Mechanism of Ethanol-Induced Insulin Secretion from INS-1 and INS-1E Tumor Cell Lines

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Key Words

Insulin secretion • Ethanol • Ca^{2+} • PKC • PTP • SNARE

Abstract

Alcohol causes reactive hypoglycemia by attenuating the release of counter regulatory hormones, redistribution of pancreatic blood flow and direct stimulation of insulin secretion. Objective of this study was characterization of ethanol-induced insulin secretion. Signaling of ethanol- and glucose-induced insulin release from INS-1 and INS-1E cells was compared. Both cell lines responded similarly to all experimental interventions. In contrast to glucose, ethanol-induced insulin secretion was not hindered in calcium depleted medium or by addition of 10 μM BAPTA/AM (intracellular chelator). Inhibitor of protein kinase C Bisindolylmaleimide (3 μM) abolished glucose- but not ethanol-induced insulin secretion. Tetanus toxin (20 nM), inhibitor of SNARE proteins complex formation, blocked ethanol-induced insulin secretion. Both 5 mM N-ethylmaleimide and 10 μM ZnCl_2 (inhibitor of protein tyrosine phosphatases), which block disassembly of SNARE complexes and their further participation in exocytosis, increased

basal insulin secretion. In contrast to glucose, already high insulin secretion was further increased after ethanol stimulation in either treatment. Conclusion: Signaling of ethanol-induced insulin secretion from INS-1 and INS-1E cell lines bypasses calcium and PKC involving steps, is sensitive to tetanus toxin but resistant to N-ethylmaleimide and ZnCl_2 . An extra pool of secretory vesicles not available for glucose is exploited for exocytosis after ethanol stimulation.

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Introduction

Extensive clinical experience indicates that unexpected, frequent, and prolonged hypoglycemia is a substantial problem after alcohol ingestion in diabetic subjects [1]. However, the mechanisms underlying the hypoglycemic properties of ethanol remain largely elusive. Acute ingestion of alcohol impairs the epinephrine response and markedly suppresses the release of GH in response to a fall in blood glucose and combination of gin and tonic can induce reactive hypoglycemia levels in healthy volunteers [2]. Recent findings [3] demonstrate that ethanol acutely exerts substantial influences on

pancreatic microcirculation by evoking a massive redistribution of pancreatic blood flow from the exocrine into the endocrine part via mechanisms mediated by nitric oxide and vagal stimuli, augmenting late-phase insulin secretion, and thereby evoking hypoglycemia. Ethanol (40, 80, and 160 mM) in isosmotic medium induced dose-dependent release of insulin and TRH from adult rat pancreatic islets *in vitro* [4]. Mechanism of this direct ethanol effect remains to be disclosed.

Secretion of insulin could be stimulated by several ways. Most important and relatively well known is conventional glucose-induced secretion. The transduction pathway for glucose-induced insulin secretion has attracted an attention of scientists for decades and is relatively well known. Glucose is metabolized through the glycolysis and Krebs (TCA) cycle. The increased ATP/ADP ratio leads to closure of the ATP-sensitive K⁺ channels and depolarizes the cell membrane. Activation of voltage-gated calcium channels increases Ca²⁺ entry and higher concentration of intracellular calcium stimulates insulin release [5].

Cell swelling-induced insulin secretion can be induced by relative hypotonicity or small permeant molecules (e.g. ethanol, urea, glycerol). Although first report appeared time ago [6], only recently this phenomenon has attracted appropriate attention [4, 5, 7-10]. Comparison of swelling induced insulin secretion with signal transduction mediating glucose-induced insulin secretion indicates the presence of novel signaling pathway [7]. Hypotonic stimulation of insulin secretion from pancreatic islets is independent from both the extracellular [6, 7, 11] and intracellular Ca²⁺ [7], resistant to various insulin release inhibitors and ion-flux modifiers [6, 11], cannot be inhibited by noradrenaline [7], does not involve G proteins or phospholipase A2 activation and is N-ethylmaleimide insensitive [8].

