

Cell Swelling-induced Insulin Secretion from INS-1E Cells is Inhibited by Extracellular Ca^{2+} and is Tetanus Toxin Resistant

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Key Words

Insulin secretion • Cell swelling • Ca^{2+} • Tetanus toxin
• Calcium channels • SNARE

Abstract

Cell swelling-induced insulin secretion represents an alternative pathway of stimulation of insulin secretion. INS-1E rat tumor beta cells do not release insulin in response to cell swelling in presence of Ca^{2+} despite a good response to glucose challenge and appropriate increase in cell volume. Surprisingly, perfusion with Ca^{2+} -depleted medium showed distinct secretory response of INS-1E cells to hypotonicity. Objective of this study was further characterization of the role of Ca^{2+} in secretory process in INS-1 and INS-1E cell lines. Ca^{2+} depleted hypotonic medium with 10 μM BAPTA/AM (intracellular chelator) induced insulin secretion from both types of cells. We demonstrated expression of L-type Ca^{2+} channel $\text{Ca}_v1.2$ and non-L-type Ca^{2+} channels $\text{Ca}_v2.1$ (P/Q-type), $\text{Ca}_v2.2$ (N-type), and $\text{Ca}_v3.1$ (T-type) in both cell lines. Inhibition of L type channel with nifedipine and/or P/Q type with ω -agatoxin IVA enabled distinct response to hypotonic medium also in INS-1E cells. Tetanus toxin (TeTx) in medium containing Ca^{2+} and

a group of calcium channel blockers inhibited hypotonicity-induced insulin secretion from INS-1 cells but not from INS-1E cells. Conclusion: Hypotonicity-induced insulin secretion from INS-1E cells is inhibited by extracellular Ca^{2+} , does not require intracellular Ca^{2+} and is TeTx resistant.

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Introduction

Exploration of cell swelling-induced insulin secretion brought interesting information on alternative pathway of stimulation of insulin release. Comparison with signal transduction mediating glucose-induced insulin secretion [1] indicates the presence of novel signaling pathway; hyposmotic stimulation of insulin secretion from pancreatic islets is independent of both the extracellular [1-3] and intracellular Ca^{2+} [1], various insulin release inhibitors and ion-flux modifiers [2, 3], cannot be inhibited by nor-adrenaline [1], does not involve protein kinase C [1], G proteins or phospholipase A2 activation and is N-ethylmaleimide insensitive [4]. Interestingly, glucose itself

induces swelling of pancreatic β -cells [5] and tumor-derived INS-1E cells [6]; therefore a possibility that swelling is integrated into glucose signaling should be also considered.

A failure to identify transduction pathway and its resistance to physiological inhibitors suggests that signaling of cell swelling-induced exocytosis bypasses conventional transduction steps and could be effective at the distal end of the cascade. It has been shown that secretory vesicle swelling is critical for exocytosis [7-10]. Stretching of vesicular and plasma membranes in the region of contact results in exposing areas of hydrophobic acyl chains is leading to subsequent merging and fusion. Fusion rates are orders of magnitude higher if an osmotic gradient is applied [7]. The externalization of hormones or transmitters upon exocytosis of vesicles is augmented by secretion of water from the vesicle membrane through the widened fusion pore [10]. Considering these data, we hypothesized that cell swelling-triggered exocytosis is a result of a direct biophysical effect of the osmotic gradient on secretory vesicles [11].

Recently we have compared pancreatic islets, INS-1 and INS-1E cell lines and discovered that INS-1E rat tumor beta cells [12] are unable to release insulin in response to cell swelling despite a good response to glucose challenge. Response to hyposmotic stimulation in presence of Ca^{2+} was absent despite appropriate increase in cell volume. Surprisingly, perfusion with Ca^{2+} -depleted medium showed distinct secretory response of INS-1E cells to hypotonicity [12].

It is the aim of present work to gain more information on the mechanism of the inhibitory role of Ca^{2+} in cell swelling-induced exocytosis in the INS-1E cell line.

Materials and Methods

Male Wistar rats (Charles River, Sulzfeld, Germany) weighing 300-350 g were kept under controlled temperature (22-24 °C) and constant 12 h light/dark cycle, fed with Purina Chow and tap water *ad libitum*. The animal-use protocols conformed to the guiding principles of the European Convention on Animal Protection and were approved by the Ethical committee of the Institute of Experimental Endocrinology SAS and National Veterinary Board.

Chemicals

Collagenase type XI, soybean Trypsin inhibitor type I-S, nifedipine, mibefradil, (Sigma, Germany), BAPTA/AM ((1,2-Bis(2-aminophenoxy)ethane- $\text{N},\text{N},\text{N}',\text{N}'$ -tetraacetic acid tetrakis (acetoxymethyl ester)) (Molecular Probes, Eugene, OR, USA), dimethyl sulfoxide (DMSO) (Sigma, Germany) was used for

dilution and BAPTA/AM, ω -agatoxin IVA (Alexis, Switzerland), SNX-482 and Tetanus toxin (Sigma, Germany).

Isolation of islets of Langerhans

Islets were isolated from adult male Wistar rats according to the method by Lacy and Kostianovsky [13]. Briefly, under pentobarbital anesthesia (3.7 mg/100 g body mass) the common bile duct was cannulated proximally near the hilus of the liver, a tie was placed adjacent to the duodenum, and pancreas was distended by injection of approximately 10 ml of Hank's Balanced Salt Solution (HBSS) with 1% BSA. The pancreas was then dissected out, minced, and digested with 1 mg/ml collagenase with 0.008 g soybean Trypsin inhibitor type I-S in 100 ml HBSS for 10 min at 37°C. The tissue was then separated from the remaining acinar tissue under a dissecting microscope and used immediately for the experiment.

Incubation media

Krebs-Ringer Hepes buffer (KRH - basal) (292 mOsm/kg H_2O) of following composition. 118 mM NaCl, 4 mM KCl, 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 , 5 mM NaHCO_3 , 25 mM HEPES, 3 mM D-glucose, 2.5 mM CaCl_2 , 0.1% BSA, pH 7.4.

Glucose medium. This medium was prepared by increasing D-glucose concentration to 20 mM. To maintain physiological osmolarity, the NaCl concentration was reduced to 109.5 mM. Other components were the same as in the basal medium.

Hyposmotic medium (204 mOsm/kg H_2O). 30% hyposmotic medium was prepared by appropriate dilution of basal medium with deionized water. In Ca^{2+} depleted medium CaCl_2 was omitted and 1mM EGTA was added.

Rat insulinoma derived β cells

Rat insulin-secreting cell lines INS-1 and INS-1E sensitive to glucose stimulation [14], were kind gift from Dr. Claes B. Wollheim (University Medical Center, Geneva, Switzerland). Cells were cultivated at 37°C in humidified atmosphere containing 5% CO_2 /95% O_2 air in complete medium composed of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 2 mM glutamine, 25 mM HEPES, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. The maintenance culture was passaged once a week by gentle trypsinization. Cells between passages 168 - 182 (INS-1) and 94 - 107 (INS-1E) were used for this study.

