EXPERIMENTAL STUDY

Reduction of insulin secretion in the insulinoma cell line INS-1 by overexpression of a $\text{Ca}_v2.3$ ($\alpha1\text{E}$) calcium channel antisense cassette

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Abstract

Objective: Multiple types of voltage-activated Ca^{2+} channels (T, L, N, P, Q and R type) coordinate a variety of Ca^{2+} -dependent processes in neurons and neuroendocrine cells. In insulinoma cell lines as well as in endocrine tissues, the non-L-type $\alpha1\text{E}$ ($\text{Ca}_v2.3$) subunit is expressed as the tissue-specific splice variant $\alpha1\text{Ee}$.

Design and Methods: To understand the functional role of $\alpha1\text{E}$ -containing Ca^{2+} channels, antisense $\alpha1\text{E}$ mRNA was overexpressed in INS-1 cells by stable transfection of an antisense $\alpha1\text{E}$ cassette cDNA. As controls, either a sense $\alpha1\text{E}$ 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 $\alpha1\text{E}$ INS-1 clones, the glucose-induced insulin release was significantly reduced as compared with wild-type INS-1 cells and with a sense $\alpha1\text{E}$ INS-1 clone. However, in the antisense INS-1 clones, the KCl-induced insulin release was less impaired by overexpressing the antisense $\alpha1\text{E}$ 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: $\alpha1\text{E}$ is involved in glucose-induced insulin secretion probably by influencing the excitability of INS-1 cells.

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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^+ channels and voltage-gated Ca^{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^+ 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^{2+} channels (1, 2).

The influx of calcium ions through voltage-gated Ca^{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^{2+} channels, but also non-L-type Ca^{2+} channels,

the members of a second subfamily of high-voltage-activated (HVA) Ca^{2+} channels, are involved in the Ca^{2+} -induced insulin secretion (4, 5).

Recently, the 'R-type' Ca^{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^{2+} channel has so far been defined by its resistance to known Ca^{2+} channel blockers such as dihydropyridines, ω -conotoxin GVIA and ω -conotoxin MVIIC (9–11). Using antisense $\alpha1\text{E}$ oligonucleotides and the $\alpha1\text{E}$ -selective antagonist SNX-482, it was shown that $\alpha1\text{E}$ is related to multiple resistant components of HVA Ca^{2+} channels in rat cerebellar granule cells (12, 13). Some, but not all of the resistant component is absent in $\alpha1\text{E}$ -depleted cerebellar granule cells isolated from $\alpha1\text{E}$ knockout mice (14). The predicted structure of $\alpha1\text{Ee}$ in the insulinoma cell line INS-1 and in rat islets of Langerhans (15) is different from the cloned $\alpha1\text{Ed}$ (16) and $\alpha1\text{E-1}/\alpha1\text{E-3}$ variants

(17). The $\alpha 1E$ 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 $\alpha 1E$ splice variant, a cassette containing the 5'-region of $\alpha 1E$ cDNA in antisense orientation was stably transfected into INS-1 cells. The glucose- and KCl-induced insulin secretion of antisense $\alpha 1E$ INS-1 cell clones was compared with those INS-1 clones which were stably transfected with sense $\alpha 1E$ 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 MVIIC, was purchased from Bachem Biochemica (Heidelberg, Germany).

