TRPV6 channel modulates proliferation of insulin secreting INS-1E beta cell line

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Abstract

Transient receptor potential channel vanilloid type 6 (TRPV6) is a non-selective cation channel with high permeability for Ca2+ ions. So far, the role of TRPV6 in pancreatic beta cells is unknown. In the present study, we characterized the role of TRPV6 in controlling calcium signaling, cell proliferation as well as insulin expression, and secretion in experimental INS-1E beta cell model. TRPV6 protein production was downregulated using siRNA by approx. 70%, as detected by Western blot. Intracellular free Ca2+ ([Ca2+]i) was measured by fluorescence Ca2+ imaging using fura-2. Calcineurin/NFAT signaling was analyzed using a NFAT reporter assay as well as a calcineurin activity assay. TRPV6 downregulation resulted in impaired cellular calcium influx. Its downregulation also reduced cell proliferation and decreased insulin mRNA expression. These changes were accompanied by the inhibition of the calcineurin/NFAT signaling. In contrast, insulin exocytosis was not affected by TRPV6 downregulation. In conclusion, this study demonstrates for the first time the expression of TRPV6 in INS-1E cells and rat pancreatic beta cells and describes its role in modulating calcium signaling, beta cell proliferation and insulin mRNA expression. In contrast, TRPV6 fails to influence insulin secretion.

1. Introduction

Calcium signaling is essential to regulate a variety of biological processes of the pancreatic beta cells. Calcium influx triggers insulin secretion [1] and modulates beta cell proliferation as well as apoptosis [2]. Notably, impaired calcium homeostasis in beta cells plays a critical role in the pathophysiology of type 1 and type 2 diabetes [3,4].

Previous studies indicated that pancreatic beta cells or experimental beta cell lines express several members of a large family of non-selective cation entry channels, such as the transient receptor potential vanilloid (TRPV) channel 2 and 4 [5–7]. TRPV channels are composed of six membrane-spanning channels (TRPV1–6) which regulate ion influx in response to thermal, hormonal, pharmacological and osmotic signals [8]. TRPV1 was shown to influence insulin sensitivity in animals as well as insulin exocytosis in INS-1E cells, but not in primary beta cells [9–11]. TRPV2 and TRPV4 modulate growth, death as well as insulin exocytosis in pancreatic beta cells as well as in experimental beta cell lines [5–7,9]. TRPV6 has the highest permeability for calcium ions among all known TRPVs [12]. Although TRPV6 mRNA is expressed in the exocrine as well as in the endocrine pancreas [13–15], the function of this channel in pancreatic beta cells is still unknown. In the present study, we therefore investigated the role of TRPV6 channel in controlling calcium homeostasis, cell proliferation as well as insulin expression, and secretion using a well-established experimental INS-1E rat beta cell model obtained from INS-1 cell line [16]. This cell line was used by numerous laboratories to characterize voltage-operated Ca2+ channels as well as other TRP channels in the past [17–21]. Our study shows that TRPV6 is expressed in INS-1E beta cell line and rat pancreatic islets. TRPV6 controls calcium homeostasis and stimulates cell
proliferation as well as insulin mRNA expression, without affecting insulin secretion.

2. Materials and methods

2.1. Materials

Media and supplements for cell culture were purchased from Biochrom AG (Berlin, Germany). Unless otherwise indicated, all other reagents were from Sigma Aldrich (Deisenhofen, Germany). Rabbit anti-TRPV6 antibody and TRPV6 blocking peptide was from Alomone Labs (Jerusalem, Israel). Mouse anti-β-actin antibody was from Sigma Aldrich. Guinea Pig anti-insulin antibody was from Dako (Santa Clara, CA, USA). Rabbit antibodies against phosphorylated ERK1/2, total ERK1/2 and secondary HRP-linked anti-rabbit and anti-mouse antibodies were from Cell Signaling Technology (Danvers, MA, USA). Secondary anti-rabbit and anti-Guinea Pig Cy3 and Alexa Fluor® 488 conjugated antibodies used for immunofluorescence were from Life Technologies (Carlsbad, CA, USA).