Static incubation of attached cells (INS-1, INS-1E)

The experiments were performed in 12-well plates in 1 ml of medium. Cell density was 3×10^5 per well. Before the experiments, cells were maintained for 2 h in glucose-free Krebs-Ringer bicarbonate HEPES buffer (KRBH) of the following composition: 135 mM NaCl, 3.6 mM KCl, 5 mM NaHCO_3 , 0.5 mM NaH_2PO_4 , 0.5 mM MgCl_2 , 1.5 mM CaCl_2 , and 10 mM HEPES, pH 7.4. BSA (0.1%) was added as an insulin carrier. The cells were then washed twice in PBS and preincubated for 30 min in glucose-free medium. Next, cells were washed once with glucose-free KRBH and then incubated for 30 min successively in basal medium and stimulating media as indicated. Insulin secretion was measured by radioimmunoassay (RIA).

Calcium channel	Target mRNA	Accession nr.	PCR primer sequence 5' → 3'
Ca _v 1.2	Cacnalc	NM_012517	F: CAGGAGGTGATGGAGAAGCCA R: CTGCAGGCGGAACCTGTTGTT
Ca _v 2.1	Cacnala	NM_012918	F: CACCAACCCTGGTCCCGCCT R: CATGGGCTTTGGGCGCTCCT
Ca _v 2.2	Cacnalb	NM_147141	F: TGAAGACACACATGGACCG R: AGTCCTGTGCATGCCGGTG
Ca _v 3.1	Cacnalg	NM_031601	F: CACCAAGTCTGAGTCAGAGC R: TGATTCATCTCATGATGGGC

Table 1. Sequences of primer pairs used for the amplification of calcium channels. F: forward primer; R: reverse primer [16].

Incubation media

Basal medium (302 mOsm/kg H₂O). Krebs-Ringer Hepes buffer (KRH) (133,75 mM NaCl, 3.6 mM KCl, 5 mM NaHCO₃, 0.5 mM NaH₂PO₄, 0.5 mM MgCl₂, 1.5 mM CaCl₂, and 10 mM HEPES, 2.5 mM glucose, BSA (0.1%), pH 7.4 [14].

Stimulating media

Glucose medium. This medium was prepared by increasing D-glucose concentration to 15 mM. To maintain physiological osmolarity, the NaCl concentration was reduced to 127.5 mM. Other components were the same as in the basal medium.

Hypotonic medium (211 mOsm/kg H₂O). 30% hypotonic medium was prepared by appropriate dilution of basal medium with deionized water.

In Ca²⁺ depleted medium CaCl₂ was omitted and 1mM EGTA was added.

Specific inhibitors (10 μM nifedipine, 100 nM ω-agatoxin IVA, 100 nM SNX-482, 10 μM mibefradil) were added to basal and stimulating medium in some experiments as indicated.

Perfusion of the cells

The flow rate was set at 0.3 ml/min, and fractions were collected every minute. Insulin secretion was measured by RIA using rat insulin as a standard.

BAPTA/AM

INS-1 or INS-1E cells (approximately 5x10⁷ per chamber) were perfused for 2 h in glucose-free culture medium at 37 °C. After this stabilization period cells were exposed to 20 min perfusion with basal medium followed by 20 min perfusion with hypotonic medium and 20 min with basal medium. All media successively applied were either without (control) or with BAPTA/AM.

Tetanus toxin

Tetanus toxin (20 nM) was present during 30 min preincubation in depolarization medium (81,35 mM NaCl, 56 mM KCl, 5 mM NaHCO₃, 0.5 mM NaH₂PO₄, 0.5 mM MgCl₂, 1.5 mM CaCl₂, and 10 mM HEPES, 2.5 mM glucose, BSA 0.1%, pH 7.4) to induce depolarization and exocytosis followed by endocytosis thus ensure toxin transport into the cells. Control cells were preincubated in depolarization medium without toxin. Preincubation was followed by 60 min perfusion with glucose-

free Krebs-Ringer bicarbonate HEPES buffer. After stabilization period cells were exposed to 20 min perfusion with basal medium followed by 20 min perfusion with hypotonic medium and 20 min with basal medium. All media successively applied were either without any inhibitors (control), with tetanus toxin (TeTx), group of calcium channel inhibitors (control + INH), or tetanus toxin and group of calcium channel inhibitors (TeTx + INH).

Radioimmunoassay for insulin

Insulin released into medium was determined directly by specific radioimmunoassay. All samples from each experiment were analyzed in the same assay to avoid interassay variation. Rat insulin standards were prepared in each type of medium we utilized and thus the correction for potential effect of different media on the antibody binding was included. ¹²⁵I-mono-iodoinsulin was prepared with the aid of lactoperoxidase [15]. Rabbit anti-insulin antibody was provided by Dr. Štolba (Institute of Endocrinology, Prague). Synthetic insulin was obtained from Novo Nordisk (USA). All assays were performed in total volume of 400 μl 0.01 M PBS (pH 7.4). After overnight incubation of samples with the antibody at 4°C, bound and free peptides were separated by cold 200 μl dextran coated charcoal (500 mg Norit + 50 mg dextran in 100 ml H₂O). After 15 min centrifugation at 3000xg, the radioactivity of pellet was measured by RIA multidetector counter (JNG-402, URVJT, Prague).

RT-PCR

RT-PCR analysis of cDNA obtained from INS-1 and INS-1E cells with specific primers for L-type Ca²⁺ channels Ca_v1.2 (315 bp) and non-L-type Ca²⁺ channels Ca_v2.1 (334 bp, P/Q-type), Ca_v2.2 (396 bp, N-type), Ca_v3.1 (271 bp, T-type). Total RNA from INS-1 and INS-1E cells was extracted using TRI-reagent (Molecular research center). Total RNA from each single well was reversely transcribed into cDNA using MULV Reverse Transcriptase and oligo (dT) primer (Fermentas). Replicated samples (first strand cDNA equivalent of 10 ng of total RNA) from each single well were amplified individually with a gene-specific primer pair (Table 1 [16]). Amplification was performed in a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA). An initial denaturation (2 min at 94°C) was followed by a number of PCR cycles (39), annealing (55°C for Ca_v 3.1 or 61°C for Ca_v1.2, Ca_v2.1, Ca_v2.2 for 1 min) and extension (72°C for 2 min), and a 10-min extension at 72°C.