RT-PCR and construction of sense and antisense $\alpha 1E$ expression vector

The rat $\alpha 1E$ 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 $\alpha 1E$ cDNA were amplified by PCR using the primer $\alpha 1E$ -as, 5'-CGGAATTCGACAGGGCGACTATGGCTTTGTA-3' (nucleotides 182–205) of rat $\alpha 1E$ cDNA sequence (GenBank L15453) as forward and $\alpha 1E$ -as-r, 5'-CGGA-ATTCCTCCGTCGGCTCCTTTTG-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 $\alpha 1E$ 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 $\alpha 1E$ (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 $\alpha 1E$ in both orientations, yielding vectors with a sense and antisense $\alpha 1E$ 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 μ mol/l 2-mercaptoethanol, 1 mmol/l sodium pyruvate, 10 mmol/l Hepes, pH 7.2–7.5, 100 IU/ml penicillin and 100 μ g/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 μ g/ml. Forty antisense $\alpha 1E$ -, eight sense- $\alpha 1E$ - 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, 2.5×10^5 cells per wells (in 1 ml medium) were incubated for 3 days at 37 °C (5% CO_2). Cells were washed twice with Krebs–Ringer bicarbonate (KRB) containing (in mmol/l) 136 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1 $CaCl_2$, 1.2 KH_2PO_4 , 5 $NaHCO_3$, 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% CO_2 . Toxins were added for 30 min in 500 μ l KRB without and with either 15 mmol/l glucose or 25 mmol/l KCl at 37 °C and 5% CO_2 . Cells were sedimented for 5 min at 1000 g, and the supernatant I (300 μ l) 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 acidified ethanol 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 [15T]; Linco Research, St Charles, MO, USA) and iodinated (^{125}I) insulin (CIS Diagnostik, Dreieich, Germany) for 20 h at room temperature. Remaining unbound insulin was precipitated by activated carbon at 30 g/l (Norit; Sigma) plus 2.5 g/l dextran FP70 (Serva, Heidelberg, Germany) in 0.2 mol/l glycine buffer, pH 8.8 and 25% normal calf serum (Biochrom KG Seromed, Berlin, Germany). The decay rate of unbound ^{125}I -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 $\alpha 1\text{E}$ 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 ($20\text{--}22^\circ\text{C}$). The bath solution contained (in mmol/l) 15 BaCl_2 , 130 N-methyl-glucamine, 10 Hepes, 5 KCl, pH adjusted with HCl to 7.4. The patch electrodes had resistances of $2\text{--}4\text{ M}\Omega$ 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 $2\text{--}5\text{ ml/min}$, as described previously (6). ($\pm$)-Isradipine was applied at a final concentration of $2\text{ }\mu\text{mol/l}$. Cells were preincubated with $0.5\text{ }\mu\text{mol/l}$ ω -conotoxin MVIIC for 30 min in the culture medium. Additionally, the bath solution was supplemented with $0.5\text{ }\mu\text{mol/l}$ of the toxin.

Data analysis

Data were used as relative insulin release and are calculated throughout as means $\pm$ 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 $\alpha 1\text{E}$ cassettes after stable transfection of INS-1 cells

In order to reduce by an antisense cassette the expression of $\alpha 1\text{E}$ voltage-gated Ca^{2+} 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 $\alpha 1\text{E}$ gene. It corresponds to the amino terminal part, the domain I and part of the I-II loop (Fig. 1B). The vector pCAGGS-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 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 $\alpha 1\text{E}$ 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 $\alpha 1\text{E}$ of wild-type RNA (see lane 15, Fig. 2A). However, the highly expressed $\alpha 1\text{E}$ 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.

Figure 1 Design of the vector construct used for the overexpression of $\alpha 1E$ 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 $\alpha 1E$ cDNA (B) in sense or antisense orientation into the EcoRI sites, thus replacing GFP by a 1146 bp fragment of rat $\alpha 1E$. The $\alpha 1E$ cDNA fragment selected for the overexpression of an $\alpha 1E$ 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 $\alpha 1E$ (22, see also 34). The sequence similarity of the $\alpha 1E$ -cDNA fragment with the structurally homologous non-L-type $\alpha 1$ subunits is depicted and is highest with $\alpha 1A$ and $\alpha 1B$, and lowest with $\alpha 1G$.

At the protein level, $\alpha 1E$ expression has been investigated by the anti- $\alpha 1E$ -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- $\alpha 1E$ serum in INS-1 cells show a faster electrophoretic mobility than the 246 kDa polypeptide detected in the HEK $\alpha 1E$ 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 $\alpha 1E$ -225 is representative for the expression of

Figure 2 Detection of sense and antisense $\alpha 1E$ 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 $\alpha 1E$ 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 HPTR (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'.

