Reduction of insulin secretion in the insulinoma cell line INS-1 by overexpression of a Ca\textsubscript{v}2.3 (α1E) calcium channel antisense cassette

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Abstract

Objective: Multiple types of voltage-activated Ca\textsuperscript{2+} channels (T, L, N, P, Q and R type) coordinate a variety of Ca\textsuperscript{2+}-dependent processes in neurons and neuroendocrine cells. In insulinoma cell lines as well as in endocrine tissues, the non-L-type α1E(Ca\textsubscript{v}2.3) subunit is expressed as the tissue-specific splice variant α1Ee.

Design and Methods: To understand the functional role of α1E-containing Ca\textsuperscript{2+} channels, antisense α1E mRNA was overexpressed in INS-1 cells by stable transfection of an antisense α1E cassette cDNA. As controls, either a sense α1E cassette or a control vector containing enhanced green fluorescent protein as an unrelated gene was stably transfected. The overexpression of each transfected cassette cDNA was recorded by RT-PCR.

Results: In three independent antisense α1E INS-1 clones, the glucose-induced insulin release was significantly reduced as compared with wild-type INS-1 cells and with a sense α1E INS-1 clone. However, in the antisense INS-1 clones, the KCl-induced insulin release was less impaired by overexpressing the antisense α1E cassette than the glucose-induced insulin release, leading to the assumption that glucose (15 mmol/l) and KCl (25 mmol/l) finally depolarize the membrane potential to a different extent.

Conclusion: α1E is involved in glucose-induced insulin secretion probably by influencing the excitability of INS-1 cells.

Introduction

The β-cells from the islets of Langerhans are electrically excitable. Elevated glucose concentrations in the blood stream are linked to insulin secretion from the β-cells through the successive activation of ATP-regulated K\textsuperscript{+} channels and voltage-gated Ca\textsuperscript{2+} channels in the plasmalemma of β-cells. The catabolism of glucose through the Embden–Meyerhof pathway raises the ratio of ATP to ADP plus AMP. Increased ATP concentrations link glucose uptake and glucose metabolism to the opening of ATP-dependent K\textsuperscript{+} channels in the β-cell. Closing of these channels at elevated ATP concentrations leads to depolarization of the β-cell and to the subsequent activation of voltage-gated Ca\textsuperscript{2+} channels (1, 2).

The influx of calcium ions through voltage-gated Ca\textsuperscript{2+} channels is the major but not the only trigger for the secretion of insulin in the β-cells from the islets of Langerhans and in the insulinoma cell line INS-1 (3). Not only dihydropyridine-sensitive L-type Ca\textsuperscript{2+} channels, but also non-L-type Ca\textsuperscript{2+} channels, the members of a second subfamily of high-voltage-activated (HVA) Ca\textsuperscript{2+} channels, are involved in the Ca\textsuperscript{2+}-induced insulin secretion (4, 5).

Recently, the ‘R-type’ Ca\textsuperscript{2+} channel was characterized as an additional candidate which might be functionally involved in the release of peptide hormones (6, 7) as well as in the secretion of hormones from chromaffin cells (8).

The R-type Ca\textsuperscript{2+} channel has so far been defined by its resistance to known Ca\textsuperscript{2+} channel blockers such as dihydropyridines, ω-conotoxin G Via and ω-conotoxin MVIIIC (9–11). Using antisense α1E oligonucleotides and the α1E-selective antagonist SNX-482, it was shown that α1E is related to multiple resistant components of HVA Ca\textsuperscript{2+} channels in rat cerebellar granule cells (12, 13). Some, but not all of the resistant component is absent in α1E-depleted cerebellar granule cells isolated from α1E knockout mice (14). The predicted structure of α1Ee in the insulinoma cell line INS-1 and in rat islets of Langerhans (15) is different from the cloned α1Ed (16) and α1E-1/α1E-3 variants.
(17). The α1E splice variant is characterized by a deletion of 19 amino acids in the II/III loop and an insertion of 43 amino acids in the carboxy terminus (15, 18, 19).

In order to identify the functional role of the new α1E splice variant, a cassette containing the 5′-region of α1E cDNA in antisense orientation was stably transfected into INS-1 cells. The glucose- and KCl-induced insulin secretion of antisense α1E stably transfected INS-1 cell clones was compared with those INS-1 clones which were stably transfected with sense α1E or control vector.

