

Different secretory response of pancreatic islets and insulin secreting cell lines INS-1 and INS-1E to osmotic stimuli

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Short title:

Glucose, cell swelling and insulin secretion

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Summary

Objective of this study was to characterize osmotically-induced insulin secretion in two tumor cell lines.

We compared response of freshly isolated rat pancreatic islets and INS-1 and INS-1E tumor cell lines to high glucose, 30% hypotonic medium and 20% hypertonic medium.

In Ca^{2+} containing medium glucose induced insulin release in all three cell types. Hypotonicity induced insulin secretion from islets and INS-1 cells but not from INS-1E cells, in which secretion was inhibited despite similar increase in cell volume in both cell types. GdCl_3 (100 $\mu\text{mol/l}$) did not affect insulin response from INS-1E cells to hypotonic challenge. Hypertonic medium inhibited glucose-induced insulin secretion from islets but not from tumor cells. Noradrenalin (1 $\mu\text{mol/l}$) inhibited glucose-induced but not swelling-induced insulin secretion from INS-1 cells. Surprisingly, perfusion with Ca^{2+} depleted medium showed distinct secretory response of INS-1E cells to hypotonicity while that of INS-1 cells was partially inhibited.

Functioning glucose-induced insulin secretion is not sufficient prerequisite for hypotonicity-induced response in INS-1E cells suggesting that swelling-induced exocytosis is not essential step in the mechanism mediating glucose-induced insulin secretion. Both cell lines are resistant to inhibitory effect of hyperosmolarity on glucose-induced insulin secretion. Response of INS-1E cells to hypotonicity is inhibited by the presence of Ca^{2+} in medium.

Key words: cell volume, insulin secretion, pancreatic islets, tumor cell line, Ca^{2+} and exocytosis

Introduction

Cell volume regulation in response to osmotic challenge is integrated into a signal transduction network regulating various cell functions (Jakab *et al.* 2002, Lang *et al.* 1998a, b, Wehner *et al.* 2003). Cell swelling-induced peptide secretion represents an important cellular reaction when material stored in secretory vesicles is expelled as a secretory burst of peptide hormones or enzymes from various types of cells including endocrine, neurons, leukocytes or those of the exocrine pancreas (Štrbák and Greer 2000). Dynamics of the secretory response to cell swelling is similar to that induced by other specific or non-specific secretagogues (Bačová *et al.* 2006a). The cell swelling as a nonspecific stimulator of protein/peptide release with limited selectivity (Bačová *et al.* 2005, Najvirtová *et al.* 2003) is a suitable tool for characterization of peptide secretion from various cells and tissues (Štrbák and Greer 2000, Štrbák 2006). Although the mechanism of swelling-induced exocytosis is not fully understood, its comparison with signal transduction mediating glucose-induced insulin secretion indicates the presence of novel signaling pathway (Bačová *et al.* 2005). Hyposmotic stimulation of insulin secretion from pancreatic islets is independent from both the extracellular and intracellular Ca^{2+} (Bačová *et al.* 2005, Blackard *et al.* 1975, Straub *et al.* 2002), various insulin release inhibitors and ion-flux modifiers (Blackard *et al.* 1975, Straub *et al.* 2002), cannot be inhibited by noradrenaline (Bačová *et al.* 2005), does not involve protein kinase C (Bačová *et al.* 2005), G proteins or phospholipase A2 activation and is N-ethyl maleimide insensitive (Bačová *et al.* 2007). Interestingly, glucose itself induces swelling of pancreatic β -cells (Miley *et al.* 1997) and tumor-derived INS-1E cells (Jakab *et al.* 2006); therefore a possibility that swelling is integrated into glucose signaling should be also considered.

Secretion induced by extracellular hyposmolarity is due to the relative reduction of extracellular compared to intracellular osmolarity and not to dilution of any essential extracellular elements. If medium is made by the addition of equivalent osmolar amount of mannitol (Greer *et al.* 1983), sorbitol (Blackard *et al.* 1975, Straub *et al.* 2002), choline chloride (Blackard *et al.* 1975) or by biologically inactive L-glucose (Nikodemova *et al.* 1999) to maintain isosmolarity on both sides of the plasma membrane, no secretion is induced. If the medium is made hyperosmolar, secretion is not stimulated and is often suppressed (Kučerová and Štrbák 2001, Miley *et al.* 1997, Najvirtová *et al.* 2003, Sato *et al.* 1990, Wang *et al.* 1992). However, on return of the cells to an isosmolar medium, there is a prompt dose-related burst of ‘off response’ secretion whose magnitude is proportional to that of the preceding hyperosmolarity (Greer *et al.* 1985). This indicates that it is the relative difference in intracellular vs. extracellular osmolar concentration which causes exocytosis rather than the absolute osmolarity of either compartment.