$\alpha 1\text{E}$, it can be concluded that $\alpha 1\text{E}$ expression in the antisense clone as84 is reduced.

Dihydropyridine- and ω -conotoxin MVIIC-sensitive inward currents

The sequence of the selected $\alpha 1\text{E}$ fragment corresponding to the 1130 bp cDNA was compared with structurally related $\alpha 1$ 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 $\alpha 1\text{A}$ and $\alpha 1\text{B}$ respectively. The sequence identity is lower when compared with $\alpha 1\text{C}$ and $\alpha 1\text{G}$, members of the L- and T-type subfamily of voltage-gated Ca^{2+} channels (Fig. 1B).

Because of the high sequence similarity between $\alpha 1\text{E}$ 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 $\alpha 1\text{E}$ -overexpressing cell clone as84 (Fig. 3). Current density of HVA inward currents was similar in wild-type, antisense $\alpha 1\text{E}$, and sense $\alpha 1\text{E}$ 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 $0.5 \mu\text{mol/l}$ 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 $2 \mu\text{mol/l}$ ($\pm$)-isradipine to inhibit dihydropyridine-sensitive L-type Ca^{2+} channels (Fig. 3D). In conclusion, the overexpressions of sense and antisense $\alpha 1\text{E}$ 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

Figure 3 Sensitivity of HVA Ca^{2+} -inward currents towards L- and non-L-type Ca^{2+} channel blockers. Peak I_{Ba} was recorded in cells derived after stable transfection of the antisense (As) or sense (S) cassette and was compared with wild-type INS-1 cells (wt). Original current traces were obtained by depolarizations of the cell membrane from a holding potential of -90 mV to the test potential of $+15 \text{ mV}$. (A) Representative current traces of total I_{Ba} for the control INS-1 cells, an antisense and a sense INS-1 clone. (B) Representative current traces of the remaining I_{Ba} for the control INS-1 cells, an antisense and a sense INS-1 clone after blocking HVA N- and P/Q-type currents by $0.5 \mu\text{mol/l}$ ω -conotoxin MVIIC. (C) In the presence of $0.5 \mu\text{mol/l}$ ω -conotoxin MVIIC, no significant difference was seen between the inhibition in the sense and the antisense clone. Peak I_{Ba} was reduced to $34 \pm 4 \text{ pA/pF}$ (S.E.M.) ($n = 10$) in wild-type, to $29 \pm 3 \text{ pA/pF}$ ($n = 15$) in as84, and to $33 \pm 4 \text{ pA/pF}$ ($n = 4$) in the S9 clone. (D) Mean data are compared for the peak I_{Ba} recorded in the presence of $2 \mu\text{mol/l}$ ($\pm$)-isradipine, a blocker of L-type calcium currents. Peak I_{Ba} was reduced to $47 \pm 5\%$ ($n = 3$) and $46 \pm 6\%$ ($n = 5$) in wild-type and antisense cells respectively.

due to the overexpression of the antisense $\alpha 1E$, 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 $\alpha 1E$ cassette reduces protein expression of $\alpha 1E$ as well as $\alpha 1E$ -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 Ca^{2+} -dependent and Ca^{2+} -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 $\alpha 1E$ 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 2.5×10^5 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.

Figure 5 Stimulus-induced insulin release (means ± S.E.M.) in wild-type, sense and antisense $\alpha 1\text{E}$ INS-1 cells. Insulin secretion was induced by 15 mmol/l glucose or 25 mmol/l KCl in wild-type INS-1 cells, in the sense clone S9 and in two independent antisense clones, as84 and as6C2. The individual numbers plotted in this figure correspond to the data in Table 1. Note that the reduction of glucose-induced insulin release in the antisense clones is partially due to an increased basal release of insulin. The difference of glucose-induced insulin release between wild-type INS-1 cells and each of the two antisense clones is highly significant ($P < 0.001$).