Materials and methods

Antagonists

The L-type Ca\(^{2+}\) channel antagonist isradipine (PN200-110) was a kind gift from Novartis (Basel, Switzerland). The selective antagonist for N- and P-/Q-type Ca\(^{2+}\) channels, ω-conotoxin MVIIIC, was purchased from Bachem Biochemica (Heidelberg, Germany).

RT-PCR and construction of sense and antisense α1E expression vector

The rat α1E cDNA fragment was generated by PCR. Total RNA was used as a template and isolated from rat brain (20). After reverse transcription of total RNA as described (19), fragments of rat α1E cDNA were amplified by PCR using the primer α1E-as, 5′-CGGAATTCGCAGAGGGCGACTATGGCTTTGTA-3′ (nucleotides 182–205) of rat α1E cDNA sequence (GenBank L15453) as forward and α1E-as-r, 5′-CGGAAATTCCCTCGAGGCGACTATGGCTTTGTA-3′ as reverse primer (nucleotides 1311-1292). Eight additional nucleotides were linked to the 5′-end of each primer containing an EcoRI site for subcloning. The annealing temperature for the α1E cassettes was 57°C during 32 cycles of amplification (21). Oligonucleotide primers were purchased from Eurogentec Bel SA (Seraing, Belgium). The 1146 bp long cDNA fragment was extracted from an agarose gel, purified and digested by EcoRI. The fragment contains 12 nucleotides from the 5′-untranslated region of the rat α1E (see also (22)). It was subcloned into the EcoRI site of the eukaryotic expression vector pCAGGS-GFP-neo containing the β-actin promoter with the cytomegalovirus (CMV) enhancer, the rabbit β-globin 3′-flanking sequence including the polyadenylation signal site, and the CMV-neo cassette as a selection marker. During subcloning, the GFP (green fluorescent protein) cassette of pCAGGS-GFP-neo was replaced by the 1146 bp long EcoRI fragment of the rat α1E in both orientations, yielding vectors with a sense and antisense α1E cassette. The vector pCAGGS-GFP-neo expressing GFP was used as a control for non-specific cloning events.

For the detection of RNA by RT-PCR in overexpressing clones, the cDNA fragments of the housekeeper enzyme hypoxanthine-phosphoribosyl transferase (HPRT) were amplified as reported (15) using the annealing temperature 57°C during 27 cycles.

INS-1 cells and cell culture

Aliquots of the wild-type INS-1 cell line were a gift from Dr S Ullrich with the kind permission of Dr C B Wollheim (Geneva, Switzerland). INS-1 cells and derived cell clones were maintained in RPMI 1640 medium (Life Technologies, Karlsruhe, Germany) containing 11 mmol/l glucose and supplemented with 10% heat inactivated fetal calf serum (Roche Molecular Biochemicals, Mannheim, Germany), 50 μmol/l 2-mercaptoethanol, 1 mmol/l sodium pyruvate, 10 mmol/l Hepes, pH 7.2–7.5, 100 IU/ml penicillin and 100 μg/ml streptomycin.

Stable transfection of INS-1 cells and culture of cell clones

Sense, antisense and control vectors were transfected into the rat insulinoma cell line INS-1 as described for stable transfection of HEK-293 cells (23) or INS-1 cells (24). Stable transformants were selected by adding geneticin (G418; Gibco-BRL, Eggenstein, Germany) in the medium at 250 μg/ml. Forty antisense α1E-, eight sense-α1E- and three GFP-vector-containing colonies were screened by RT-PCR (for sense and antisense vectors) and by fluorescence (for the control vector).