The aim of the present study was to compare glucose- and osmotically-induced insulin secretion from different insulin secreting systems: freshly isolated pancreatic islets and rat insulinoma cell lines INS-1 and INS-1E. The differences in secretory responses between these cells may provide an important insight into the mechanisms of insulin secretion induced by cell swelling.

Material and Methods

All 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 the national Veterinary Board.

Male Wistar rats (Charles River, Sulzfeld, Germany) (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*.

Tumor cell lines

Rat insulinoma derived β cells

Rat insulin-secreting cell lines INS-1 and INS-1E sensitive to glucose stimulation (Merglen *et al.* 2004), were kind gift from Dr. Claes B. Wollheim (University Medical Center, Geneva, Switzerland). Cells were cultivated at 37°C in humidified atmosphere containing 5% CO₂/ 95% O₂ air in complete medium composed of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 1 mmol/l sodium pyruvate, 50 μ mol/l 2-mercaptoethanol, 2 mmol/l glutamine, 25 mmol/l HEPES, 100 U/ml penicillin, and 100 μ g/ml streptomycin (all from PAA, Switzerland). The maintenance culture was passaged once a week by gentle trypsinization.

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 mmol/l NaCl, 3.6 mmol/l KCl, 5 mmol/l NaHCO₃, 0.5 mmol/l NaH₂PO₄, 0.5 mmol/l MgCl₂, 1.5 mmol/l CaCl₂, and 10 mmol/l 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 and stimulating media as indicated. Insulin secretion was measured by radioimmunoassay (RIA).

Cell clusters

INS-1E and INS-1 cells ($4 \times 10^5/\text{ml}$) were seeded in nonadherent bacterial 10 cm Petri dishes and cultured in complete medium for 2-3 days before use.

Perifusion of cell clusters

Spheroid clusters composed of INS-1 or INS-1E cells were preincubated for 2 h in glucose-free culture medium. After centrifugation ($1000 \times g$), cell clusters were resuspended in glucose-free KRBH and counted, and about 500 spheroids were distributed per chamber in a 250 μl media thermostated at 37 °C. The flow rate of media was set at 0.3 ml/min, and fractions were collected every minute after a 20 min washing period with 2.5 mmol/l glucose. Insulin secretion was measured by RIA using rat insulin as a standard.

Incubation media

Basal medium (292 mOsm/kg H_2O): Krebs-Ringer Hepes buffer (KRH) (133,75 mmol/l NaCl, 3.6 mmol/l KCl, 5 mmol/l NaHCO_3 , 0.5 mmol/l NaH_2PO_4 , 0.5 mmol/l MgCl_2 , 1.5 mmol/l CaCl_2 , and 10 mmol/l HEPES, 2.5 mmol/l glucose, BSA (0.1%), pH 7.4 (23).

Stimulating media:

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

Hypotonic medium (202 mOsm/kg H_2O): 30% hypotonic medium was prepared by appropriate dilution of basal medium with deionized water.

Hypertonic medium (352 mOsm/kg H_2O): 60 mmol/l mannitol was added to glucose medium.

In Ca^{2+} depleted medium CaCl_2 was omitted and 1mmol/l EGTA was added.

Specific inhibitors noradrenaline and GdCl_3 (SIGMA, Germany) were added to stimulating medium in some experiments as indicated.

Confocal laser scanning measurement

A confocal laser scanning microscope LSM510 META on Axiovert 200 (Zeiss, Germany) was used to investigate proportion parameters of INS-1 and INS-1E cells before and after stimulation by hypotonic medium. All measurements were taken in time series scanning mode using C-Apochromat 40× objective with water immersion (numeric aperture 1.2). He-Ne-laser line (633 nm) was used as an excitation source; emission was detected at 620-680 nm (BP filter, reflection contrast).

Pancreatic islets

Isolation of islets of Langerhans

Islets were isolated from adult male Wistar rats according to the method by Lacy and Kostianovsky (1967). 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 of islets in vitro

Incubations were performed in Eppendorf tubes at 37°C. Groups of 5 islets per tube were preincubated for 60 min in Krebs-Ringer Hepes buffer (KRH) followed by the incubation four subsequent 30 min periods in basal or stimulating media in final volume of 200 µl. At the end of each incubation period medium was saved for insulin radioimmunoassay.

Incubation media

Basal medium: Krebs-Ringer Hepes buffer (KRH) (118 mmol/l NaCl, 4 mmol/l KCl, 1.2 mmol/l MgSO₄, 1.2 mmol/l KH₂PO₄, 5 mmol/l NaHCO₃, 25 mmol/l HEPES, 3 mmol/l D-glucose, 2.5 mmol/l CaCl₂, 0.1% BSA, pH 7.4 (Bačová *et al.*, 2005). This medium was used for preincubation and non-stimulated incubation periods.

Stimulating media:

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

Hypotonic medium: 30% hypotonic medium (202 mOsm/kg H₂O) was prepared by appropriate dilution of basal medium with deionized water.

Hypertonic medium: 60 mmol/l mannitol was added to basal medium.