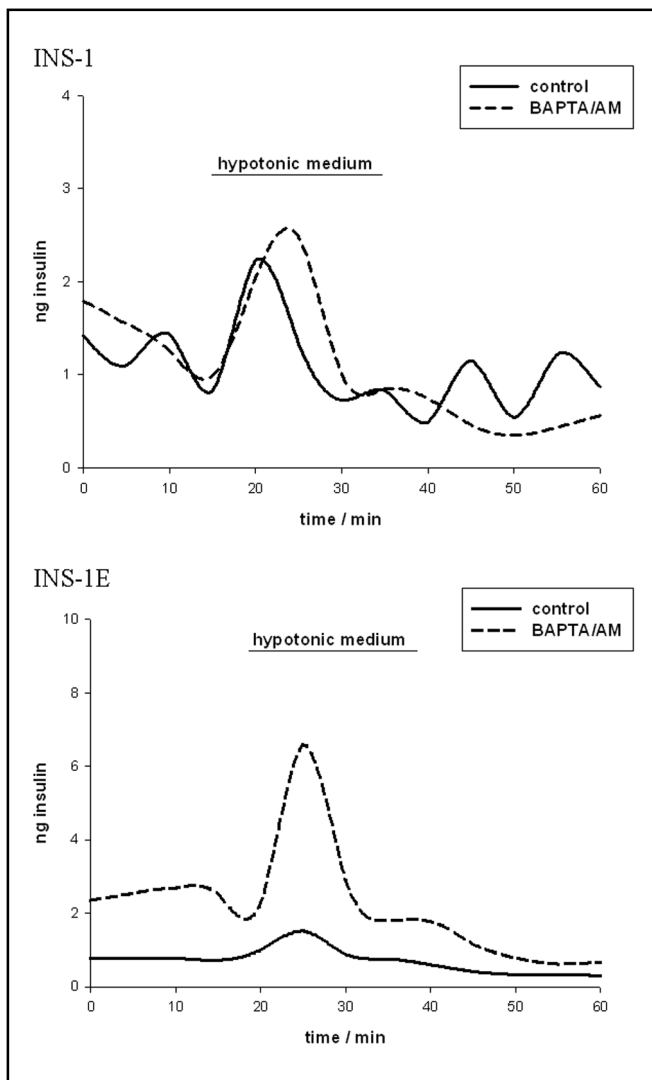


Fig. 1. Secretory response of INS-1 and INS-1E cells to perfusion with hypotonic medium in Ca^{2+} containing (control) or Ca^{2+} depleted medium with intracellular chelator (BAPTA/AM). Typical results of repeated experiments are shown. Cells were perfused at flow rate 0.3 ml/min alternately for 20 min with basal medium, stimulating 30% hypotonic medium and basal medium. Ca^{2+} containing hypotonic medium induced insulin secretion only from INS-1 cells. Hypotonic Ca^{2+} depleted medium with 10 μM BAPTA/AM induced insulin secretion from both types of cells.

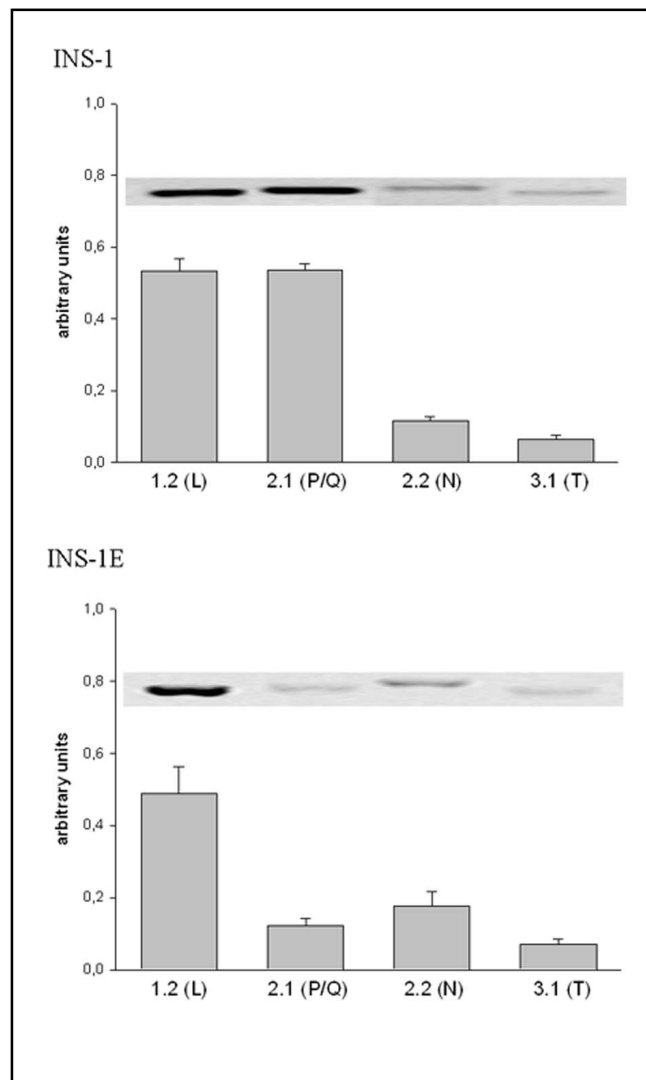


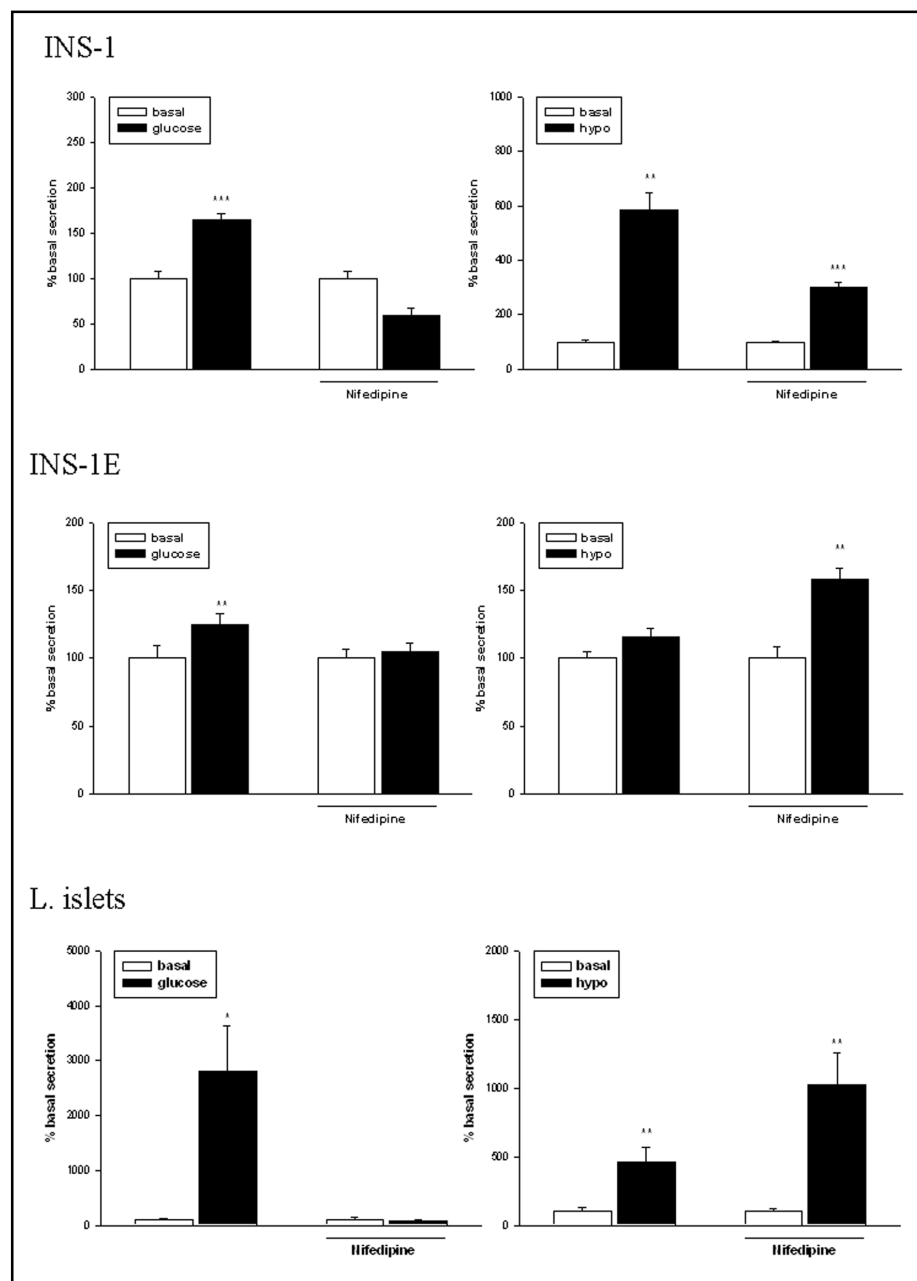
Fig. 2. Expression of various calcium channels in INS-1 and INS-1E cells after 30 min incubation in basal medium. L-type (1.2), and non L-types P/Q-type (2.1), N-type (2.2) and T-type (3.1) were tested. The density of calcium channels bands was normalized relative to S18 bands amplified from the same cDNA. Data in columns are given as means \pm S.E.M (n=3); representative bands are shown.