Insulin secretion

The secretion of insulin in INS-1 cells was analyzed as reported (7). In 24-well plates, 2.5 × 10⁵ cells per well (in 1 ml medium) were incubated for 3 days at 37°C (5% CO₂). Cells were washed twice with Krebs–Ringer bicarbonate (KRB) containing (in mmol/l) 136 NaCl, 4.7 KCl, 1.2 MgSO₄, 1 CaCl₂, 1.2 KH₂PO₄, 5 NaHCO₃, 10 Hepes, pH 7.4 and 0.5% BSA (fraction V; Sigma Chemical Co., München, Germany). They were sensitized to glucose in KRB for 30 min at 37°C and 5% CO₂. Toxins were added for 30 min in 500 μl KRB without and with either 15 mmol/l glucose or 25 mmol/l KCl at 37°C and 5% CO₂. Cells were sedimented for 5 min at 1000 g, and the supernatant (300 μl) was decanted and stored at −20°C. In order to determine remaining cellular insulin content, the cells were extracted overnight at 4°C with 1 ml acetylated ethyl alcohol containing 75% (v/v) ethanol plus 0.5% HCl. The extract was diluted 1:2000 up to 1:5000 in 0.2 mol/l glycine/NaOH buffer (pH 8.8) plus 0.25% BSA, while the supernatant I was diluted from 1:25 up to 1:100 depending on the amount of insulin secreted.
RIA

The insulin content was quantified as reported (7) using a modified coated charcoal RIA (25). The diluted solutions were incubated with antiserum (anti-rat insulin from guinea pig) (Lot ARI-02 [1ST]; Linco Research, St Charles, MO, USA) and iodinated (125I) insulin (CIS Diagnostik, Dreieich, Germany) for 20 h at room temperature. Remaining unbound 125I-insulin was determined in a counter (LKB-Wallac, Freiburg, Germany).

For each well, both secreted insulin and the remaining cellular insulin were determined in duplicate. The percentage of insulin secreted per remaining insulin was taken as a measure of the relative insulin release. In the final normalization, the release in the absence of antagonist was taken as 100%. The insulin release in the presence of antagonists was related to this 100% value. A calibration curve was determined in parallel with known amounts of rat insulin (Novo Nordisk, Copenhagen, Denmark).

Isolation of membrane proteins and immunodetection of α1E splice variants by Western blotting

Stably transfected HEK-293 cells were cultivated as described (19). Microsomal membrane proteins from untransfected and stably transfected HEK-293 cells as well as from the rat insulinoma cell line INS-1 (26) were isolated according to Pereverzev et al. (19). Aliquots of microsomal membranes were stored at −80°C.

The membrane proteins were separated by PAGE according to standard protocols and immunoblotting was performed as reported previously (19). The enhanced chemiluminescence detection kit was purchased from Amersham Corp. (Braunschweig, Germany).

Protein concentrations were determined with the bicinchoninic acid method (Pierce, Rockford, IL, USA).

Voltage-clamp recordings

Electrophysiological recordings from untransfected and transfected INS-1 cells were performed in the whole cell configuration of the patch clamp technique at room temperature (20–22°C). The bath solution contained (in mmol/l) 15 BaCl2, 130 N-methyl-glucamine, 10 Hepes, 5 KCl, pH adjusted with HCl to 7.4. The patch electrodes had resistances of 2–4 MΩ when filled with an electrode solution composed of (in mmol/l) 130 CsCl, 5 oxalacetate, 5 creatine, 5 pyruvate, 5 EGTA, 10 Hepes/CSOH (pH 7.4). The cells were placed on a 12 mm wide round cover slip in a recording chamber and were continuously superfused at a rate of 2–5 ml/min, as described previously (6). (±)-Isradipine was applied at a final concentration of 2 μmol/l. Cells were preincubated with 0.5 μmol/l ω-conotoxin MVIIIC for 30 min in the culture medium. Additionally, the bath solution was supplemented with 0.5 μmol/l of the toxin.

Data analysis

Data were used as relative insulin release and were calculated throughout as means±S.E.M. Significance was estimated by the Student’s t-test and levels of P < 0.05 were considered as statistically significant. The data are given as normalized values by taking the insulin release in the absence of antagonists as the 100% value.

Results

Overexpression of α1E cassettes after stable transfection of INS-1 cells

In order to reduce by an antisense cassette the expression of α1E voltage-gated Ca2+ channels in the insulinoma cell line INS-1, and to compare its effect on insulin secretion with control cells, three different recombinant mammalian expression vectors were constructed (Fig. 1A), bearing a 1130 bp sense or antisense sequence of the 5'-end from the rat α1E gene. It corresponds to the amino terminal part, the domain I and part of the I-II loop (Fig. 1B). The vector pCACGS-GFP-neo, expressing GFP, was stably transfected into INS-1 cells, to control non-specific effects of the cloning procedure.