Glucose/Hypertonic medium: 60 mmol/l mannitol was added to glucose medium.

Specific inhibitors noradrenaline and GdCl₃ (Sigma, Germany) were added to stimulating medium.

All media were gassed with 5% CO₂/95% O₂ atmosphere for 30 min and pH was 7.4.

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-monoiodoinsulin was prepared with the aid of lactoperoxidase (Zorad *et al.* 1985). 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 μ l 0.01 mol/l PBS (pH 7.4). After overnight incubation of samples with the antibody at 4°C, bound and free peptides were separated by cold 200 μ l 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).

Data analysis

Results of static incubations are shown as percentage of basal secretion (mean \pm SE). Data from the same samples before and after stimulation were compared by paired Student's t-test. Data from different experiments were evaluated by unpaired t-test. Differences were considered significant at level $p < 0.05$.

Results

A high concentration of extracellular glucose induced distinct insulin release from pancreatic islets and both tumor cell lines by 5 times and 2 times, respectively, showing that all three cell types respond normally to glucose stimulation (Fig. 1A).

Hypotonic medium (30% diluted basal medium) increased insulin secretion from pancreatic islets by 4 times and from INS-1 cells by 5 times (Fig. 1B). In contrast, INS-1E cells responded to hypotonic medium by a 50% decrease in insulin secretion. The same results were obtained when hypotonicity was achieved by reducing of NaCl concentration to 55 mM (data not shown). The absence of secretory response to hypotonicity in INS-1E cells prompted us to measure changes of cell volume after osmotic stimulus. Direct measurements of cell size showed a significant increase in 2D cell area by 9 and 13.1% of INS-1E and INS-1 cells, respectively, in hypotonic medium (Tab.1) indicating cell swelling. The width (shorter parameter) increased more than the length in both cell lines suggesting their shape was more

rounded after swelling. Although measured parameters were larger in INS-1E than in INS-1 cells, there was no significant difference between the changes of 2D area (%) suggesting similar responsiveness of both cell lines to osmotic challenge.

To explain the inability of hypotonic medium to induce insulin secretion from INS-1E cells, we examined the role of stretch sensitive channels in these effects. A presence of 100 $\mu\text{mol/l}$ GdCl_3 , an inhibitor of stretch sensitive channels, in the medium did not affect basal or hypotonicity-induced insulin secretion from INS-1E cells (Fig. 2) suggesting that they do not play a significant role in the mechanism of insulin secretion.

The atypical secretory reaction to hypotonic stimulus in INS-1E cells suggested irregular response of these cells to osmotic and/or cell volume changes. Therefore, we tested the effects of hypertonic stimulus on insulin secretion. As expected, in pancreatic islets, the glucose-induced insulin secretion was prevented by the hypertonic medium (Fig. 3). However, in both tumor cell lines, the hypertonicity did not have any effect on glucose-induced insulin secretion further underlying differences between normal and tumor cells.

Noradrenalin, a physiological inhibitor of insulin release, prevented glucose-induced insulin secretion (Fig. 4A) from INS-1 cells; however, it did not prevent the swelling-stimulated insulin secretion (Fig. 4B). The response of INS-1E cells to both stimuli was not compared because of the absence of the response to swelling at conditions necessary for glucose stimulation (Ca^{2+} containing medium).

It was not possible to test responses of tumor cell lines in Ca^{2+} depleted medium in static incubations as described above because the cells do not adhere to the surface of plates at this condition. Therefore experiments with perfusion of cell clusters were used. Typical result from repeated experiments is shown in Fig. 5. Perfusion confirmed inability of INS-1E cells to release insulin in response to hypotonicity in the presence of extracellular Ca^{2+} (Fig. 5A). Surprisingly, distinct insulin release appeared during perfusion of INS-1E cells with Ca^{2+}

depleted hypotonic medium (Fig. 5B); this response was even higher than that seen in INS-1 cells in which the release was partially inhibited.

Discussion

Clonal INS-1E cells were isolated from parental INS-1 based on insulin content and secretory response to glucose (Merglen *et al.* 2004). INS-1E cell line has been provided to many investigators including our laboratory. We have taken an advantage of having two insulin secreting cell lines responsive to glucose and have compared their secretory properties in response to osmotic stimuli with freshly isolated pancreatic islets.

All three *in vitro* models e.g. isolated pancreatic islets and both tumor cell lines released insulin in response to glucose challenge, demonstrating a functional glucose-activated signal transduction pathway. Hypotonic medium containing Ca^{2+} induced insulin secretion from the pancreatic islets and INS-1 cells, whereas insulin secretion was inhibited from INS-1E cells. A difference between insulin response to glucose and hypotonicity in this cell line indicates that a complete signaling activated by glucose stimulation is not a sufficient prerequisite for secretory response to cell swelling suggesting that some extra step(s) is required to trigger swelling-induced exocytosis.