More complex may be mechanism of insulin secretion induced by permeants. In addition to effects mediated by specific receptors, permeant molecules (ethanol, urea, glycerol) when added in hypo- or isosmolar medium act also by a general unspecific mechanism; as small uncharged molecules - permeants diffuse rapidly across the cell membrane down their concentration gradient [12]. An increase of their concentration inside the cell results promptly in cell swelling due to secondary water transport. Resulting cell swelling evokes an immediate secretory burst of hormones stored in secretory vesicles. Cell swelling induced *in vitro* by permeant molecules in isotonic medium stimulate prompt high amplitude hormone secretion from various type of cells

[4, 13-20], but mechanism of its effect inducing exocytosis remains unclear [8, 9]. Recently Best et al. [21] showed that glycerol and urea can activate beta-cells via their rapid uptake across the beta-cell plasma membrane, possibly via AQP7. This results in cell swelling, VRAC activation, electrical activity and insulin release. The secretory process induced by permeant molecules is similar to that induced by specific secretagogues in many respects, including response dynamics and refractory periods [22]. It has been believed, that ethanol and hypotonicity induce exocytosis via the same mechanism (for review see [23]). Our recent experiments indicate, however, that the mechanism of ethanol-induced insulin secretion in the tumor cells INS-1E differs from that induced by hypotonicity; INS-1E cells are able to release insulin in response to ethanol [20] but not to hypotonicity [9]. This is of special interest since hypotonicity induces membrane depolarization and electrical activity in these cells [24].

Insulin release is ultimately achieved when β -granules fuse with the plasma membrane. This process, called exocytosis, is mediated by a set of highly conserved membrane proteins known as SNARE proteins (SNAREs), [for soluble NSF attachment protein (SNAP) receptors] [25] which form the core of the fusion machinery [26]. β -cells contain several types of SNARE proteins including SNAP-25, syntaxin 1-3, VAMP-2 and synaptotagmin [27].

Glucose-induced insulin release is controlled by a balance between protein phosphorylation and dephosphorylation [28] and involves the activation of protein kinase C (PKC) [29]. Various PKC isoforms have been implicated in diverse cellular functions, including growth, differentiation, and apoptosis, as well as specialized responses, such as secretion and muscle contraction [30-32]. With respect to the effect on the Ca²⁺ signaling of β -cells induced by glucose stimulation, PKC appears to sensitize the secretory machinery to the Ca²⁺ responsible for increased insulin secretion [33-35]. On the other hand, the effect of PKC on the glucose-induced Ca²⁺ influx is still a matter of debate [36]. PKC is also involved in cell response to volume changes [37, 38]. Pancreatic β -cells express several PKC isoforms including PKC- α , - β , - δ , - ϵ , and - ζ [39]. Due to their great variety, it is difficult to predict the involvement of a particular isoform and its mode of action. Published data indicates that the association of PKC- ϵ with insulin granules is essential for insulin exocytosis [29].

On the other hand dephosphorylation can be mediated by protein tyrosine phosphatases (PTPs). PTPs

represent a large family of enzymes. The fact that one of PTPs (PTP1B) dephosphorylates NSF and elicits SNARE complex disassembly [40] suggests that a role of PTP in exocytosis of insulin containing secreting granule should be considered.

The aim of the present study was to compare some steps of the signaling pathway and exocytotic mechanism involved in the insulin secretion induced by ethanol and glucose. The role of extra- and intracellular Ca^{2+} in ethanol-induced insulin secretion from both types of cells was explored. In addition we tested the participation of protein kinase C (PKC) and protein tyrosine phosphatases (PTPs) using their inhibitors Bisindolylmaleimide VIII and ZnCl_2 respectively. The role of SNARE proteins in ethanol-induced exocytosis of insulin containing granules was tested by the application of inhibitors of SNARE protein complex formation tetanus toxin (TeTx) and N-ethylmaleimide (NEM).

Materials and Methods

Chemicals

Bisindolylmaleimide VIII (BIM), tetanus toxin (TeTx), N-ethylmaleimide (NEM) (all Sigma, Germany, BAPTA/AM ((1,2-Bis(2-aminophenoxy)ethane- N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester)) (Molecular Probes, Eugene, OR, USA), Dimethyl sulfoxide (DMSO) (Sigma, Germany) was used for dilution of BAPTA/AM.

Rat insulinoma derived β cells

Rat insulin-secreting cell lines INS-1 and INS-1E sensitive to glucose stimulation [41], were kind gift from Prof. Claes B. Wollheim (University Medical Center, Geneva, Switzerland). Cells were cultured in humidified atmosphere containing 5% CO_2 / 95% O_2 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.