Geneticin-resistant colonies were randomly selected, isolated and expanded. The integration of the sense and antisense cassette as well as the transcription of each vector was monitored by RT-PCR. Therefore, total RNA was extracted from each clone and taken as a template for RT-PCR. Transcripts of the HPRT gene were used to confirm equal loading of DNA on the individual lanes. In Fig. 2A, 4 out of 15 antisense INS-1 cell clones are shown displaying variable amounts of amplified α1E cDNA fragments. In parallel, two sense clones were selected and the RNA from wild-type INS-1 cells was used for RT-PCR. Annealing temperature and cycle number during PCR were adapted to those conditions such that no cDNA fragment was amplified from endogenous α1E of wild-type RNA (see lane 15, Fig. 2A). However, the highly expressed α1E cassette cDNA was clearly amplified. The clones with high expression of each cassette as the antisense clones as6C2, as66, as84 and as90, and the sense clones S9 and S6B2 were selected for further experiments.
At the protein level, α1E expression has been investigated by the anti-α1E-specific serum in the reference system of stably transfected HEK-293 cells (19) as well as in INS-1 cells (7). The major polypeptides stained by the anti-α1E serum in INS-1 cells show a faster electrophoretic mobility than the 246 kDa polypeptide detected in the HEK α1E reference system. After loading maximal amounts of microsomal protein, the largest proteolytic fragment, a protein of 225 kDa, was quantified and its amount was reduced in the antisense clone as84 as compared with wild-type and sense S9. In three independent Western blots the reduction was $41 \pm 15\%$ ($n = 3$). As far as the truncated α1E-225 is representative for the expression of α1E cDNA fragments in sense and antisense orientation. Three different vectors were constructed starting from the eukaryotic expression vector pCAGGS-GFP-neo. It contains the β-actin promoter with CMV enhancer, the last exon of the rabbit β-globin gene, and the CMV-neo cassette as a selection marker. The GFP-containing vector was used for mock transfection. The sense and antisense vectors were derived from the former vector by subcloning the α1E cDNA (B) in sense or antisense orientation into the EcoRI sites, thus replacing GFP by a 1146 bp fragment of rat α1E. The α1E cDNA fragment selected for the overexpression of an α1E fragment in sense and antisense orientation in INS-1 cells was amplified as the mouse and human homologue, which is different from the cloned rat α1E (22, see also 34). The sequence similarity of the α1E-cDNA fragment with the structurally homologous non-L-type α1 subunits is depicted and is highest with α1A and α1B, and lowest with α1G.

Figure 1 Design of the vector construct used for the overexpression of α1E cDNA fragments in sense and antisense orientation. Three different vectors were constructed starting from the eukaryotic expression vector pCAGGS-GFP-neo. It contains the β-actin promoter with CMV enhancer, the last exon of the rabbit β-globin gene, and the CMV-neo cassette as a selection marker. The GFP-containing vector was used for mock transfection. The sense and antisense vectors were derived from the former vector by subcloning the α1E cDNA (B) in sense or antisense orientation into the EcoRI sites, thus replacing GFP by a 1146 bp fragment of rat α1E. The α1E cDNA fragment selected for the overexpression of an α1E fragment in sense and antisense orientation in INS-1 cells was amplified as the mouse and human homologue, which is different from the cloned rat α1E (22, see also 34). The sequence similarity of the α1E-cDNA fragment with the structurally homologous non-L-type α1 subunits is depicted and is highest with α1A and α1B, and lowest with α1G.

Figure 2 Detection of sense and antisense α1E cassettes by RT-PCR in stably transfected INS-1 cell clones. The full-length cDNA of sense and antisense cassettes was amplified after reverse transcription (see lanes with ‘+’) of total RNA from four antisense (lanes 2–9) and two sense clones (lanes 11–14). Temperature and cycle number were selected to obtain optimal conditions for a selective amplification of only overexpressed transcripts. Therefore, no endogenous α1E was amplified when using the primers designed for the vector construct (lane 15). The names of individual clones are abbreviated. In parallel, the cDNA of HPRT (GenBank M63983) was amplified as housekeeper gene. Individual lanes are labeled ‘-‘, when no reverse transcription was performed. The lanes loaded with marker are labeled ‘M’.

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α1E, it can be concluded that α1E expression in the antisense clone as84 is reduced.