RT-PCR products were electrophoresed on 1.5% agarose gels, which were stained with ethidium bromide and photographed. Photographs were scanned by using an image analysis system (Kodak Digital Science 1D). The density of calcium channels bands was normalized relative to S18 bands amplified from the same cDNA.

Data analysis

Results are shown as means \pm SE. Data from the same samples before and after stimulation were compared by paired Student's t-test, one or two tailed when appropriate. Data from different experiments were evaluated by unpaired t-test. Differences were considered significant at level $p < 0.05$.

Fig. 3. Effect of Nifedipine, inhibitor of L-type calcium channels on glucose- and swelling-induced insulin secretion from isolated islets of Langerhans (L. islets) and INS-1, INS-1E cell lines. After preincubation, islets (5 per tube) or cells were incubated for two subsequent 30 min periods in control (basal) and stimulating medium containing either 20 mM glucose (glucose) or 30% hypotonic medium (hypo). Insulin secretion under basal conditions was considered to be 100 %. Values are shown as mean \pm SE (n=10); *P<0.05; **P<0.01; ***P<0.001 compared with preceding basal secretion. Nifedipine inhibited stimulating effect of glucose in all three systems, significant secretory response to hypotonicity in presence of nifedipine appeared in INS-1E cells.



Results

Role of Intracellular calcium

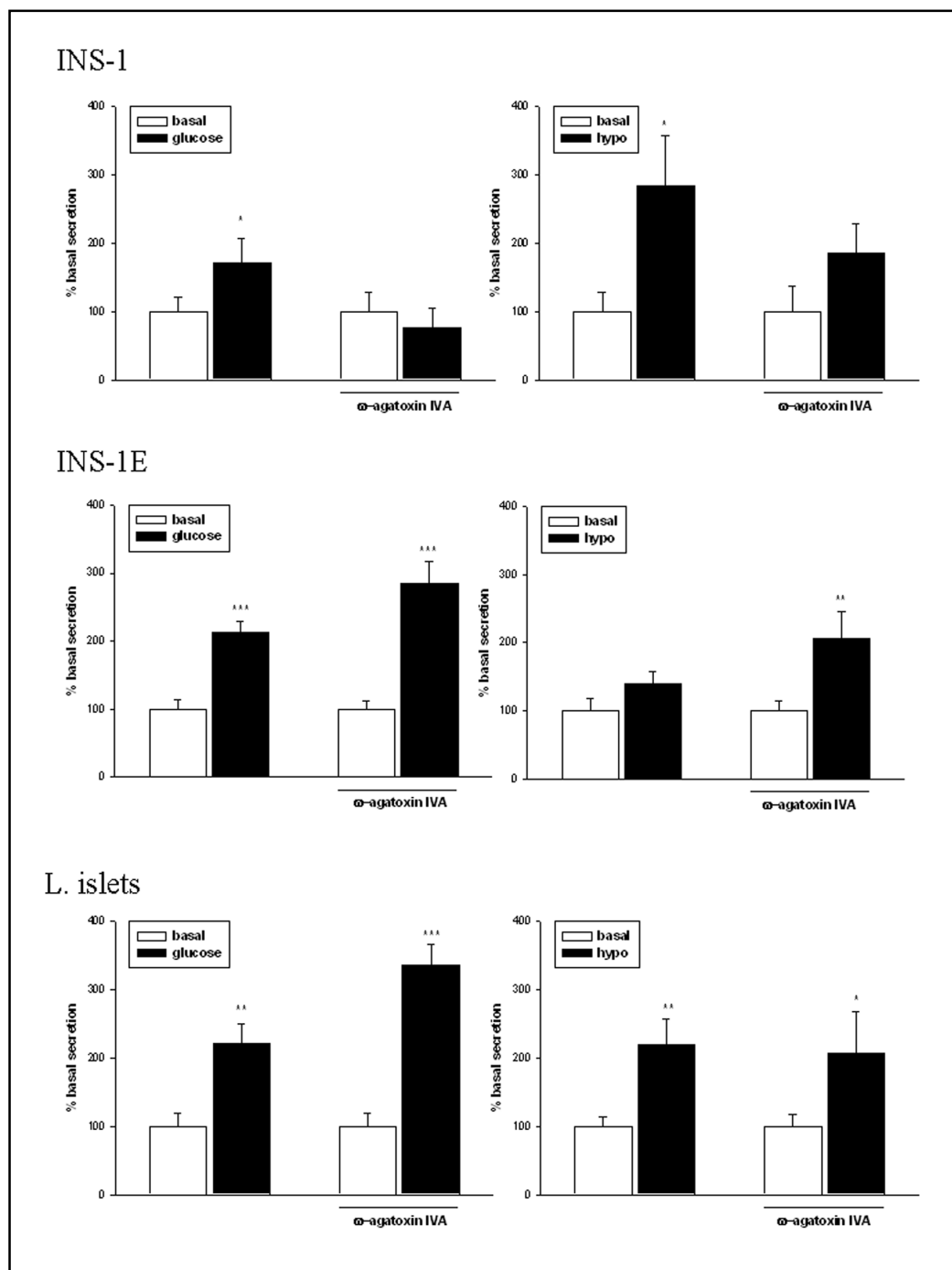
Hypotonic (30%) medium containing Ca^{2+} was a powerful stimulator of insulin secretion from INS-1 cells but not from INS-1E cells (Fig. 1). To specify a role of the intracellular Ca^{2+} in the hypotonicity-induced insulin secretion 10 μM BAPTA/AM, a membrane permeable Ca^{2+} chelator, was added to the calcium free and 1 mM EGTA containing medium. Clear stimulating effect of hypotonic solution on insulin release from INS-1E cells appeared (Fig. 1); the stimulating effect of hypotonicity in INS-1 cells was not affected (Fig. 1).