2.2. Cell culture

The experimental model of beta cells (rat insulinoma INS-1E cell line) was kindly provided by Dr. Pierre Maechler (Médical Universitaire, Genève, Switzerland). Cells were cultured as previously published [6]. In brief, RPMI medium was used supplemented with 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES buffer and 50 μM β-mercaptoethanol at 37 °C in a humidified atmosphere (5% CO2, 95% O2).

2.3. siRNA transfection

ON-TARGETplus SMARTpool of four individual TRPV6 siRNAs or non-targeting (nt) siRNA were obtained from Thermo Scientific Dharmacon (Waltham, MA, USA). In brief, fast-forward transfection either with 30 nM TRPV6 siRNA or 30 nM nt siRNA was performed using HiPerfect reagent (Qiagen, Hilden, Germany) according to the user’s protocol. The extent of TRPV6 downregulation by siRNA was assessed after 48 h using HiPerfect. Calcineurin activity was assessed after 48 h by Calcineurin Cellular Activity Assay Kit (Qiagen) according to manufacturer’s protocol. TIDR-software was used for data acquisition and evaluation (HEKA, Lamprecht, Germany). Results are shown as mean traces of [Ca2+]i (nM) ± SEM with n values indicating the number of experiments per data point. The measurements lasted 8 min.

2.4. Calcium imaging

Fura-2 fluorescence was used to detect very small changes of cytosolic free Ca2+ ([Ca2+]i). Two days after nt or TRPV6 siRNA transfection, INS-1E cells were pre-incubated with culture medium containing 1 μM fura-2/AM for ≈ 30 min at 37 °C. Thereafter, cells were washed with a Ringer-like solution containing (mM): 120 NaCl, 6 CsCl, 1 MgCl2, 10 glucose, and 10 HEPES acid and 1.5 CaCl2 (pH 7.4), to remove any cell debris or dead cells. Washed coverslips were mounted in a chamber containing the Ringer-like solution on the inverted microscope (Olympus BW50WI, Olympus Europa Holding GmbH, Hamburg, Germany) and equilibrated to room temperature (≈ 22 °C). A digital imaging system (TILL Photonics, Munich, Germany) was used to monitor changes in [Ca2+]i, based on fura-2 fluorescence. Fura-2 fluorescence was alternately excited at 340 and 380 nm wavelength and emission was detected every 500 ms at 510 nm. The detection field contained a cluster of fura-2-loaded cells. The fluorescence ratio (I340 nm/I380 nm) corresponds to changes in [Ca2+]i, which was calculated according to Grynkiewicz et al. (1985) [22]. For calibration of free Ca2+ concentration, the fluorescence level in the presence of EGTA (2 mM) (Rmin) or calcium (1.5 mM) (Rmax) was measured. For functional detection of TRPV6, a similar protocol was used as described in the literature [23]. In brief, measurements of the basal levels of [Ca2+]i were obtained during the first 3 min, followed by an isosmotic replacement with Ca2+-free Ringer-like solution (2 mM EGTA). Three minutes later, 1.5 mM Ca2+ was added to increase [Ca2+]i. The reversibility of Ca2+ changes is an indicator of cell viability and functional relevance of the Ca2+ sensing TRPV6 channel [22–26].

2.5. Detection of cell proliferation

INS-1E cells proliferation was evaluated using a Cell Proliferation ELISA BrdU colorimetric kit (Roche Diagnostics, Penzberg, Germany). Cells were seeded in 96-well plates and transfected either with 30 nM nt or TRPV6 siRNA and subsequently cultured in a standard growing medium. After 24, 48 and 72 h BrdU solution (10 μM) was added and cells were incubated for an additional 2 h. The incorporation of BrdU into the DNA was quantified according to the manufacturer’s protocol.

2.6. Detection of cell viability by MTT assay

INS-1E cells were seeded in 96-well plate and transfected either with nt or TRPV6 siRNA and cultured for 48 h. Thereafter, MTT solution (0.5 mg/ml) was added and cells were incubated for 30 min. Formazan crystals were then dissolved in 100 μl DMSO and optical density at 570 nm and 650 nm (reference wave length) was measured as previously described [27].