Preparation of microcapsules

For the experiment shown in Figure 1 encapsulated cells were used. The cells were washed from culture medium (RPMI 1640 modified medium) in cold PBS buffer. They were gently dispersed in the filter-sterilized solution of 2 wt. % sodium alginate (Protanal LF 10/60, FMC BioPolymer, USA) in 0.9 wt. % NaCl, pH 7.4. The cells dispersion ($\sim 10^9$ cells/ml) was air-stripped by speed of 1.0 ml/min using the in-house made coaxial nozzle. The droplets were falling into 150 ml of filter-sterilized gelling solution consisting of 30 mM BaCl_2 in 0.9 wt.% NaCl with pH adjusted to 7.4. The time for microcapsule formation was 10 min. Microcapsules were washed in a few

Fig. 1. Effect of Ca^{2+} free medium and 10 μM BAPTA/AM on ethanol-induced insulin secretion from INS-1 and INS-1E cells. Encapsulated cells were incubated for two subsequent 30 min periods in medium containing alternately basal and stimulating medium (80 mM ethanol). Media without Ca^{2+} contained 1 mM EGTA with/without DMSO and BAPTA/AM. Results are shown as ng of insulin released by cells during 30 min incubation, mean $\pm$ SE, $n = 10$. *** $P < 0.001$ compared to previous (basal) incubation (paired t-test).

cycles in 0.9 wt. % NaCl and then kept in the cell culture medium (RPMI 1640 modified medium). The diameter of such prepared microcapsules was $\sim 900 \mu\text{m}$.

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

Incubations were performed in Eppendorf tubes at 37°C . Groups of 5 microcapsules per tube were preincubated for 60 min followed by the incubation for two subsequent 30 min periods in basal or stimulating media in final volume of 200 μl . When indicated, calcium free medium with 1 mM EGTA and/or 10 μM BAPTA/AM (a cellular Ca^{2+} scavenger) was present throughout the experiment. For solvent control, medium with 0.1% DMSO was used. At the end of each incubation period medium was saved for insulin radioimmunoassay.

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 stimulated for two subsequent 30 min period in KRBH and stimulating media as indicated. Insulin released into medium was measured by radioimmunoassay (RIA).

Perfusion of cells with TeTx

Tetanus toxin (20 nM) was present during cells preincubation in depolarization medium to induce depolarization and exocytosis followed by endocytosis thus ensure toxin transport into the cells. Control cells were preincubated in depolarization medium without toxin. After incubation with TeTx, cells were perfused for 1 h by glucose-free culture medium followed by five subsequent periods by scheme (30 min KRBH, 20 min ethanol, 20 min KRBH 20 min ethanol, 6 min KRBH), were performed in calcium containing or calcium depleted medium. The flow rate of media was set at 0.25 ml/min, and fractions were collected every minute from 1 to 96 minute of experiment. Insulin secretion was measured by RIA using rat insulin as a standard.

Incubation media

Glucose free medium: (292 mOsm/kg H_2O): 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, BSA (0.1%), pH 7.4.

Basal medium: Krebs-Ringer Hepes buffer (KRH): 133.75 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, 2.5 mM glucose, BSA (0.1%), pH 7.4.

Ethanol medium: This medium was prepared by ethanol addition to 80 mM concentration. To maintain physiological osmolarity, the NaCl concentration was reduced to 93.75 mM. Other components were the same as in the basal medium. Media without calcium ($-\text{Ca}^{2+}$) contained 1 mM EGTA.

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.

Depolarization medium: Plasma membrane-depolarizing medium contained 50 mM KCl, whereas NaCl concentration was reduced to 72 mM to maintain constant osmolarity. Other components were the same as in the basal medium.

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

Fig. 2. Effect of PKC inhibition on insulin secretion from INS-1 and INS-1E cells. Attached cells; after preincubation with or without Bisindolylmaleimide VIII (BIM) they were incubated in two subsequent 30 min period in basal and stimulating media according to scheme i) control group: basal medium → 15 mM glucose or 80 mM ethanol; ii) testing group: basal medium + 3 μM BIM → 15 mM glucose or 80 mM ethanol + 3 μM BIM. Results are shown as ng of insulin released by cells during 30 min incubation, mean $\pm$ SE, $n = 10$. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ compared to previous (basal) incubation (paired t-test); # $P < 0.05$, ## $P < 0.005$, ### $P < 0.001$ compared to basal incubation without BIM (unpaired t-test); ^^ $P < 0.005$ compared to ethanol incubation without BIM (unpaired t-test).