Direct measurements of cell size showed that hypotonic medium induced swelling of both INS-1 and INS-1E cells. Miley *et al.* (1997) reported that both glucose and hypotonic medium induced swelling of β -cells. Recently (Jakab *et al.* 2006) it was shown that both high glucose and hypotonicity induced swelling in INS-1E cells and superfusion with an isotonic solution containing 20 mmol/l glucose or a 30% hypotonic solution led to the activation of chloride conductance. These studies have implied that swelling of β -cells may be an integral part of glucose-activated signaling inducing insulin secretion. However, our present 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 suggest that cell swelling-induced exocytosis is not an essential step in glucose-induced stimulation of 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.

A failure to identify transduction pathway of cell swelling-induced exocytosis and its resistance to physiological inhibitors like noradrenalin suggests that this signaling pathway bypasses conventional transduction steps and/or may be effective at the distal end of the cascade. It has been shown that swelling of secretory vesicles is critical step of exocytosis (Alvarez de Toledo *et al.* 1993, Finkelstein *et al.* 1986, Lin *et al.* 1996, Tsuboi *et al.* 2002). Stretching of vesicular and plasma membranes in the region of contact results in exposure of hydrophobic acyl chains leading to subsequent merging and fusion. The fusion rates are order of magnitude higher if an osmotic gradient across plasma membrane is present (Finkelstein *et al.* 1986). The externalization of hormones or neurotransmitters upon exocytosis of vesicles is augmented by water efflux from the vesicle membrane through the widened fusion pore (Tsuboi *et al.* 2002). Based on these data, we had hypothesized that exocytosis triggered by cell swelling is a result of direct biophysical effect of the osmotic gradient on secretory vesicles (Štrbák *et al.* 2004). However, our present data demonstrating that rat INS-1E tumor β cells swelling in response either to glucose or hypotonicity (Jakab *et al.* 2006) are unable to release insulin in response to cell swelling despite a good response to glucose, challenge this hypothesis. It is well documented that swelling of secretory vesicles precedes exocytosis (Hattori *et al.* 1990, Landgraf and Ludwig 1991, Landgraf *et al.* 1990, Pow and Morris 1989). Therefore, if the vesicle swelling alone was the only essential prerequisite of hypotonicity

induced exocytosis, it is unlikely that secretory vesicles with normal response to glucose stimulation would not release insulin in response to cell swelling.

It is possible that INS-1E cells either do not adequately perceive the change of cell volume or they react specifically to osmotic stimuli as it has been shown for oxytocin (OT) and vasopressin secreting cells. Direct stimulation of the hypothalamic paraventricular (PVN) or supraoptic nucleus (SON) with hypertonic saline increases vasopressin and OT secretion (Hattori *et al.* 1990, Hattori *et al.* 1992, Landgraf and Ludwig 1991, Landgraf *et al.* 1990, Pow and Morris 1989). It has been also shown that hypotonic stimuli hyperpolarize magnocellular neurosecretory cells via stretch-inactivated channels (Bourgue and Oliet 1997). In our previous studies (Bačová *et al.* 2006b, Najvirtová *et al.* 2002), we used tissue explants of hypothalamic PVN and posterior pituitary for simultaneous thyrotropin releasing hormone (TRH) and OT examination. While TRH secretion was stimulated by cell swelling, OT, a neurohormone engaged in water and salt regulation, was not released after cell swelling-inducing stimuli (Najvirtová *et al.* 2003). We have shown (Bačová *et al.* 2006b) that an unspecific intranuclear OT response to hypotonic stimulation (e.g. hypotonicity stimulated OT release) within the PVN and the SON could be unmasked by inhibiting the specific response in the presence of 50 or 100 $\mu\text{mol/l}$ GdCl_3 , an inhibitor of stretch sensitive channels (Oliet and Bourgue 1996, Yang and Sachs 1989) and a potent blocker of voltage-sensitive Ca^{2+} channels (Romano-Silva *et al.* 1994). INS-1E cells responded to hypotonic stimulation by decreased insulin secretion suggesting possibility of stretch sensitive channels mediated specific response to osmotic stimulus. Hypotonic medium did not induce, however, insulin release even in presence of 100 $\mu\text{mol/l}$ GdCl_3 . These results suggest that the GdCl_3 sensitive mechanisms (involving mechanoreceptors regulated channels and/or voltage gated Ca^{2+} channels) are not responsible for atypical secretory behavior of INS-1E cells in hypotonic

medium. However, possibility that mechanosensitive receptors and their sensitivity are comprised in this tumor cell line although unlikely, can not be excluded.