2.7. Cell cycle analysis by PI staining

Propidium iodide (PI) staining was performed as previously reported [28] with several modifications. In brief, 1.5 × 105 INS-1E cells were transfected either with nt or TRPV6 siRNA and cultured in growing medium for 48 h. Then cells were trypsinized and centrifuged (600 × g, 5 min). Cell-reach pellet was then resuspended in 900 μl of PBS and mixed with ethanol (70%). This mixture was then incubated overnight at −20 °C. Thereafter, cells were centrifuged (600 × g, 5 min) and washed with 1 ml of PBS, and centrifuged again (600 × g, 5 min). The pellet was resuspended in 500 μl of staining buffer containing PI (20 μg/ml) and RNase A (20 μg/ml) and incubated at 37 °C for 20 min. At least 1 × 104 cells were measured by FACs Calibur (BD Biosciences, Heidelberg, Germany) using a FL2 channel. Apoptotic and proliferating cells were counted by BD CellQuest Pro™ Software.

2.8. NFAT activity

NFAT reporter assay (Qiagen) was used. In brief, cells were seeded into black 96 wells plates and cotransfected with nt or TRPV6 siRNA (each at the concentration of 30 nM) and a mixture of inducible NFAT-responsive firefly luciferase construct and constitutively expressing Renilla luciferase construct (40:1) using Attractene Reagent (Qiagen) according to manufacturer’s protocol. After 48 h cells were lysed and luciferase assays were performed using a dual-luciferase reporter assay system (Promega, Witchburg, WI, USA). NFAT-responsive firefly luciferase activity was normalized to the Renilla luciferase activity.

2.9. Calcineurin activity

INS-1E cells were plated in a Petri dish and immediately transfected either with 30 nM nt or TRPV6 siRNA using HiPerfect. Calcineurin activity was assessed after 48 h by Calcineurin Cellular Activity Assay Kit (Enzo Life Sciences, Farmingdale, NY, USA).

2.10. Western blot

Protein isolation and Western blot detection of TRPV6 as well as ERK1/2 phosphorylation were carried out as recently described [29]. Blocking
Fig. 1. TRPV6 mRNA expression and protein production in INS-1E cells and rat pancreatic islets. A, real time PCR detection of TRPV6 mRNA expression in rat duodenum (positive control), rat pancreatic islets and INS-1E cells. B, Western blot detection of TRPV6 in rat pancreatic islets and INS-1E cells. C–E, immunofluorescence detection of TRPV6 in INS-1E cells (C: nuclei stained with DAPI, D: TRPV6 positivity, E: merged). F–I, immunofluorescence detection of TRPV6 in rat pancreatic islets (F: insulin positive cells, G: TRPV6 positive cells, H: nuclei stained with DAPI, I: merged). Note TRPV6 positivity in beta cells. J, TRPV6 mRNA expression in INS-1E cells transfected with nt (white bars) or TRPV6 siRNA (gray bars). K, Western blot detection of TRPV6 protein in INS-1E cells transfected with nt or TRPV6 siRNA and quantification of TRPV6 protein (normalized to beta-actin) in cells transfected with nt (white bars) or TRPV6 siRNA (gray bars). Results are the mean ± SEM, obtained from at least three replicates.
TRPV6 peptide was used at the ratio of 3 μg per 1 ml of primary antibody, to detect the specific and non-specific signals. Signals were visualized using ChemiDoc MP Imaging System (BioRad, Hercules, CA, USA).