**Dihydropyridine- and ω-conotoxin MVIIC-sensitive inward currents**

The sequence of the selected α1E fragment corresponding to the 1130 bp cDNA was compared with structurally related α1 subunits of the non-L-type subfamily of voltage-gated Ca\(^{2+}\) channels. At the amino acid level, the sequence reveals 75 and 76% sequence identity with rat α1A and α1B respectively. The sequence identity is lower when compared with α1C and α1G, members of the L- and T-type subfamily of voltage-gated Ca\(^{2+}\) channels (Fig. 1B).

Because of the high sequence similarity between α1E and members of the non-L-type subfamily, the sensitivity of the Ba\(^{2+}\) inward currents towards Ca\(^{2+}\)-channel-selective antagonists was tested in wild-type and in the antisense α1E-overexpressing cell clone as84 (Fig. 3). Current density of HVA inward currents was similar in wild-type, antisense α1E, and sense α1E control cells. The minor reduction of inward currents observed in the sense and the antisense clone as compared with wild-type cells was not significant. The sensitivity towards 0.5 μmol/l ω-conotoxin MVIIC was not significantly impaired in sense or antisense INS-1 cell clones as compared with wild-type INS-1 cells (Fig. 3C). Similar results were recorded when using 2 μmol/l (±)-isradipine to inhibit dihydropyridine-sensitive L-type Ca\(^{2+}\) channels (Fig. 3D). In conclusion, the overexpressions of sense and antisense α1E cassettes are specific and do not impair the sensitivity of Ba\(^{2+}\) inward currents towards P/Q-type, N-type and L-type voltage-gated Ca\(^{2+}\) channels.

**Insulin content and basal insulin secretion**

Insulin content and the basal insulin release were determined in sense and antisense clones (Fig. 4). Wild-type INS-1 cells contain the highest amount of insulin similar to the sense clone S9 (Fig. 4A). Both the sense clone S6B2 and the GFP control clone contain less than half of the amount of insulin of the wild-type cells and similar to the antisense clones as90, as84 and as6C2 (Fig. 4A). On the other hand, basal insulin release is lowest in wild-type INS-1 cells, and increased to different extents in sense, control and antisense clones (Fig. 4B).

In conclusion, both insulin content and basal insulin secretion are changed during the cloning procedure. The changes for the insulin content, which are significant in S6B2, GFP3D1, as90, as84 and as6C2 clone, as compared with wild-type INS-1 cells, are not
due to the overexpression of the antisense α1E, because basal release and insulin content are changed in both antisense and sense or control clones.

**Stimulus-induced insulin secretion**

The glucose- and KCl-induced insulin secretion was investigated in wild-type, as well as in sense and antisense INS-1 cell clones. The percentage of insulin secretion was determined as insulin released per remaining intracellular insulin. At 15 mmol/l glucose, the insulin secretion was increased 3.5-fold from a basal rate of 0.50 to 1.71% (Table 1). Compared with wild-type INS-1 cells, no significant difference was observed in the S9 sense clone showing a 3.9-fold increase from a basal rate of 0.45 to 1.71% (Table 1). The two antisense clones as84 and as6C2 revealed a reduced glucose-induced stimulation at 2.0-fold and 1.7-fold. Such a clear reduction of stimulus-induced insulin secretion was not observed for the KCl-induced insulin secretion (Fig. 5), which showed a 3.7- and 3.0-fold increase for the wild-type and the S9 clone respectively, and a 2.9- and 2.6-fold increase for the two antisense clones as84 and as6C2 respectively (Table 1). KCl-induced insulin release was not significantly different between sense and antisense INS-1 clones, probably because 25 mmol/l KCl depolarizes the INS-1 cells more strongly than 15 mmol/l glucose does through ATP-dependent K⁺ channels. The ratio of glucose-induced insulin release is significantly lowered in the two antisense INS-1 clones, favoring the conclusion that overexpression of the antisense α1E cassette reduces protein expression of α1E as well as α1E-mediated insulin secretion.