media on the antibody binding was included. 125I-monoiodoinsulin was prepared with the aid of lactoperoxidase [42]. 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 mol/l PBS (pH 7.4). After overnight incubation of samples with the antibody at 4°C, bound and

Fig. 3. Effect of SNARE proteins inhibition on insulin secretion induced by ethanol from INS-1 and INS-1E cells. INS-1 and INS-1E cells were divided into three groups; control was incubated for 30 min in 100 μ l depolarization medium and TeTx groups were incubated in the same medium with 20 nM tetanus toxin in presence/absence of calcium. After 60 min preincubation in 200 μ l glucose free medium cells were perfused at flow rate 0.25 ml/min with basal medium and stimulating ethanol medium with (TeTx + Ca^{2+}) or without 1.5 mM CaCl_2 (TeTx - Ca^{2+}) in scheme (30 min basal medium $\rightarrow$ 20 min ethanol $\rightarrow$ 20 min basal medium $\rightarrow$ 20 min ethanol). Calcium depleted medium contained 1 mM EGTA.

free peptides were separated by cold 200 μ l dextran coated charcoal (500 mg Norit +50 mg dextran in 100 ml H_2O). After 15 min centrifugation at 3000g, the radioactivity of pellet was measured by RIA multidetector counter (JNG-402, URVJT, Prague).

Data analysis

Results are shown as means $\pm$ SE. Data were compared by paired (for comparison with data from previous incubation) or unpaired Student's t-test where applicable and were considered significantly different at level $P < 0.05$.

Fig. 4. Effect of N-ethylmaleimide on insulin secretion from INS-1 and INS-1E cells. Cells after preincubation with or without N-ethylmaleimide (NEM) were incubated in two subsequent 30 min period in basal and stimulating media in scheme i) control group: basal medium $\rightarrow$ 15 mM glucose or 80 mM ethanol; ii) testing group: basal medium +5 mM NEM $\rightarrow$ 15 mM glucose or 80 mM ethanol +5 mM NEM. Results are shown as ng of insulin released by cells during 30 min incubation, mean $\pm$ SE, $n = 10$, *** $P < 0.001$ compared to previous (basal) incubation (paired t-test); ### $P < 0.001$ compared to basal incubation without NEM (unpaired t-test).

Results

In term of insulin secretion both INS-1 and INS-1E cell lines responded essentially the same way to all experimental interventions.

Extra- and Intracellular Calcium

Since it was not possible to test the responses of tumor cell lines in Ca^{2+} -depleted medium in static incubation, because cells do not adhere to the surface of

Fig. 5. Effect of PTP inhibition on insulin secretion from INS-1 and INS-1E cells. Cells after preincubation were incubated in two subsequent 30 min period in basal and stimulating media in scheme i) control group: basal medium → 15 mM glucose or 80 mM ethanol; ii) testing group: basal medium +10 μ mol/l ZnCl_2 → 15 mM glucose or 80 mM ethanol +10 μ mol/l ZnCl_2 . Results are shown as ng of insulin released by cells during 30 min incubation, mean $\pm$ SE, n = 10. ** $P < 0.005$, *** $P < 0.001$ compared to previous (basal) incubation (paired t-test); ## $P < 0.005$, ### $P < 0.001$ compared to basal incubation without ZnCl_2 (unpaired t-test).

plates under these conditions, we used encapsulated cells for this experiment. Depletion of calcium from extracellular medium did not affect ethanol-induced insulin secretion (Fig. 1). To specify role of the intracellular Ca^{2+} a membrane permeable Ca^{2+} chelator, BAPTA/AM was added to the calcium free, 1 mM EGTA containing medium. The stimulating effect of ethanol was not hindered by the addition of 10 μ M BAPTA/AM (Fig. 1).

Protein kinase C

Role of PKC was tested by the application of BIM (present in the medium with attached cells throughout

experiment, including preincubation). BIM at 3 μ M concentration increased basal insulin secretion. Stimulation by 15 mM glucose was completely abolished by BIM, while that induced by ethanol was not inhibited (Fig. 2).

SNARE complex

Tetanus toxin: Ethanol-induced insulin secretion was inhibited by preincubation with 20 nM tetanus toxin, (clostridial neurotoxin which cleaves SNARE proteins) if calcium was present in the medium. Tetanus toxin in calcium depleted medium (with 1mM EGTA) did not affect ethanol-induced insulin secretion (Fig. 3) neither from INS-1 or INS-1E cells.