2.11. Immunofluorescence detection of TRPV6

INS-1E cells were cultured in 4 chamber tissue culture slides for 24 h. Thereafter, cells were washed with PBS and fixed in 4% paraformaldehyde in PBS for 10 min. Fixed cells were washed three times with ice cold PBS and permeabilized by incubating in PBS containing 0.1% Triton X-100 for 10 min. After three 5 min washes in PBS, unspecific binding of antibodies was blocked by incubating with blocking solution containing 0.2% gelatine and 15 mM glycerine for 1 h at RT. Next, cells were incubated overnight at 4 °C with anti TRPV6 antibody (dilution 1:500 in blocking solution). To confirm the specificity of the primary antibody blocking TRPV6 peptide was used at the ratio of 3 μg per 1 ml of primary antibody. After incubation, cells were washed three times in PBS, each time for 5 min. Thereafter, cells were incubated with the secondary antibody (dilution 1:250 in blocking solution) for 1 h at RT in the darkness. Next, cells were washed with PBS (3 × 5 min.). To stain nuclei, cells were incubated with DAPI (1 μg/ml in PBS) for 1 min. Then, cells were washed in PBS and cover glass was mounted on culture slides using Fluoromount G (SouthernBiotech, Birmingham, AL, USA). Cells were photographed at 400× magnification using a Zeiss Axiosvert 510 Meta Confocal Laser Scanning Microscope Fluorescence (Carl Zeiss, Oberkochen, Germany). Immunofluorescence detection of TRPV6 in pancreatic islets was performed on paraffin-embedded tissue. The slides (6 μm) were baked in laboratory oven at 58 °C for 45 min. Next, the sections were deparaffinized in xylene and rehydrated through a graded series of ethanol (96%, 85%, 80%, 70%, 50%, 30%). Then, slides were placed in a microwave oven to unmasked antigenic sites by pre-treatment for 3 × 5 min in 0.01 M citrate buffer (pH 6.0). The next steps were the same as for INS-1E cells except incubations with primary and secondary antibodies with or without the blocking peptide (slides were co-incubated with anti-TRPV6 diluted 1:200 and anti-insulin antibody diluted 1:500 and then with secondary Cy3 and Alexa Fluor® 488 conjugated antibodies diluted 1:500).

2.12. Real time PCR

RNA was isolated using Tripure Reagent (Roche Diagnostics) according to the manufacturer’s instruction. cDNA was generated using 1 μg of RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). Real-time PCR was prepared using SYBR Green Master Mix with specific primers on QuantStudio 12 K Flex™ Real-Time system (Life Technologies, Grand Island, NY, USA). Gene expression was evaluated by Delta Delta CT (ΔΔCT) method, using GAPDH as a reference gene. Sequences of gene-specific primers are given in Supplementary Table 1.

2.13. Insulin secretion assay

INS-1E cells were seeded in 24-well plates (1 × 10^5 cell per well) and transfected either with 30 nM nt or TRPV6 siRNA. After 48 h cells were preincubated in glucose-free KRHB containing in mM: 136 NaCl, 4.7 KCl, 1 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 2 NaHCO₃, 10 HEPES (pH 7.4) for 1 h. Thereafter, cells were washed in glucose-free KRHB and incubated with the same buffer supplemented either with 2.8 or 16.7 mM glucose for 20 or 60 min. The concentration of released insulin (normalized to intracellular insulin content [30]) was determined using a High Range Rat Insulin ELISA Kit (DRG Instruments GmbH, Germany, Marburg). The detection limit of the assay was 1.5 μg/l.

2.14. Statistical analysis

The Student’s t-test (parametric two-tailed t-test) was used for statistical significance determination. If the normality failed Mann–Whitney test was used. p-values < 0.05 (*), p < 0.01 (**) were considered significant. Data are means ± SEM and show a representative experiments performed in triplicates at least.

3. Results

3.1. TRPV6 gene and protein expression in INS-1E cells and rat pancreatic islets

TRPV6 mRNA expression was detected in INS-1E cells, rat pancreatic islets as well as in rat duodenum (positive control [31]) (Fig. 1A). TRPV6 protein detection in INS-1E cells and pancreatic islets by Western blot technique revealed two signals of approximately 90 and 70 kDa corresponding to glycosylated and non-glycosylated TRPV6 protein [32] (Fig. 1B). Presence of TRPV6 in INS-1E cells was confirmed by immunofluorescence (Fig. 1C-e). Double immunofluorescence in rat pancreatic sections revealed that TRPV6 is expressed in beta cells (Fig. 1F–I). TRPV6 immunoreactivity detected by Western blot and immunofluorescence using a TRPV6 blocking peptide showed that the antibody was specific. As shown in Supplementary Fig. 1a, preincubation of TRPV6 antibody with the blocking peptide, at the ratio of 3 μg per 1 ml of primary antibody, caused a loss of TRPV6 signals, whereas a non-specific signal was still detectable in INS-1E cells. Furthermore, preincubation of pancreatic sections with the immunizing control peptide completely blocked TRPV6 immunoreactivity, as detected by immunofluorescence analysis.