We conclude that swelling-induced insulin secretion requires extra step(s) which does not occur in signaling of glucose-induced insulin secretion and is not active in INS-1E cells in presence of extracellular Ca^{2+} . However, specific response of neuropeptides within hypothalamic nuclei involves Ca^{2+} dependent mechanisms (Hattori *et al.* 1990, Ludwig and Landgraf 1992, Bačová *et al.* 2006b). When GdCl_3 was used, EGTA was omitted to prevent chelator neutralization in our experiments. In perfusion experiments Ca^{2+} depletion was more complete (both CaCl_2 omission and EGTA added to medium). It can be speculated that a specific response insensitive to GdCl_3 and requiring Ca^{2+} was involved in the mechanism of inhibiting effect of hypotonicity on insulin secretion in INS-1E cells. However, hypothalamic magnocellular neurons increase cell size in response to hyperosmolar conditions and decrease cell size in response to hyposmolar conditions (Zhang *et al.* 2001) and hypotonic stimuli hyperpolarize magnocellular neurosecretory cells via stretch-inactivated channels (Bourque and Oliet 1997). In contrast, INS-1E cells swelled in response to hypotonic medium in our experiments. Although we can not exclude a specific reaction, we consider this possibility unlikely.

Negative modulation by Ca^{2+} influx of osmotically induced secretion was found in many types of secretory cells (for review Štrbák and Greer 2000) and is most likely related to effective regulatory volume decrease. When medium Ca^{2+} is depleted or Ca^{2+} influx prevented by Ca^{2+} channel blockers, the amplitude of cell swelling is greater and the regulatory volume decrease (RVD) slower. The apparent negative modulation by Ca^{2+} influx of osmotically induced secretion in normal pituitary cells may simply be due to an augmented signal intensity resulting from the greater magnitude and duration of cell swelling from an osmotic stimulus when Ca^{2+} influx is prevented than when it occurs (Štrbák and Greer 2000).

It could be speculated that bigger INS-1E cells are less sensitive to volume changes and perceive only more profound change in absence of Ca^{2+} . If this was a true, secretory response of INS-1E cells to swelling would be smaller but not inhibited as in our case. The mechanism and role of Ca^{2+} in swelling-induced insulin secretion of INS-1E cells remains therefore to be clarified.

The opposite osmotic manipulation inducing cell shrinking has most often suppressing effect on peptide/protein secretory response (for review see Štrbák and Greer 2000, Štrbák 2006). Therefore, we tested whether stimulating effect of glucose on insulin secretion can be affected by hypertonic medium. As expected, hypertonicity inhibited response of pancreatic islets to glucose stimulation. In contrast, both tumor cell lines responded with distinct release of insulin. A similar effect was observed in HIT-T 15 cells which released insulin in response to hypertonic glucose, although the response was decreased as compared to isotonic conditions (Sato *et al.* 1996). These data suggest different sensing and/or sensitivity to the effects of osmolarity in insulin secreting tumor cell lines compared to pancreatic islets.

We have shown in previous study that noradrenalin, a physiological inhibitor of insulin secretion, fully abolished glucose-induced insulin secretion from isolated pancreatic islets but did not inhibit hypotonic stimulation (Bačová *et al.* 2005). Since INS-1 cells, in contrast to INS-1E, release insulin in response to hypotonicity in the presence of extracellular Ca^{2+} , we examined the effect of noradrenalin on the both glucose and hypotonicity stimuli of insulin secretion. We found similar response to that observed in freshly isolated pancreatic islets; the secretory response of INS-1 cells to glucose but not that to hypotonicity was inhibited by noradrenalin.

In term of insulin secretory response, INS-1E cells react atypically to cell swelling-inducing stimulus despite a good response to glucose. Both INS-1 and INS-1E cell lines were found to be resistant or at least less sensitive to the inhibitory effect of hypertonicity. The

mechanism by which an increase or a decrease in cell volume are sensed and subsequently transduced into activation of cell volume sensitive mechanisms is subject of intense studies (Hoffmann *et al.* 2006). In higher eukaryotes, the available evidence indicates that there are probably multiple mechanisms by which volume changes are initially sensed (Hoffmann *et al.* 2006). However, those related to a swelling-induced peptide secretion remain unknown. We have found that there are alternative signaling pathways for hypotonicity-induced insulin secretion in these INS cell lines; one of them was inactivated by the presence of extracellular Ca^{2+} . Interestingly, Straub *et al.* (Straub *et al.* 2002) suggested two different mechanisms leading to swelling-induced exocytosis in the presence and absence of Ca^{2+} in the medium in insulin secreting βHC9 cells. Similar phenomenon (e.g. Ca^{2+} dependence) was described previously in tumor-derived rat pituitary cells (Sato *et al.* 1991). 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+} (e.g. is active either in the presence or absence of Ca^{2+}) (Bačová *et al.* 2005, Štrbák and Greer 2000, Štrbák 2006). Further analysis of mechanism by which Ca^{2+} inhibits swelling-induced insulin secretion in INS-1E cells may provide an effective tool to unravel signal transduction pathway(s) of swelling induced peptide secretion.