Calcium channels

RT-PCR analysis of cDNA obtained from INS-1 and INS-1E cells. Insulinoma cell lines INS-1 and INS-1E expressed (Fig. 2) tested calcium channels; L-type $\text{Ca}_v1.2$ and non-L-type Ca^{2+} channels $\text{Ca}_v2.1$ (P/Q-type), $\text{Ca}_v2.2$ (N-type), $\text{Ca}_v3.1$ (T-type). While expression of L and P/Q channels was highest and similar in INS-1 cells, expression of P/Q was much lower in INS-1E cells.

Role of various calcium channels in response to glucose and hypotonicity. To determine role of different calcium channel(s) in various reaction, we tested effect of specific inhibitors of high voltage activated

Fig. 4. Effect of 100 nM ω -agatoxin IVA, an inhibitor of P/Q type calcium channels on glucose- and swelling-induced insulin secretion from isolated islets of Langerhans (L. islets) and INS-1, INS-1E cell lines. Scheme of experiment and group designation as in Fig. 3. Insulin secretion under basal conditions was considered to be 100 %. Values are shown as mean \pm SE (n=10); *P< 0,05; **P< 0.01; ***P< 0.001 compared with preceding basal secretion. Agatoxin inhibited stimulating effect of glucose in INS-1 but not in INS-1E or islets. Distinct response to hypotonicity appeared in INS-1E cells.



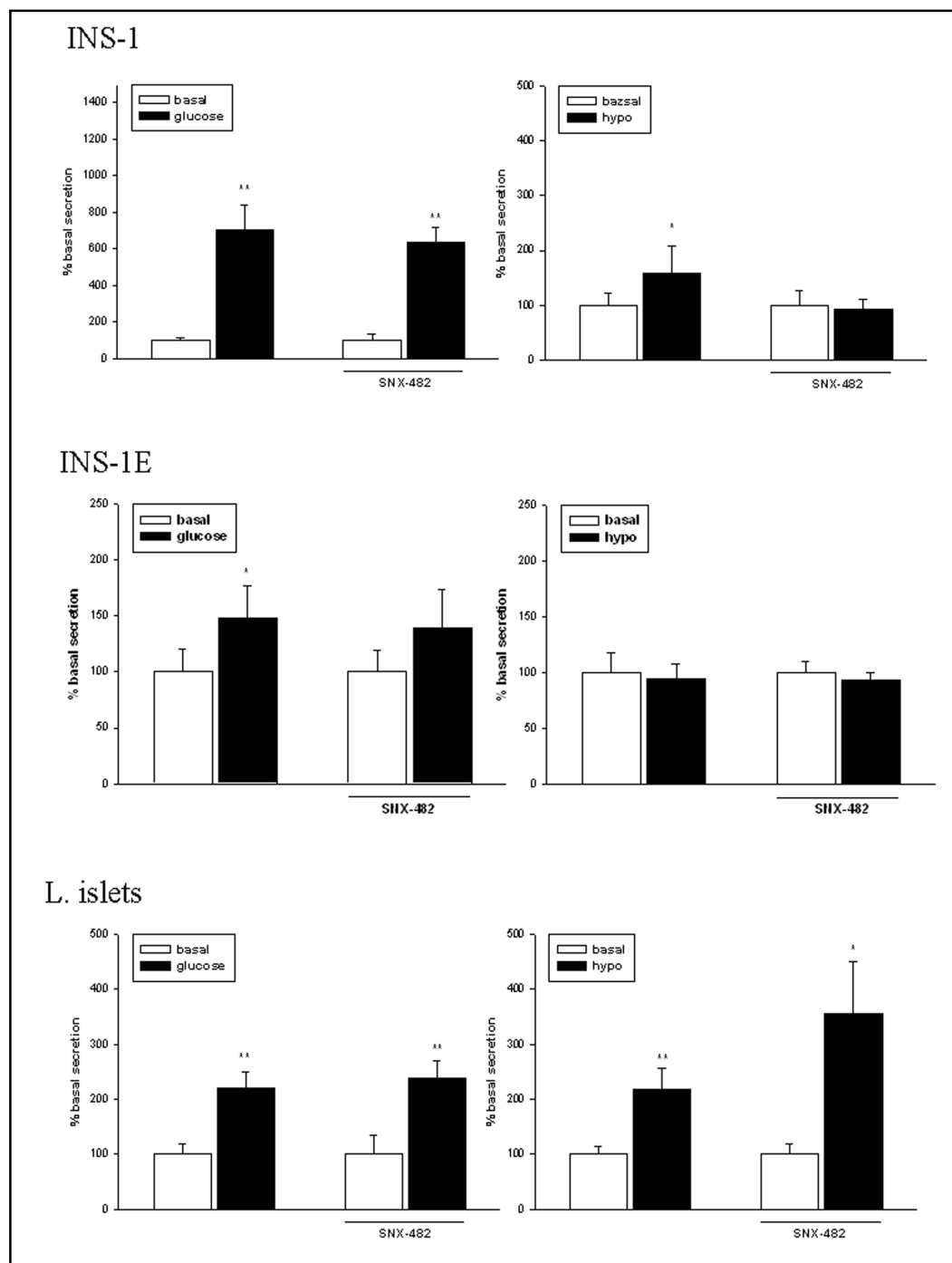
(HVA) and low voltage activated (LVA) channels on the response of both insulinoma cell lines and fresh isolated rat pancreatic islets to glucose and hypotonic stimulation.

L-type. Nifedipine, inhibitor of L-type calcium channels, inhibited (Fig. 3) glucose-induced insulin secretion from all three systems; INS-1 and INS-1E cells and pancreatic islets. Swelling-induced insulin secretion from INS-1E cells emerged in presence of nifedipine;

the positive response of INS-1 cells and islets was not affected.

P/Q type. Inhibitor of P/Q type calcium channels ω -agatoxin IVA prevented glucose-induced insulin secretion from INS-1 cell line, response of INS-1E cells and islets to glucose was not affected (Fig. 4). Secretory response of INS-1 cells to hypotonic solution was inhibited, while 2-fold increase from INS-1E cells appeared.

Fig. 5. Effect of inhibition of R-type calcium channels by 100 nM SNX-482 on glucose- and swelling-induced insulin secretion from isolated islets of Langerhans (L. islets) and INS-1, INS-1E cell lines. Scheme of experiment and group designation as in Fig. 3. Insulin secretion under basal conditions was considered to be 100 %. Values are shown as mean \pm SE (n=10); *P< 0.05; **P< 0.01 compared with preceding basal secretion. SNX-482 inhibited stimulating effect of glucose in INS-1E. Response to hypotonicity in INS-1 cells was inhibited, that in INS-1E cells remained absent.