To study the consequences of TRPV6 downregulation on INS-1E cells physiology, we used siRNA. TRPV6 mRNA expression in INS1-1E cells was downregulated by approximately 65% (Fig. 1J), whereas protein production was reduced by approximately 70%, as compared to nt siRNA-transfected cells (Fig. 1K).
3.2. Downregulation of TRPV6 protein production is accompanied by impaired calcium homeostasis

To investigate the contribution of TRPV6 to the intracellular calcium influx, we recorded the responses of INS-1E cells to rapid changes of [Ca\(^{2+}\)]\(_i\) from a Ca\(^{2+}\)-free to a 1.5 mM Ca\(^{2+}\) containing extracellular solution. A similar protocol was used by Lehen’kyi et al. for TRPV6 detection. Notably, there was a significant lower basal level of cytosolic Ca\(^{2+}\) (control) in cells with TRPV6 downregulation (e.g. 112 ± 7 nM vs. 171 ± 20 nM at 150 s) (Fig. 2B). In a Ca\(^{2+}\)-free solution, [Ca\(^{2+}\)]\(_i\) decreased from 170 ± 20 nM (150 s) to 33 ± 11 nM (n = 10; p < 0.005; t = 350 s) in nt siRNA-transfected INS-1E cells (Fig. 2B–C). In the presence of extracellular Ca\(^{2+}\), [Ca\(^{2+}\)]\(_i\) increased above the baseline (285 ± 51 nM; n = 10; t = 420 s). In cells with downregulated TRPV6, however, [Ca\(^{2+}\)]\(_i\) decreased from 112 ± 7 nM (150 s) to 10 ± 2 nM, only (n = 10; p < 0.001; t = 420 s). After replacement with the Ca\(^{2+}\) solution, [Ca\(^{2+}\)]\(_i\) was still below the basal level of cytosolic Ca\(^{2+}\) (-20 ± 10 nM; n = 10). Thus, changes of [Ca\(^{2+}\)]\(_i\) in Ca\(^{2+}\)-free and Ca\(^{2+}\)-solution were significantly reduced in TRPV6 siRNA-transfected cells as compared to wild type INS-1E cells (n = 10; p < 0.01 vs nt-transfected cells; Fig. 2C). In summary, activation of the Ca\(^{2+}\) sensing TRPV6 channel stimulates Ca\(^{2+}\) influx in INS-1E cells.

3.3. TRPV6 modulates calcineurin/NFAT activity

It was shown before that TRPV6 modulates NFAT activity in LN-CAP cells [34]. Since NFAT and the calcium-dependent activator calcineurin can stimulate beta cells growth and increase insulin production [35], we therefore measured the activities of calcineurin and NFAT in TRPV6 siRNA-transfected cells. Suppression of TRPV6 mRNA expression resulted in strongly reduced calcineurin and NFAT activities in INS-1E cells (Fig. 3A and B, p < 0.05).

3.4. TRPV6 affects the expression of pdx-1, Ccnd1–2, Ccdk4, Irs2 and regulates ERK1/2 phosphorylation

Calcineurin/NFAT pathway was implicated in controlling beta cell growth-controlling genes such as pdx-1, cyclin D1, cyclin D2 (Ccnd1–2), cyclin-dependent kinase 4 (Ccdk4) and insulin receptor substrate 2 (Irs2) [35–37]. Since decreased TRPV6 protein production led to reduced activity of calcineurin/NFAT, we next evaluated the consequences of TRPV6 downregulation on the mRNA expression of the above listed genes. Reduction of TRPV6 protein production resulted in
reduced pdx1 (p < 0.05), Ccnd2 (p < 0.05), Cdk4 (p < 0.01) and Irs2 (p < 0.05), whereas Ccnd1 expression was not affected (Fig. 4A–E).