In conclusion: A functional glucose-activated signal transduction pathway is not a sufficient prerequisite for swelling-induced insulin release, thus demonstrating the necessity of extra signaling step(s) mediating responses to osmotic effects. Swelling-induced exocytosis is not an essential step in glucose-induced signaling mediating insulin secretion. The paradoxical response of INS-1E cells to swelling in the presence of extracellular Ca^{2+} is not mediated by GdCl_3 (stretch) sensitive channels. Both, INS-1 and INS-1E cell lines are resistant to the inhibitory effects of hypertonicity on glucose-induced insulin secretion. The depletion of Ca^{2+} from medium had permissive effects on hypotonicity-induced insulin

secretion from INS-1E cells. Further studies of these cell lines may provide an important insight into mechanisms of cell swelling-induced peptide secretion.

Conflict of interest

There are no conflicts of interest.

Acknowledgements

The work was supported by the project 2/6158/26 of the Grant Agency of Ministry of Education and Slovak Academy of Sciences (VEGA), project APVV 0235-06 and project of CE SAV CENDO. Authors thank to Dr. Claes B. Wollheim and Pierre Maechler (University Medical Center, Geneva) for providing kind gift of INS-1 and INS-1E rat insulin secreting cell lines and to Institute of Endocrinology (Prague) for providing us with specific rabbit anti rat insulin antibody prepared by Dr. Štolba.

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Legend to figures.

Fig. 1: Insulin secretion in response to glucose and 30% hypotonic medium (hypo) from isolated Langerhans's islets and cell lines (INS-1, INS-1E). After preincubation islets (5 per tube) or cells were incubated for two subsequent 30 min periods in control (basal) medium and stimulating medium **A:** high glucose medium; **B:** 30 % hypotonic medium (detailed composition see in Material and Methods). Insulin secretion during under basal conditions was considered to be 100%. Values are shown as mean \pm SE (n=10); *P< 0.05; **P< 0.005; ***P< 0.001 compared to previous control period. All three systems released insulin in response to glucose challenge. INS-1E cells do not release insulin in response to hypotonicity.

Fig. 2: Effect of GdCl₃, an inhibitor of stretch sensitive channels, on insulin secretory response to hypotonic medium in INS-1E cells. After preincubation cells were incubated in basal KRBH and then in 30% hypotonic medium (hypo) with or without 100 μ mol/l GdCl₃ over 30 minutes periods. Insulin secretion during control period was considered to be 100%. Values are shown as mean \pm SE (n=10); *P< 0.05 compared to previous control period. GdCl₃ did not affect basal and hypotonicity-induced secretion.

Fig. 3: Insulin secretion in response to hypertonic medium from isolated Langerhans's 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) medium and stimulating (glucose medium, hypertonic medium and glucose/hypertonic medium (see Material and Methods)). Insulin secretion under basal condition was considered to be 100%. Values are shown as mean \pm SE (n=10); *P< 0.05; ***P< 0.001 compared to previous control period.

Hypertonicity of medium inhibited stimulating effect of glucose in islets (**A**) but not in pancreatic cell lines (**B**: INS-1; **C**: INS-1E).

Fig 4: Effect of physiological inhibitor noradrenaline (NA) on stimulated insulin secretion from INS-1 cell line. After preincubation cells were incubated for two subsequent 30 min periods in control (basal) medium and stimulating medium - **A**: glucose medium; **B**: hypotonic medium (see Material and Methods) with or without 1 $\mu\text{mol/l}$ NA. Insulin secretion during control period was considered to be 100%. Values are shown as mean \pm SE (n=10); * $P < 0.05$; *** $P < 0.001$ compared to control period. Stimulating effect of glucose but not that of hypotonicity was prevented by NA.

Fig. 5: Secretory responses of spheroid clusters composed of INS-1E and INS-1 cells. Typical results of repeated experiments are shown. Clusters of INS-1E and INS-1 cells were perfused at flow rate 0.3 ml/min with basal medium containing 2.5 mmol/l glucose (20 min) and stimulating 30% hypotonic medium (30% hypo, 20 min), **A**: in presence of 1.5 mmol/l CaCl_2 ; **B**: in Ca^{2+} depleted medium containing 1mmol/l EGTA. Ca^{2+} containing hypotonic medium induced insulin secretion only in INS-1 cells. In Ca^{2+} depleted medium hypotonicity induced burst of insulin secretion in INS-1E cells which was even higher than partially inhibited response of INS-1 cells.