**Discussion**

The insulinoma cell line INS-1, representing a permanent β-cell line, was established in the laboratory of Prof. Dr C B Wollheim (26) and has become a useful model for studying the molecular mechanism of Ca²⁺-dependent and Ca²⁺-independent insulin secretion. INS-1 cells have retained the capability to respond to an increase in the glucose concentration with a biphasic insulin secretion (3). Most important is the observation that INS-1 cells secrete insulin in response to glucose concentrations in the physiological range. In addition, INS-1 cells can be genetically engineered, which is useful for basic research as well as for exploring their use as an alternative to isolated islets for transplantation therapy of type 1 diabetes (27).

**Figure 4** Insulin content (A), and basal insulin-release (B) in wild-type, sense and antisense α1E INS-1 cell clones. The remaining insulin content was determined after stimulus-induced secretion by extraction with acidified ethanol. The insulin content was quantified from 2.5 × 10⁵ cells which were grown during 3 days in 24-well plates in 1 ml medium. The insulin content is significantly reduced in S6B2 and GFP3D1 cells as well as in all three antisense clones compared with wild-type INS-1 cells (P < 0.05). Basal insulin release was determined after a 30 min incubation without adding any secretagogue. The basal release is significantly increased in GFP3D1 and as6C2 cells compared with wild-type INS-1 cells (P < 0.05). The data from between 3 and 6 (A) and 3 and 16 (B) independent experiments are summarized and the S.E.M. is shown.
Ca\(^{2+}\) influx for the Ca\(^{2+}\)-dependent insulin secretion is mediated through more than one voltage-gated Ca\(^{2+}\) channel. INS-1 cells express dihydropyridine-sensitive L-type (6, 28) as well as non-L-type Ca\(^{2+}\) channels (4, 5). In addition, islets of Langerhans, as well as INS-1 cells, contain \(\alpha_{1E}\), which is a candidate for the toxin-resistant Ca\(^{2+}\) channel (R-type). This current is defined as the component which remains after the block of L-, N- (29) and P-/Q-type Ca\(^{2+}\) channels (30). Its physiological function is incompletely understood and was investigated in the present study by using an overexpressing antisense \(\alpha_{1E}\) cassette. In addition, the \(\alpha_{1E}\)-selective peptide toxin SNX-482 was used as an antagonist of \(\alpha_{1E}\) Ca\(^{2+}\) channels and thus as a partial blocker of insulin secretion (7).

Insulinoma cells, capable of overexpressing sense and antisense \(\alpha_{1E}\) mRNA fragments, were generated from parental INS-1 cells. The cDNA of rat \(\alpha_{1E}\) was selected from the 5\(^{\text{'}}\)-end containing part of the non-coding region, the domain I as well as part of the linker between domains I and II. The amplified cDNA fragment corresponds to a transcript which is different from the cloned rat \(\alpha_{1E}\) (31) but corresponds to the cDNA fragment also found in mouse and human \(\alpha_{1E}\) (22). The 1146 bp cDNA fragment was introduced into the eukaryotic expression vector pCAGGS, which keeps the cDNA under the control of the CMV immediate early enhancer and the chicken \(\beta\)-actin promoter.

The expression of the rat \(\alpha_{1E}\) cDNA fragments was monitored by RT-PCR. Eleven out of 15 analyzed antisense clones and both sense clones showed a high concentration of the cassette cDNA as compared with the endogenous \(\alpha_{1E}\). The endogenous \(\alpha_{1E}\) transcript was not amplified because of an increased annealing

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**Table 1** Glucose- and KCl-induced insulin secretion in wild-type, control, sense and antisense INS-1 cell clones. Cells (2.5 x 10\(^5\)) were incubated under identical conditions in 24-well plates. The percentage of insulin secreted per remaining insulin was taken as a measure of the relative insulin release. The control clone overexpressed GFP (GFP3D1). One sense (S9) and two or three antisense clones (as90, as84 and as6C2) were compared with wild-type INS-1 cells. Only the ratio of the glucose-induced secretion per basal secretion was significantly reduced in all three antisense clones compared with wild-type INS-1 cells.