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 $\alpha 1\text{E}$, 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 $\alpha 1\text{E}$ cassette. In addition, the $\alpha 1\text{E}$ -selective peptide toxin SNX-482 was used as an antagonist of $\alpha 1\text{E}$ Ca^{2+} channels and thus as a partial blocker of insulin secretion (7).

Insulinoma cells, capable of overexpressing sense and antisense $\alpha 1\text{E}$ mRNA fragments, were generated from

parental INS-1 cells. The cDNA of rat $\alpha 1\text{E}$ was selected from the 5'-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 $\alpha 1\text{E}$ (31) but corresponds to the cDNA fragment also found in mouse and human $\alpha 1\text{E}$ (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 β -actin promoter.

The expression of the rat $\alpha 1\text{E}$ 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 $\alpha 1\text{E}$. The endogenous $\alpha 1\text{E}$ transcript was not amplified because of an increased annealing

Table 1 Glucose- and KCl-induced insulin secretion in wild-type, control, sense and antisense INS-1 cell clones. Cells (2.5×10^5) 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 (as 90, 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.

Cell clone	Basal secretion (%)			Stimulus-induced secretion (%)			Ratio of stimulus-induced to basal secretion		
	Mean	S.E.M.	n	Mean	S.E.M.	n	Mean	S.E.M.	n
Glucose-induced stimulation (15 mmol/l)									
INS-1, wild-type	0.50	0.04	9	1.71	0.16	9	3.5	0.4	9
GFP3D1	0.93*	0.15	3	2.96	0.48	3	3.2	0.3	3
S9	0.45	0.08	4	1.71	0.28	4	3.9	0.3	4
as90	0.62	0.09	3	0.90	0.13	3	1.5*	0.1	3
as84	0.64	0.07	6	1.29	0.12	6	2.0**	0.1	6
as6C2	1.00*	0.22	5	1.63	0.27	5	1.7**	0.2	5
KCl-induced stimulation (25 mmol/l)									
INS-1, wild-type	0.49	0.05	7	1.72	0.16	7	3.7	0.5	7
S9	0.42	0.06	4	1.16	0.20	3	3.0	0.3	3
as84	0.61	0.05	6	1.72	0.18	6	2.9	0.3	6
as6C2	0.79	0.05	3	2.04	0.25	3	2.6	0.3	3

* 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 $\alpha 1E$ cassette should be highly expressed in the selected INS-1 clones.

On the protein level, a reliable quantification of the $\alpha 1E$ expression level was impaired because of a putative proteolysis of the $\alpha 1E$ Ca^{2+} channel. Low amounts of a full-length $\alpha 1E$ could be detected (7); however, a major part of $\alpha 1E$ seems to be degraded. Sequence comparison of rat $\alpha 1E$ 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 $\alpha 1E$ beginning at position 934 (GenBank L15453), QDLRRTNSLMVP, 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 RERRRRHHMSVWEQRTSQL. 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 $\alpha 1E$ corresponds to an expected one if proteolysis occurs in the linker between domains II and III. Using the convertase inhibitor LLRVKR at concentrations which inhibit convertases, failed to prevent the assumed proteolysis (R Vajna & T Schneider, unpublished observations).

Although the sequence similarity between the $\alpha 1E$ cassette and the homologous region of $\alpha 1A$ or $\alpha 1B$ is about 75%, we can exclude a prominent interaction of $\alpha 1E$ antisense cassette with the endogenous $\alpha 1A$ and $\alpha 1B$ transcripts because of Ba^{2+} inward currents. No significant reduction but a tendency was observed in the sensitivity of inward currents towards $\alpha 1A$ - and $\alpha 1B$ -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 $\alpha 1E$ 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 $\alpha 1E$ 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 $\alpha 1E$ as a mid-voltage activating Ca^{2+} channel comes from Ca^{2+} current measurements in palaeocortical neurons (32).

In conclusion, voltage-gated Ca^{2+} channels containing $\alpha 1E$ 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 $\alpha 1E$ is involved in the successive activation of voltage-gated Ca^{2+} channels, then its blocking by an antagonist should be helpful for therapeutic purposes. The importance of resistant-type Ca^{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).

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