Fig. 1:

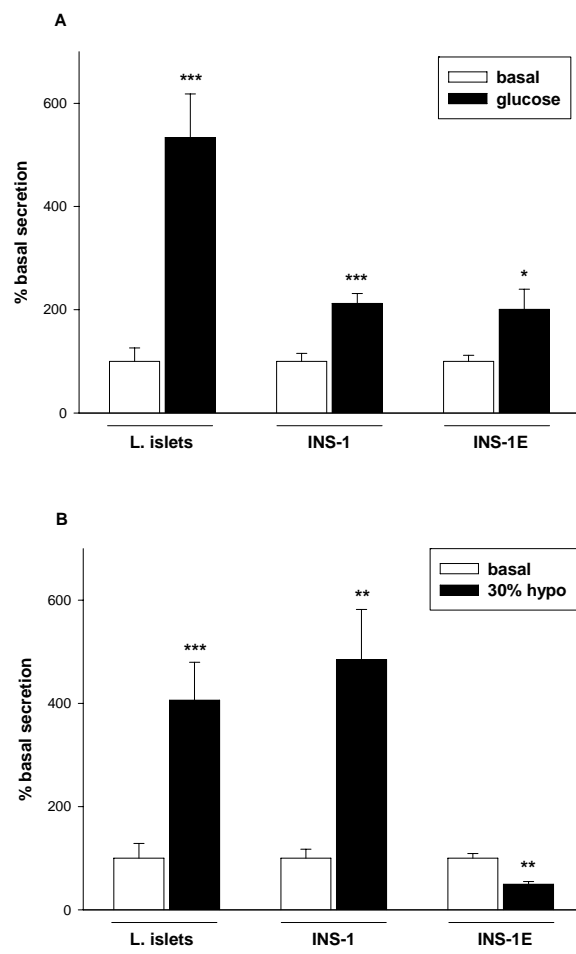


Fig. 2:

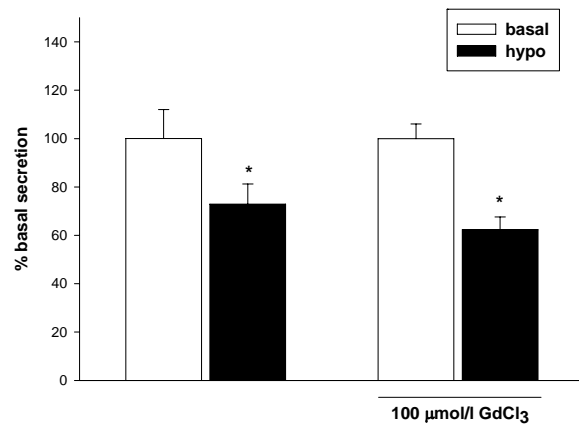


Fig. 3:

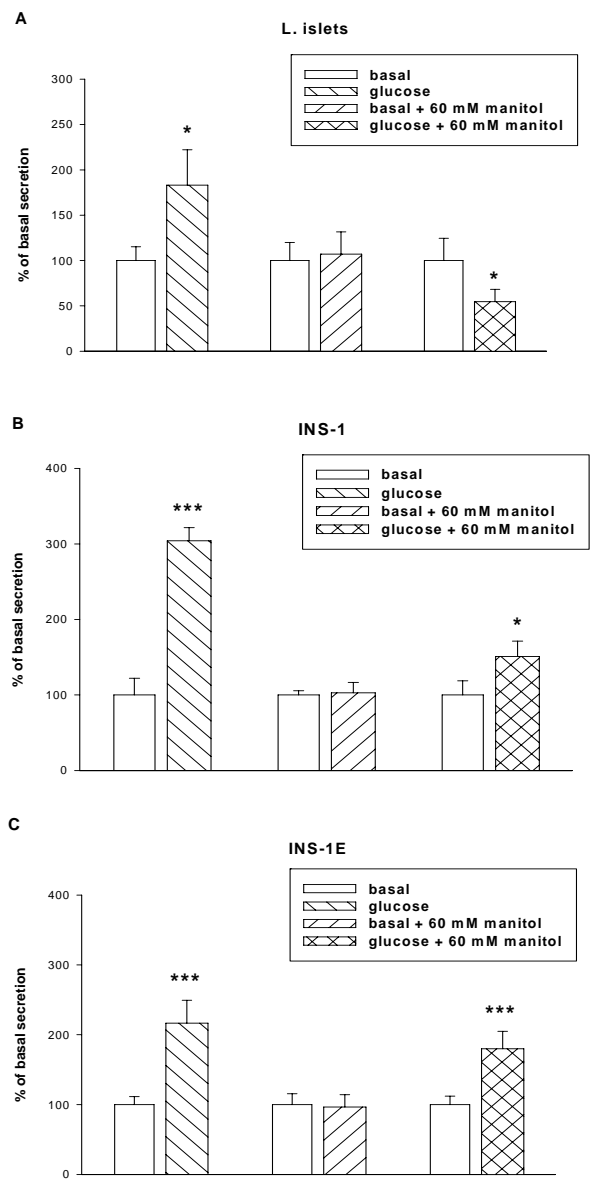


Fig. 4:

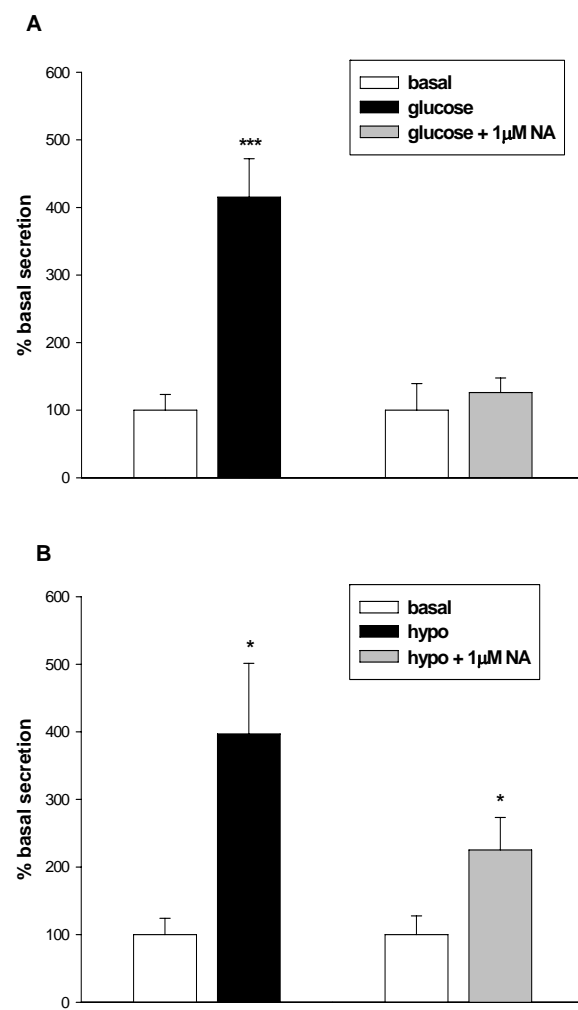
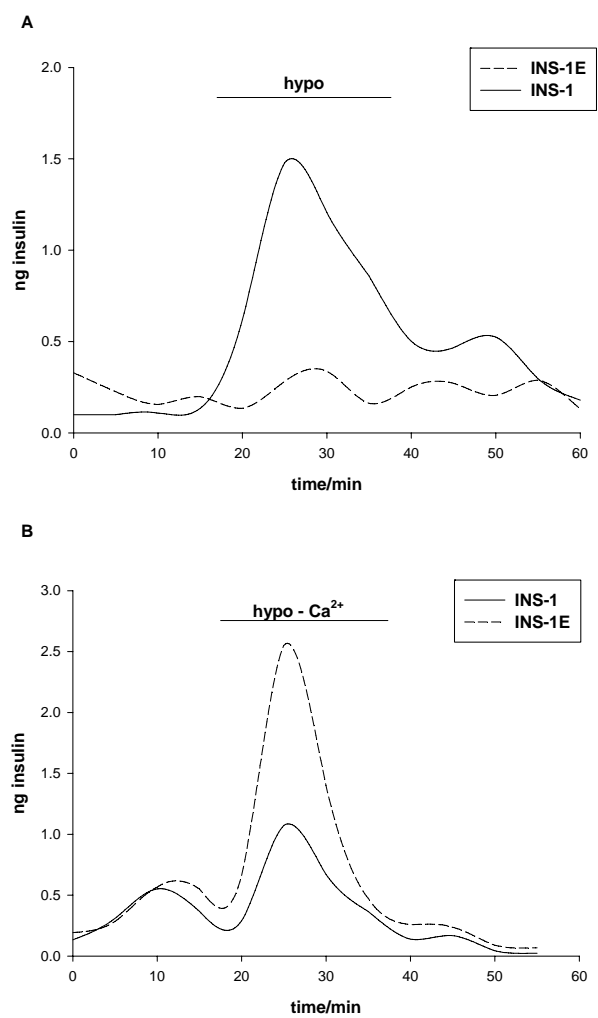


Fig. 5:



Tab. 1: Volume changes of INS-1E and INS-1 cells after hypotonic stimulation.

Measurements were taken in time series scanning mode using a confocal laser-scanning microscope. Parameters were measured before and 90 s after hypotonic stimulation. Values are shown as mean \pm SE *P< 0.05, **P< 0.005, ***P< 0.001, compared to parameters before stimulation (paired t-test).

	n	Width			Length			Area		
		Before (μm)	After (μm)	Changes (%)	Before (μm)	After (μm)	Changes (%)	Before (μm^2)	After (μm^2)	Changes (%)
INS-1	7	14.5 ± 1.3	16.1 $\pm 1.5^{***}$	10.3 ± 0.9	32.2 ± 4.9	38.9 $\pm 4.8^{**}$	2.7 ± 0.9	345.6 ± 51.1	387 $\pm 54.8^{***}$	13.1 ± 2.1
INS-1E	4	21.1 ± 2	22.6 ± 2	7.51 ± 2.50	41.1 ± 2.6	44.6 $\pm 2.5^*$	3.5 ± 0.7	632.5 ± 50.5	687 $\pm 45.7^*$	9.0 ± 2.83