R-type. SNX-482, blocker of R-type calcium channels (Fig. 5) inhibited glucose-induced insulin secretion only from INS-1E cells. Swelling-induced insulin secretion from INS-1 cells was inhibited while that from INS-1E cells was not affected. Stimulatory effect in islets persisted.

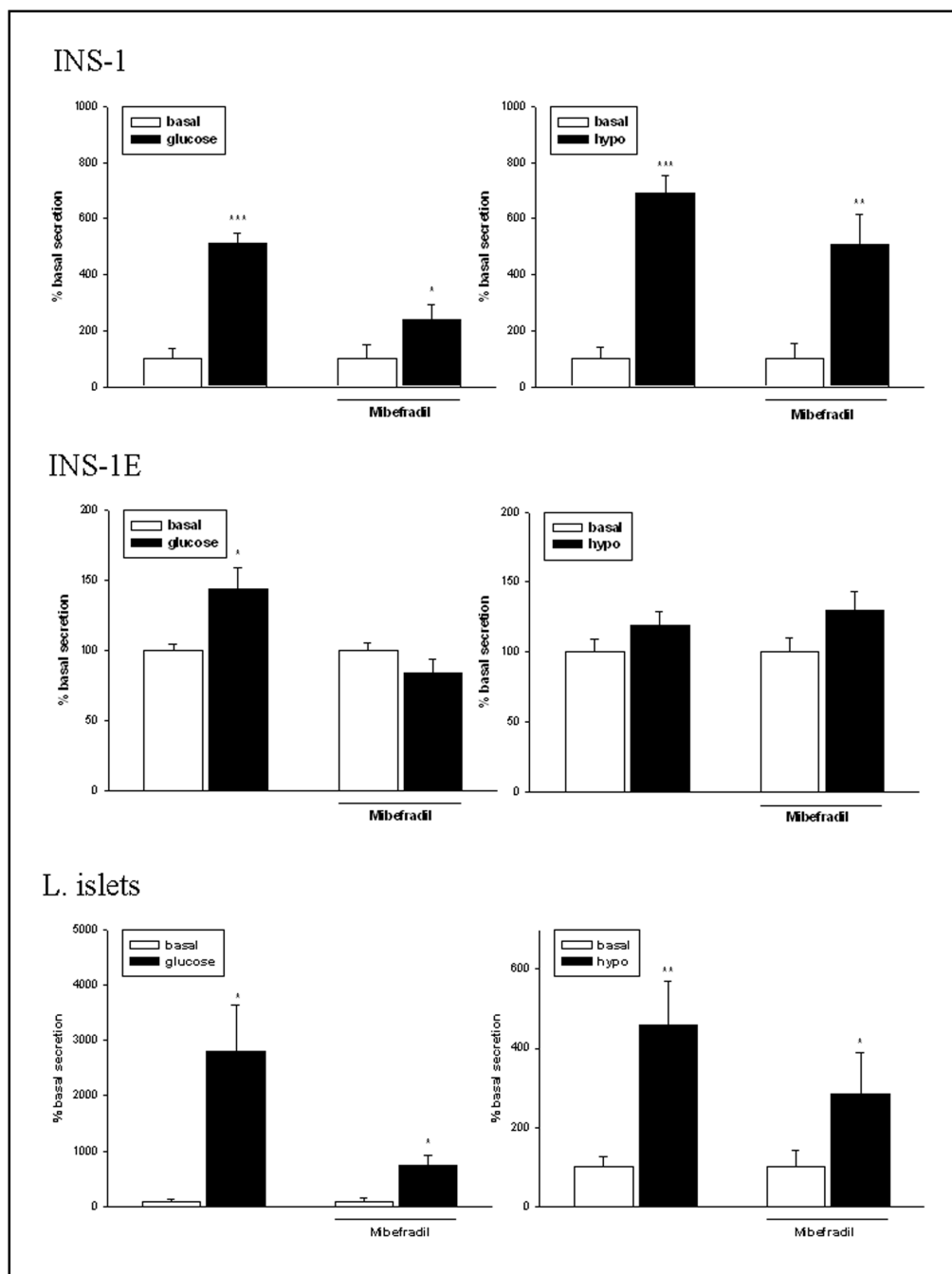
T-type. Low voltage activated channels T-type was inhibited by mibefradil (Fig. 6). Presence of inhibitor in

the medium prevented glucose-induced insulin secretion from INS-1E cells. Swelling-induced secretion was not affected in any of the tested systems.

SNARE proteins

Role of SNARE proteins in stimulated insulin secretion was tested by TeTx inhibition (Fig. 7). This zinc dependent metalloproteinase is inactive

Fig. 6. Effect of inhibition of low voltage activated T-type calcium channels by 10 μ M mibefradil on glucose- and swelling-induced insulin secretion from isolated islets of Langerhans (L. islets) and INS-1, INS-1E cell lines. Scheme of experiment and group designation as in Fig. 3. Insulin secretion under basal conditions was considered to be 100 %. Values are shown as mean \pm SE (n=10); *P<0.05; **P<0.01; ***P<0.001 compared with preceding basal secretion. Mibefradil inhibited stimulating effect of glucose in INS-1E and in INS-1 but not in islets. Response to hypotonicity was not affected.



in calcium depleted EGTA containing medium. In present experiments its effect was therefore tested in complete basal medium. To enable hypotonicity-induced secretion also from INS-1E cells we used group of calcium channel blockers (10 μ M nifedipine, 100 nM ω -agatoxin, 10 μ M mibefradil) instead of Ca^{2+} depletion. Tetanus toxin inhibited hypotonicity-induced secretion from INS-1 cells (Fig. 7). In contrast, presence of group of Ca^{2+} channel blockers enabled hypotonicity-stimulated insulin secretion from INS-1E cells even in presence of TeTx (Fig. 7).

