 2004). In these studies, the pore-forming units of the channels were over-expressed on top of normal expression patterns without taking into account constitutive expression of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$. By contrast, our model does not rely on over-expression of calcium channels, and the discrepancy of the results could thus be explained by the differences in the methodology used.

The $\text{Ca}_v2.3$ calcium channel was blocked by SNX-482, which previously has been shown to inhibit 25% of

insulin secretion in mouse β -cells as well as 20% in a knock-out mouse model (Jing *et al.* 2005). This study indicated the importance of this channel in the second phase of insulin secretion. In our cells, SNX-482 did not exert a pronounced effect on either insulin secretion or $[Ca^{2+}]_i$ influx. This is in accordance with our previous results from the experiments performed in INS-1 cells, where no effect of SNX-482 on exocytosis could be detected (MacDonald *et al.* 2005). On the other hand, Pereverzev *et al.* (2002) studied the effect of a *Ca_v2.3* antisense expression cassette and reported a reduction in insulin secretion. In this study, the transfection procedure lowered the insulin content of the cells significantly. Glucose-stimulated insulin secretion was expressed as percentage of basal insulin secretion, which in turn was expressed as percentage of insulin content. Since the insulin content was very different in the transfected and the untransfected cells, it is difficult to compare these results with those obtained by us.

About 832/2 cells were found to have functional dihydropyridine-sensitive calcium channels, since insulin secretion in response to high KCl concentrations could be inhibited by isradipine. The failure of 832/2 cells to respond to increased glucose concentrations can thus not be attributed to dysfunctional calcium channels. Most likely, a metabolic coupling signal is lacking in 832/2 cells. In fact, we have previously examined mitochondrial metabolism in different 832 clones derived from INS-1 cells. We found that pyruvate cycling, where pyruvate fed into the tricarboxylic acid (TCA) cycle from a distinct cellular pool of the triose, correlated strongly with glucose responsiveness (Lu *et al.* 2002). Thus, 832/2 cells exhibit a much less pronounced activity of this anaplerotic pathway than 832/13 cells. The high basal insulin secretion might also be a reflection of this disturbed metabolism.

Both *Ca_v1.2* and *Ca_v1.3* calcium channels and sodium channels have been reported in humans (Seino *et al.* 1992, Barnett *et al.* 1995). The sodium channel was not found to affect insulin secretion after stimulation with 10 mM glucose because of rapid inactivation (Barnett *et al.* 1995). This is likely to be the same in our study since the half-maximal inactivation of the sodium channel in 832/13 cells is similar to that reported in human islets (Barnett *et al.* 1995). In the same study, around 70% of the calcium channels in human β -cells were sensitive to dihydropyridines, indicating the importance of these channels in β -cells. Also, the rapid first phase of insulin secretion in mouse β -cells has been coupled to the close association between the secretory granules and the calcium channels (Barg *et al.* 2001). The relative contribution of *Ca_v1.2* and *Ca_v1.3* calcium channels to insulin secretion has not been fully elucidated in most species (Yang & Berggren 2006). Nevertheless, it has been

established in mouse β -cells that *Ca_v1.2* contributes to calcium influx coupled to insulin secretion (Barg *et al.* 2001). In fact, a missense mutation in the *Ca_v1.2*-subunit has been identified in humans (Splawski *et al.* 2004). This reduces the inactivation of the calcium channels and results in episodic hypoglycaemia in the patients, emphasizing the importance of *Ca_v1.2* for insulin secretion. Moreover, both *Ca_v1.2* and *Ca_v1.3* mRNA levels are reduced in the Zucker diabetic fatty rat, which results in reduced calcium currents (Roe *et al.* 1996). In humans, insulin secretion is defective in the pre-diabetic stage of impaired glucose tolerance (Hosker *et al.* 1989, Tripathy *et al.* 2000, Abdul-Ghani *et al.* 2006), and an absolute decompensation of β -cell function characterises the transition to mild diabetes (Tripathy *et al.* 2000). Voltage-gated calcium channels could be involved in this transition. Therefore, the expression pattern of voltage-gated calcium channels is of interest to study in different animals and cell models.

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