N-ethylmaleimide: Another inhibitor of SNARE proteins NEM (present in the medium with attached cells throughout experiment, including preincubation) at 5 mM concentration induced high basal insulin secretion (Fig. 4) in both types of cells. Addition of glucose (Fig. 4) did not further increase insulin secretion. In contrast, high insulin secretion from NEM treated cells was further increased after ethanol stimulation (Fig. 4).

Protein tyrosine phosphatases and ZnCl_2 : ZnCl_2 , an inhibitor of PTPs, added into medium after preincubation in 10 μ M concentration induced high basal insulin secretion (Fig. 5). Glucose stimulation (Fig. 5) did not further increase insulin secretion in the presence of ZnCl_2 . In contrast, high insulin secretion from ZnCl_2 treated cells was further increased by ethanol stimulation (Fig. 5).

Discussion

Signaling

The stimulation of insulin secretion by glucose requires metabolism of the sugar by the β - cell and involves at least two mechanisms. The best characterized and major one serves to produce the triggering signal of exocytosis, a rise in the cytoplasmic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), thus inducing cascade of events [43-45]. Some studies showed the existence of a Ca^{2+} -independent pathway of regulation by glucose which can be induced in a Ca^{2+} -independent manner by poorly hydrolyzable GTPyS [46, 47]. While it is generally accepted that physiologically relevant ethanol concentrations increase intracellular calcium levels [48-50], effect of ethanol on inducing release of intracellular Ca^{2+} is not clear and may differ in various types of tissue [51]. On the one hand, ethanol induces release of intracellular Ca^{2+} from the microsomes in the absence of extracellular Ca^{2+} [52], on the other hand some reports have shown an inhibitory

effect of ethanol on voltage-gated calcium channels [53, 54].

Direct measurements of cell size revealed cell swelling in both cell lines after exposure to the ethanol [20]. The independence of cell swelling-induced insulin release from the extracellular Ca^{2+} has been repeatedly demonstrated [4, 7, 11], here we tested also importance of Ca^{2+} recruited from intracellular stores in ethanol induced insulin secretion from insulinoma cell lines. Since these cells do not adhere to the surface of plates in Ca^{2+} depleted medium, we used encapsulated cells for static incubations. Encapsulation has been intensively tested as a perspective tool for immunoisolation of transplanted pancreatic islets [55]. It was shown that encapsulation in alginate beads does not affect the insulin secretion profile *in vitro* [55, 56].

As supposed, Ca^{2+} depleted medium did not affect stimulating effect of ethanol. To eliminate the possibility of the intracellular Ca^{2+} involvement in the ethanol-induced insulin secretion, we used a membrane permeable Ca^{2+} chelator BAPTA/AM in combination with the calcium free medium. This treatment is very powerful [57] as confirmed by Ca^{2+} monitoring in INS-1E cells [7]. Again, no effect on ethanol-induced insulin secretion was noted. This characteristic is similar to that found in hypotonic stimulation of insulin secretion from rat pancreatic islets [7]. Similarly to hypotonic stimulation [7], neither PKC inhibition by Bisindolylmaleimide VIII prevented ethanol-induced insulin secretion in present study. We therefore conclude that like in hypotonic stimulation of natural β -cells, signaling pathway of the ethanol-induced insulin secretion in INS-1 and INS-1E cells does not involve extra- or intra-cellular Ca^{2+} or PKC. An increase of the basal insulin secretion from BIM treated tumor cell lines in present study is of interest. We had not seen this effect in native rat pancreatic islets [7]. We can only speculate about the reason for the difference; an effect of BIM interfering with regulation of exocytosis in tumor cells may take a place.

Exocytosis

Further part of our study was aimed at mechanism of exocytosis. Exocytotic secretion depends on the functioning of a mechanism involving membrane SNARE proteins. β -cells contain several types of SNARE proteins including SNAP-25, syntaxin 1-3, VAMP-2 and synaptotagmin [27]. SNARE proteins are proteolytic substrates for the clostridial neurotoxins [27], particularly their light chain subunit. We showed that ethanol-induced insulin secretion was blocked only in presence of Ca^{2+} in

the incubation medium. In Ca^{2+} free medium TeTx did not affect ethanol-induced secretion. It is likely that TeTx as a Zn^{2+} dependent metalloproteinase [58] was not active in the presence of chelator EGTA in the Ca^{2+} depleted medium. Hausinger et al. [59] observed similar results when EGTA and EDTA blocked proteolysis of synaptotagmin by endogenous protease. We therefore conclude that tetanus toxin sensitive SNARE proteins are involved in the ethanol-induced insulin secretion from INS-1 and INS-1E cells.