Calcineurin is able to modulate ERK1/2 phosphorylation [38]. Since ERK1/2 modulates cell proliferation and insulin gene expression [39,40] we next evaluated the consequences of TRPV6 reduction on ERK1/2 phosphorylation. We found that the basal level of ERK1/2 phosphorylation was reduced in TRPV6 siRNA transfected cells as compared to TRPV6 wild type INS-1E cells (p < 0.05) (Fig. 4F).

3.5. TRPV6 controls INS-1E cell proliferation

Since TRPV6 modulates the expression of beta cell growth-regulating genes, we asked next whether TRPV6 affects INS-1E cells proliferation. Indeed, two and three days after TRPV6 siRNA transfection, INS-1E cell proliferation was reduced as compared to nt transfected cells (Fig. 5A; p < 0.01).

Moreover, TRPV6 downregulation led to reduced cell viability (Fig. 5B, p < 0.01). The promitogenic activity of TRPV6 in INS-1E cells was confirmed by cell cycle analysis. The number of cells in S-phase decreased in cells with downregulated TRPV6 protein levels (p < 0.05), whereas the number of death cells increased (Fig. 5C, p < 0.05). Overall, these data reveal the importance of TRPV6 in controlling INS-1E cell proliferation.

3.6. TRPV6 affects insulin expression but not insulin secretion

As shown in our current study TRPV6 regulates calcineurin/NFAT activities (Fig. 3A–B). Calcineurin/NFAT signaling is involved in modulation of insulin mRNA expression [36]. We therefore examined whether TRPV6 modulates insulin mRNA expression and secretion in INS-1E cells. Downregulation of TRPV6 protein production was associated with a decrease of ins mRNA expression (p < 0.05) mRNA by approximately 17% (Fig. 6A). However, no effects on insulin secretion, neither at low (2.8 mM) nor high (16.7 mM) glucose concentration (p > 0.05, Fig. 6B–C) were detected. These data suggest that TRPV6 plays a role...
in stimulating insulin mRNA expression, without affecting insulin secretion.

4. Discussion

In the present study, we identify TRPV6 expression and protein production in INS-1E cells and rat pancreatic beta cells. TRPV6 mRNA has been already detected in human beta cells [15]. However, the function of this channel in beta cells is still unknown. In the current study, we show that the Ca^{2+} sensing TRPV6 channel regulates intracellular calcium as well as INS-1E cell proliferation and insulin mRNA expression.

Previous studies in prostate cancer demonstrated that TRPV6 triggers cell growth, possibly via transcriptional factor NFAT [34]. The link between TRPV6 and NFAT activity was also reported in the human colon carcinoma Caco-2 cells [41]. It is known that NFAT interacts with calcium-signaling. Calcium activates a protein phosphatase calcineurin. Activated calcineurin can dephosphorylate NFAT which then translocates into the nucleus to initiate expression of various target genes [42]. NFATs are also expressed in pancreatic beta cells [35]. Our study demonstrates a possible link between TRPV6, Ca^{2+} and calcineurin/NFAT pathway in our experimental beta cell model INS-1E cells.

Notably, there is evidence from previous studies showing that both, NFAT as well as calcineurin can affect various functions of pancreatic beta cells such as cell proliferation and viability [35,37]. Mechanisms encompass modulation of cell cycle as well as survival-controlling genes such as pdx1, cyclins and cyclin-dependent kinases [35,36]. Additionally, calcineurin can modulate beta cell survival via Irs2 [37] and ERK1/2-pathway [38]. In agreement with these reports, we found that TRPV6 downregulation led to a reduction of calcineurin/NFAT activity which was accompanied by the reduction of Pdx1, Ccdn2, Cdk4 and Ins2 mRNA expression. In contrast, Ccdn1 expression was not affected by TRPV6 downregulation. Furthermore, suppression of calcineurin/NFAT activity following TRPV6 downregulation was associated with reduced ERK1/2 phosphorylation. Since all above listed genes as well as ERK1/2 signaling are crucially important for the growth of pancreatic beta cells [43,44], we assessed the role of TRPV6 in controlling INS-1E cells proliferation. As a result, we found that TRPV6 stimulates cell proliferation. This observation is not surprising in the light of data showing the importance of ions and calcium channels in modulating pancreatic beta cell growth [2]. Importantly, the loss of TRPV6 protein production caused a maximal 30% decrease in INS-1E cell proliferation despite the presence of 10% serum. Thus, it is rationale to speculate that the role of TRPV6 in modulating cell growth may be physiologically relevant. Noteworthy, highly efficient stimuli of INS-1 cell proliferation such as GLP-1 or hepatocyte growth factor can increase beta cell replication by approximately 80–100% [45,46]. However, these experiments were conducted in a serum-free condition, which is of questionable physiological significance.