<table>
<thead>
<tr>
<th>Cell clone</th>
<th>Basal secretion (%)</th>
<th>Stimulus-induced secretion (%)</th>
<th>Ratio of stimulus-induced to basal secretion</th>
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<tr>
<td></td>
<td>Mean S.E.M. n</td>
<td>Mean S.E.M. n</td>
<td>Mean S.E.M. n</td>
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<td>glucose-induced stimulation (15 mmol/l)</td>
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<tr>
<td>INS-1, wild-type</td>
<td>0.50 0.04 9</td>
<td>1.71 0.16 9</td>
<td>3.5 0.4 9</td>
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<td>1.71 0.28 4</td>
<td>3.9 0.3 4</td>
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<tr>
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<td>2.0** 0.1 6</td>
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<td>1.63 0.27 5</td>
<td>1.7** 0.2 5</td>
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<td>KCl-induced stimulation (25 mmol/l)</td>
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<td>2.6 0.3 3</td>
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</table>

* Significantly different from the corresponding value of wild-type cells (\(P < 0.05\)).
** Significantly different from the corresponding value of wild-type cells (\(P < 0.01\)).
temperature and a lowered cycle number. Stable INS-1 clones were selected with the highest numbers of transcript amplified. Based on the strength of the selected promoter, we assume that the α1E cassette should be highly expressed in the selected INS-1 clones.

On the protein level, a reliable quantification of the α1E expression level was impaired because of a putative proteolysis of the α1E Ca\textsuperscript{2+} channel. Low amounts of a full-length α1E could be detected (7); however, a major part of α1E seems to be degraded. Sequence comparison of rat α1E with synthetic peptides used for testing proprotein convertases revealed an increased number of basic amino acid residues in the linker between domain II and III. The amino acid sequence of rat α1E beginning at position 934 (GenBank L15453), QDLRRTFNSLMVP, contains a pair of basic amino acid residues as well as additional amino acids used as the recognition site of ‘paired basic amino acid converting enzymes’, also called proprotein convertases. Furthermore, beginning at position 699, additional arginine-rich regions are present, as RERRRRHHMSWVWEORTSQL. This sequence beginning with R-E-R-R is quite similar to the consensus site for the furin proprotein convertase, R-X-K/R-R. The fragment size of degraded α1E corresponds to an expected one if proteolysis occurs in the linker between domains II and III. Using the convertase inhibitor LLRKVR at concentrations which inhibit convertases, failed to prevent the assumed proteolysis (R Vajna & T Schneider, unpublished observations).

Although the sequence similarity between the α1E cassette and the homologous region of α1A or α1B is about 75%, we can exclude a prominent interaction of α1E antisense cassette with the endogenous α1A and α1B transcripts because of Ba\textsuperscript{2+} inward currents. No significant reduction but a tendency was observed in the sensitivity of inward currents towards α1A- and α1B-selective antagonists.

The insulin content as well as the basal insulin release was not only changed in antisense clones but also in the sense clone S6B2 and the GFP control clone GFP3D1. This result points rather to non-specific effects due to the cloning procedure.

The three α1E antisense clones as90, as84 and as6C2 show a lower glucose-induced insulin release than the wild-type and the sense S9 clone, although the KCl-induced secretion is not impaired. This result can be explained by the assumption that α1E is acting as a mid-voltage-gated calcium channel during glucose-induced insulin secretion, which is overcome by the strong depolarization mediated by 25 mmol/l KCl. Additional support for the interpretation of α1E as a mid-voltage activating Ca\textsuperscript{2+} channel comes from Ca\textsuperscript{2+} current measurements in palaeocortical neurons (32).

In conclusion, voltage-gated Ca\textsuperscript{2+} channels containing α1E as an ion-conducting pore probably influence the excitability of the insulin-secreting cells. This channel can speed up or slow down the time course for the depolarization of the membrane potential necessary for the activation of HVA L-type calcium channels. If α1E is involved in the successive activation of voltage-gated Ca\textsuperscript{2+} channels, then its blocking by an antagonist should be helpful for therapeutic purposes. The importance of resistant-type Ca\textsuperscript{2+} channels for secretion of hormones is supported by recent findings from chromaffin cells where 22% of resistant-type currents are responsible for 55% of secretion (8) and by gene inactivation in mice (33).

Acknowledgements

We are grateful to Prof. Dr J Miyazaki (Osaka, Japan) for the vector pCAGGS-GFP. We thank Mrs R Clemens, Mrs M Henry and Mrs S Schulze for their excellent technical assistance. The work was financially supported by the Center of Molecular Medicine Cologne/Zentrum für Molekularbiologische Medizin Köln (Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie, Förderkennzeichen 01 KS 9502) to T S and J H.

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Received 9 October 2001
Accepted 12 March 2002