NEM added into medium induced high basal insulin secretion in both types of cells and the addition of glucose did not further increase insulin secretion. It is of special interest that high insulin secretion from NEM treated cells further increased after ethanol stimulation. We had received similar results after hypotonic stimulation of NEM treated rat pancreatic islets [8]. N-ethylmaleimide-sensitive-factor (NSF) and soluble NSF-attachment factor (α SNAP) are soluble proteins that act to disassemble SNARE complexes. They are sensitive to NEM [60], which blocks disassembly of complexes and their further participation in exocytosis [61]. Although at the beginning the numbers of vesicles available for exocytosis increases [62], subsequent steps are blocked by NEM [63]. This effect was apparently responsible for initial increase of basal secretion and subsequent absence of the response to glucose stimulation in presence of NEM in present experiments. Interestingly, despite high basal insulin secretion, cells were still able to release more insulin in response to ethanol. This additional stimulation suggests the presence of a separate pool of secretory granules available for ethanol-induced but not glucose-induced exocytosis after treatment. NEM in low concentration stimulates [64] and in concentration over 0.5 mM inhibits K/Cl co-transport [65] which is important for the regulatory volume decrease to occur. Therefore, it is likely that 5 mM NEM used in our experiments resulted in the inhibition of the RVD in swollen cells and increased the number of swollen secretory vesicles in the proximity of plasma membrane. NEM inhibits also V-type H^{+} ATPase [66] which keeps pH in secretory vesicles of β -cells close to or below 6 [67, 68]. Increase of pH resulting from enzyme inhibition is connected by changes of solubility of stored insulin and acceleration of insulin release [66]. We can speculate that these mechanisms are more likely to promote insulin secretion in swollen than in glucose stimulated cells. Alternatively, a novel NEM insensitive pathway of exocytosis can be involved after cell swelling.

ZnCl_2 was shown to be effective inhibitor of dephosphorylation of phosphotyrosyl residues [69].

We have received essentially the same results as with NEM after inhibition of protein tyrosine phosphatases (PTPs) by 10 $\mu\text{mol/l}$ ZnCl_2 . PTP1B dephosphorylates NSF, once dephosphorylated, NSF together with α -SNAP, disassembles *cis* SNARE complexes. Free SNAREs are then able to reassemble in *trans* complexes [40]. Again an increase of basal insulin secretion from ZnCl_2 treated cells was found. This secretion could be further increased by ethanol but not glucose stimulation. We suppose similar mechanism as after NEM treatment - a transient increase of number of vesicles available for exocytosis, subsequent steps are however blocked in glucose-stimulated cells. Further increase of insulin secretion after ethanol stimulation strengthens our hypothesis that an extra pool of secretory vesicles is available for exocytosis in this type of stimulation.

Although permeants and hypotonicity have been believed to induce exocytosis via the same mechanism - cell-swelling (for review see [23, 70]), sensitivity of ethanol induced insulin secretion to noradrenalin in both tumor cell lines in our recent study [20] indicates different properties of the mechanism of ethanol-induced insulin secretion in these cell lines compared to hypotonicity; therefore its mediation by permeant effect, at least in tumor cells, has been questioned [20]. Moreover, INS-1E cells do respond to ethanol but not to hypotonicity at basal conditions [9]. Simultaneous stimulation with hypotonicity in this cell line does not change secretory

response to ethanol (10 - 80 mM) in presence of Ca^{2+} (unpublished observation). On the other hand, present study demonstrates characteristics shared by hypotonic and ethanol stimulation which differs from that utilized by glucose: independence from calcium and bypassing PKC involvement [7], sensitivity to tetanus toxin but N-ethylmaleimide insensitivity [8].

In conclusion, present study showed that signaling of ethanol-induced insulin secretion from INS-1 and INS-1E cell lines bypasses calcium and PKC involving steps is sensitive to tetanus toxin but resistant to N-ethylmaleimide and ZnCl_2 . An extra pool of secretory vesicles not available for glucose is exploited for exocytosis after ethanol stimulation.

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