The relevance of TRPV6 in controlling cell proliferation is in agreement with the notion that TRPV6 augments HEK-293 cell growth [47] and is highly expressed in rapidly proliferating cells e.g. prostate [13] or breast cancers [48]. In LNCaP prostate cancer cells, TRPV6 stimulates cell proliferation and survival [34], which was inferred from experiments using TRPV6 siRNA. Notably, the extent of suppression of LNCaP cell proliferation by TRPV6 siRNA (approximately 25% three days after siRNA transfection) [23,34] was highly comparable to that detected in our experiments using INS-1E cells (30% percent vs. nt siRNA-transfected cells after three days).

Several studies reported that NFAT/calcineurin and ERK1/2 pathways are required for insulin mRNA expression [40,49]. Concordantly, we found that TRPV6 siRNA-transfected cells had lower Ins mRNA expression. Overall, these results indicate that TRPV6 stimulates INS-1E cell proliferation as well as insulin mRNA expression.

In contrast to the trophic activity of TRPV6 in INS-1E cells, we found that the downregulation of TRPV6 failed to affect insulin secretion. This finding may suggest that other Ca^{2+} permeable channels (e.g. L-type voltage operated channels) are sufficient for maintaining normal insulin exocytosis in response to glucose. In this context it is worth to notice that in our earlier work we did not observe any changes in insulin secretion in INS-1E cell with downregulated TRPV4 protein production [6]. Noteworthy, likewise TRPV6, TRPV4 is also active in the optimal temperature of living organism [50]. However, it is possible that TRPV6 deficiency in INS-1E cells was compensated by increased expression of other calcium-permeable channels which could result in normal insulin secretion. Overall, these results suggested that TRPV6 is rather not required for insulin secretion in INS-1E cells. In the literature, it is known that other TRPs such as TRPV2 and TRPM4 are relevant for the regulation of insulin secretion [7,19].

An important limitation in our studies is the use of TRPV6 siRNA technique as the only molecular tool for TRPV6 detection. Reason is the lack of specific pharmacological antagonists for TRPV6. However, this limitation is also a hallmark of earlier studies [34,51]. Nevertheless, it is worth to notice that TRPV6 can be activated by vitamin D as well as estrogen [23,52,53]. These TRPV6 modulators were shown to improve human and rodent pancreatic beta cells endocrine and metabolic function.
functions [54,55]. Since vitamin D and estrogen can activate numerous of TRPV6-independent pathways [56,57], the results obtained with these modulators of pancreatic beta cells need to be interpreted cautiously. Furthermore, our study was performed in experimental INS-1E cell model and not in isolated primary single pancreatic beta cells. Despite detection of TRPV6 in insulin-immunoreactive cells in rat pancreatic islets, we cannot exclude that the expression levels of TRPV6 may differ between INS-1E cells, as compared to primary beta cells. Also the function of TRPV6 may differ in primary beta cells as compared to INS-1E cell line. However, INS-1E is a well-established cell line to study the intracellular beta cell signaling, which was the major aspect of our in vitro study. Further studies of TRPV6 function in primary beta cells or in animals with genetically engineered TRPV6 will be therefore required to verify the relevance of our original findings reported in the experimental INS-1E cell line.

In conclusion, TRPV6 is expressed in rat INS-1E cell line as well as in beta cells in pancreatic islets. TRPV6 controls calcium influx. Intracellular calcium modulates calcineurin/NFAT activity which likely modulates genes expression as well as cell proliferation (Fig. 7). TRPV6 fails to affect insulin secretion. These findings are compatible with the notion that TRPV6 has a relevance for the neoplastic growth and transformation of neuroendocrine cells.

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Transparency document

The Transparency document associated with this article can be found, in the online version.

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