Discussion

Recently we have found [12] that in contrast to natural pancreatic islets and INS-1 cells; extracellular calcium prevents cell swelling-induced insulin secretion from INS-1E tumor cells. Present experiments also confirmed hypotonicity-induced insulin secretion in calcium containing medium only in INS-1 cells. When tumor cells were perfused with Ca^{2+} depleted medium containing intracellular chelator BABTA/AM, response to hypotonic medium emerged also in INS-1E cell line. These results

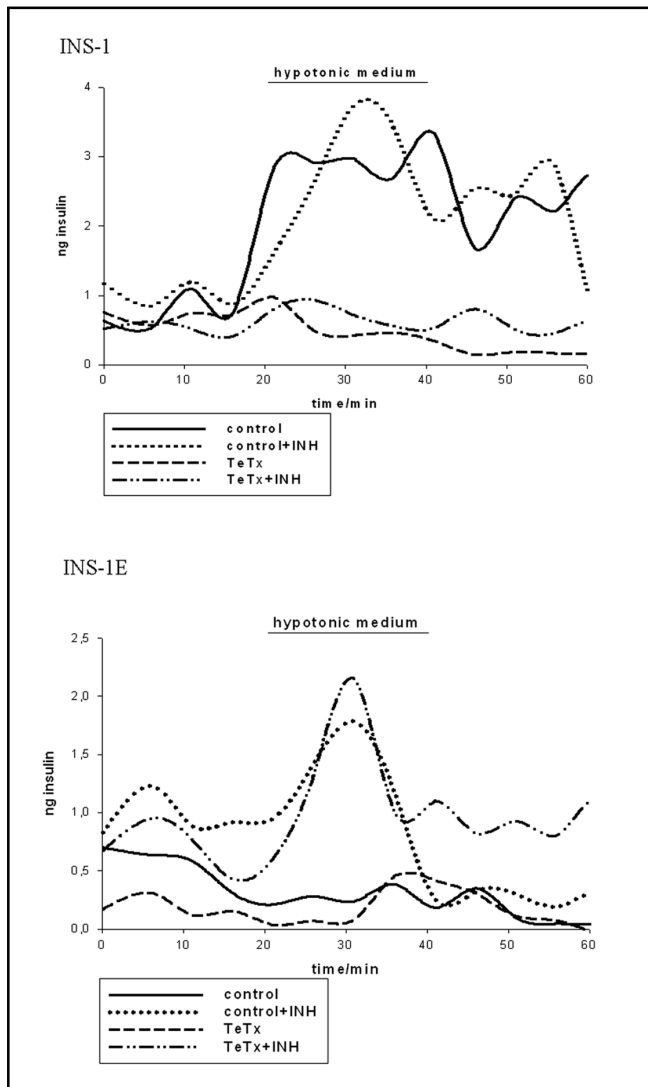


Fig. 7. Secretory response of INS-1 and INS-1E cells to hypotonic stimulus in presence of tetanus toxin (TeTx) and calcium channel inhibitors. Typical results of repeated experiments are shown. INS-1 and INS-1E cells were perfused at flow rate 0.3 ml/min with basal medium containing 2.5 mM glucose (20 min) and stimulating 30% hypotonic medium (30% hypo, 20 min), in presence or absence of 20 μ M TeTx and combination of calcium channels inhibitors (10 μ M nifedipine, 100 nM agatoxin, 10 μ M mibefradil). TeTx inhibits hypotonicity-induced secretion from INS-1 cells. Hypotonicity-induced insulin secretion from INS-1E cells appeared only in presence of group of calcium channel blockers, this secretion was resistant to TeTx.

demonstrate that similarly as in pancreatic islets [1], swelling-induced insulin secretion from INS-1 cells is independent from both extracellular and intracellular calcium. On the other hand, this type of secretion from INS-1E cells is hindered by extracellular calcium and could be promoted by Ca^{2+} depleted medium either alone

[12] or in combination with BABTA/AM (present experiments). Interestingly, Straub et al. [3] suggested two different mechanisms leading to swelling-induced exocytosis in the presence and absence of Ca^{2+} in the medium in insulin secreting β HC9 tumor cells. Ca^{2+} dependence was described previously in tumor-derived rat pituitary cells [17]. This may be a specific property of some tumor cell lines; swelling-induced insulin secretion in natural cells is independent from either extracellular or intracellular Ca^{2+} , i.e. is active either in the presence or absence of extracellular Ca^{2+} [1, 18, 19]. However, in INS-1E tumor cell line Ca^{2+} inhibits swelling-induced insulin secretion. This unusual effect of calcium was described to affect swelling-induced parathyroid hormone secretion in cells specialized in body calcium regulation, e.g. parathyroid cells [9] and is most likely mediated by extracellular calcium sensing receptor [20]. Similarly a decrease in osmolality elicits episodic release of renin from single arterioles, stimulates renin release from isolated glomeruli [21] and a decrease in the extracellular calcium concentration results in sustained stimulation of renin secretion [21]. Recently Madsen et al. [22] found that calcineurin and calcium/calmodulin suppress exocytosis of renin from juxtaglomerular cells independent of PKA. It is of interest that ethanol effect which in many aspects shares common mechanisms and properties with hypotonic stimulation [18, 23, 24] on insulin secretion from INS-1E cell line is not hindered by the presence of calcium [25]. It is likely that swelling and secretion-inducing effect of various permeants utilizes different pathways.

There is size-related and size-unrelated functional diversity among pancreatic islets [26, 27]. Only 60% islets responded to high glucose in a concentration-dependent and 32% in an all-or-none manner, and 8% islets did not respond at all [26]. We used 5 islets per tube, consequently the secretion may be subjected to high variability. Similar variability was seen also in groups of tumor cells. It has been therefore extremely important to compare secretion before and after stimulation in the same samples. For better comparison the response to stimulation in Fig. 3-6 is presented as % basal secretion from the same cells. Comparison of secretion from one group of cells before stimulation with that from different cells after stimulation might be the reason of some controversial results in the literature.

The mechanism by which calcium containing medium inhibits swelling-induced insulin secretion from INS-1E tumor cells remains to be clarified. To put more light on mechanism of the specific response of INS-1E cells we compared expression of some calcium channels in both

cell lines. According to reviews [28, 29] rat islets contain $\text{Ca}_v1.2$, $\text{Ca}_v1.3$, (L), $\text{Ca}_v2.1$, (P/Q), $\text{Ca}_v2.2$, (N) $\text{Ca}_v2.3$, (R), $\text{Ca}_v3.1$ (T) channels. Similar findings are reported also in rat cell line INS-1, except controversial presence of $\text{Ca}_v1.2$. We did not find any information on expression of Ca^{2+} channels in INS-1E cell line. Protein expression of only $\text{Ca}_v2.1$ was reported in rat islet β cells, while that of $\text{Ca}_v1.3$ $\text{Ca}_v2.1$ $\text{Ca}_v2.3$ was shown in INS-1 cells [29]. In our experiments inhibiting effect of nifedipine on glucose-induced insulin secretion in all three systems - islets, INS-1 and INS-1E cells showed that L type $\text{Ca}_v1.2$ channel was active, therefore expression of both RNA and protein should be supposed. Expression of $\text{Ca}_v2.1$, (P/Q) in INS-1E cells was shown and that in INS-1 cells confirmed in present work. Inhibiting effect of agatoxin on glucose-induced insulin secretion in INS-1 cells and promoting effect on hypotonicity-induced insulin secretion in INS-1E cells suggest also presence of active proteins. Expression of $\text{Ca}_v3.1$ (T) channels in both cell lines was demonstrated in present study, inhibiting effect on glucose-induced insulin secretion confirmed presence of active proteins in INS-1E cells.

To examine the role of particular channels we tested the effect of respective channel inhibitors. Besides L, P/Q and T channels also R channel was tested. For technical reasons N channel was not tested.

Presence of either nifedipine or ω -agatoxin in the calcium containing medium promoted hypotonicity-induced insulin secretion from INS-1E cells suggesting that L-type and P/Q channels play a role in inhibiting swelling-induced secretion from this cell type. It is of interest that inhibition of either one enabled secretory response. Apparently both L-type and P/Q channels should be simultaneously active to ensure inhibitory effect of extracellular calcium in INS-1E cells. Alternatively, a common mechanism blocked by both inhibitors is engaged. It is of interest that SNX-482, inhibitor of $\text{Ca}_v2.3$, (R) channel did not promote response to hypotonicity in INS-1E cells and surprisingly inhibited response also in INS-1 cells. Calcium may play multiple functions in β -cells; calcium sensing receptor is required for β -cell to β -cell interactions within islet-like structures [30]. Beneficial effect of some plant extracts on diabetes are mediated by effect on calcium system; resveratrol (a phytoestrogen found abundantly in grape skins, olive oil, peanuts) dose-dependently inhibited voltage-dependent Ca^{2+} currents and blunts glucose-induced, but not basal insulin release [31]. In contrast secretory response to hypotonicity emerged in INS-1E cells in presence of resveratrol (Bacová, Jakab et al. to be published). On the other hand,

extract of leaves of the *Gymnema sylvestre* (GS) plant increased β -cell Ca^{2+} levels [32], an effect that was mediated by Ca^{2+} influx through voltage-operated calcium channels [32]. Calcium promoting various secretory processes might be coming by influx from extracellular space or from various intracellular sources [33]. Hypotonicity-induced secretion of insulin from islets [1] is independent from both intra- and extracellular Ca^{2+} . Secretion from INS-1 cells (present experiments) was also independent from intracellular Ca^{2+} but surprisingly, blockers of P/Q and R type channels inhibited swelling-induced secretion from INS-1 cells (Fig. 4 and 5) although in Ca^{2+} depleted medium without [12] or with BAPTA/AM (present experiments) the response was present. Apparently, in this tumor cell line some interplay of different calcium pools in signaling swelling-induced secretion should be considered. Quite opposite situation was seen in INS-1E cell line. Secretion appeared only in Ca^{2+} depleted medium without [12] or with BAPTA/AM (present experiments) suggesting inhibitory role of calcium. Since swelling-induced insulin secretion emerged in Ca^{2+} containing medium if L and/or P/Q channels were blocked, the inhibiting role of extracellular calcium is unambiguous.

Variability in response to stimulation and inhibitors in two types of cells remind us that immortalized cell lines have many specific features absent in natural cells. Cell line could be advantageous to analyze specific mechanism and its dependence/connection with some mechanisms (e.g. signaling). However any broader interpretation should be supported by testing in natural cells. This is obvious also from comparison with freshly isolated pancreatic islets (Fig. 3-6). Many arguments and premisses generally accepted by scientific community based on data obtained on tumor cell lines should be considered with caution and their general validity might be often subjected to revision.

Exocytotic secretion depends on the functioning of a mechanism involving membrane SNARE proteins. TeTx inhibits glucose induced insulin secretion [4, 34]. We have shown previously that swelling-induced insulin secretion by TeTx was blocked only in presence of Ca^{2+} in the incubation medium [4]. In Ca^{2+} free medium TeTx did not affect hypotonicity-induced secretion. It is likely that TeTx as a Zn^{2+} dependent metalloproteinase [35] was not active in the presence of chelator EGTA in the Ca^{2+} depleted medium. To test the effect of active TeTx we used group of calcium channel blockers instead of calcium depleted medium. From the blockers tested SNX-482 was omitted because of inhibiting effect on hypotonicity-

induced secretion from INS-1 cells and no effect in INS-1E cells. TeTx was active at this conditions - hypotonicity-induced insulin secretion from INS-1 cells was inhibited by TeTx either in presence or absence of calcium channel inhibitors. In contrast, TeTx did not inhibit secretion from INS-1E cells in presence of channel blockers. Mechanism of this secretion is an enigma. Previously it was shown that preincubation of β -cells with neurotoxins TeTx and botulotoxin B blocks Ca^{2+} -induced insulin secretion but not that induced by $\text{GTP}\gamma\text{S}$ [36]. These data suggested parallel existence of Ca^{2+} activated pathway involving VAMP-2 and cellubrevin as well as existence of G proteins-induced pathway bypassing SNARE proteins. If this or another alternative pathway is engaged in calcium sensitive swelling-induced secretion in INS-1E cells remains to be uncovered. In pancreatic islets we have shown, however, that G proteins are not involved in osmotically induced insulin secretion [4]. It is of interest that an extra pool of secretory vesicles not available for glucose is exploited for exocytosis after swelling-induced insulin secretion: after hypotonic stimulation from islets [4] and ethanol stimulation from INS-1 and INS-1E tumor cell lines [29]. Recently it was shown that other permeants urea and glycerol activate rat β -cells via their rapid uptake across the β -cell plasma membrane via AQP7 [37]. We can speculate that at least part of signaling of hypotonicity-induced cell activation is shared by permeants.

Both glucose and hypotonic medium induce swelling of β cells [5] and INS-1E cells [6]. Superfusion of INS-1E cells with an isotonic solution containing 20 mmol/l glucose or a 30 % hypotonic solution led to the activation of chloride conductance [6]. These studies have implied that swelling of β cells may be an integral part of glucose-activated signaling inducing insulin secretion. This would be of clinical importance; dehydration frequently present in hyperglycemic patients might hinder cell swelling thus contributing to worsening of the secretory response of β cells to glucose.

Our data showing that INS-1E cells in the presence of extracellular Ca^{2+} have a normal secretory response

to glucose but are unable to release insulin in response to hypotonicity [12] suggest that cell swelling-induced exocytosis may be important but not exclusive critical step (at least not in INS-1E cells) in glucose-induced insulin release. However, the possibility that swelling is involved in signal transduction of glucose-induced insulin secretion by inducing other mechanism(s) not comprising exocytosis remains possible. Alternatively, Ca^{2+} inhibits secretory response to swelling only in this cell line.

In conclusion, present study showed that cell swelling-induced insulin secretion from INS-1E cells is inhibited by extracellular Ca^{2+} . We have shown expression of L-type Ca^{2+} channel $\text{Ca}_v1.2$ and non-L-type Ca^{2+} channels $\text{Ca}_v2.1$ (P/Q-type), $\text{Ca}_v2.2$ (N-type), and $\text{Ca}_v3.1$ (T-type) in INS-1 and INS-1E tumor cell lines. The inhibition of L type channel with nifedipine and/or P/Q type with ω -agatoxin IVA revealed distinct response to hypotonic medium also in INS-1E cells. TeTx was active in Ca containing medium with a group of calcium channel blockers and inhibited hypotonicity-induced insulin secretion from INS-1 cells. In contrast, at these conditions, secretion from INS-1E cells was TeTx resistant. Further analysis of mechanism(s) by which Ca^{2+} inhibits swelling-induced insulin secretion in INS-1E cells may provide valuable information on signaling and mechanism(s) of the exocytosis.

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