Different Signaling Pathways Involved in Glucose- and Cell Swelling-Induced Insulin Secretion by Rat Pancreatic Islets *in Vitro*

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Key Words

Insulin secretion • Glucose • Cell swelling • Signaling • Ca^{2+} • PKC • Noradrenaline

Abstract

Background: The objective was to compare signal transduction pathways exploited by glucose and cell swelling in stimulating insulin secretion. **Methods:** Isolated rat (Wistar) pancreatic islets were stimulated *in vitro* by 20 mmol/l glucose or 30% hypotonic medium (202 mOsm/kg) in various experimental conditions. **Results:** Glucose did not stimulate insulin release in calcium free medium. Cell swelling-induced insulin release in calcium free medium, even in the presence of the membrane permeable calcium chelator BAPTA/AM (10 $\mu\text{mol/l}$). Protein kinase C (PKC) inhibitor bisindolylmaleimide VIII (1 $\mu\text{mol/l}$) abolished the stimulation of insulin secretion by glucose but did not affect the swelling-induced insulin release. PKC activator phorbol 12-13-dibutyrate (1 $\mu\text{mol/l}$) stimulated insulin secretion in medium containing Ca^{2+} and did not potentiate insulin secretion stimulated by hypotonic extracellular fluid. Dilution of the medium (10-30%) had an additive effect on the glucose-induced insulin secretion. Noradrenaline (1 $\mu\text{mol/l}$)

abolished glucose-induced insulin secretion but did not inhibit hypotonic stimulation either in presence or absence of Ca^{2+} . **Conclusion:** Glucose- and swelling-induce insulin secretion through separate signal transduction pathways. Hypotonic stimulation is independent from both the extracellular and intracellular Ca^{2+} , does not involve PKC activation, and could not be inhibited by noradrenaline. These data indicate a novel signaling pathway for stimulation of insulin secretion exploited by cell swelling.

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Introduction

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 calcium voltage-gated channels increases Ca^{2+} entry and higher concentration of

intracellular calcium stimulates insulin release [1]. Much less is known about cell swelling-induced insulin secretion [2-7]. Both stimuli evoke a fast insulin release from β -pancreatic cells, nevertheless substantial differences indicate alternative signaling pathways leading to exocytosis following glucose and hyposmotic stimulus.

Besides the efflux of inorganic and organic osmolytes [8, 9] cell swelling evokes an immediate secretory burst of various hormones with the dynamics indistinguishable from those induced by specific secretagogues [7]. Extracellular Ca^{2+} is not required and in fact negatively modulates the osmotically-induced secretion as demonstrated in various types of secretory cells (for review see [7]) including pituitary cells [10], pancreatic islet cells [11, 12], cardiac myocytes which produce atrial natriuretic peptide [13], kidney cells which produce renin [14], neurons which produce GnRH and TRH [15, 16].

Glucose-induced insulin release is controlled by a balance between protein phosphorylation and dephosphorylation [17] and involves the activation of protein kinase C (PKC) [18]. Pancreatic β -cells express several PKC isoforms including PKC- α , - β , - δ , - ϵ , and - ξ [19, 20]. Due to great variety of isoforms, it is difficult to predict the involvement of a particular isoform and its mode of action. Recent data indicate that the association of PKC- ϵ with insulin granules is essential for insulin exocytosis [18]. Insulin secretion induced by known stimulators as glucose (both Ca^{2+} dependent and independent), KCl in presence of forskolin and 12-O-tetradecanoylphorbol or α latrotoxin could be inhibited by physiological inhibitors including noradrenaline [21-24].

The aim of the present study was to compare signaling pathways involved in the insulin secretion induced by cell swelling and glucose. We investigated the role of Ca^{2+} and PKC as well as the inhibitory effect of noradrenaline on both types of stimulations. Since two different mechanisms were reported to be activated in the stimulation of insulin secretion by hyposmotic shock in presence and absence of extracellular Ca^{2+} respectively [6], effects of all maneuvers were tested at both conditions.

Materials and Methods

Male Wistar rats (specific pathogens free, Charles River, Sulzfeld, Germany) were kept in a 12-h light-dark cycle in a temperature-controlled (22°C) animal room. The animal procedures were performed in accordance with national law and were approved by ethical committee.

Chemicals

Collagenase type XI, soybean Trypsin inhibitor type I-S, bisindolylmaleimide VIII (BIM), phorbol 12,13-dibutyrate (PDBu), noradrenaline (all Sigma, Germany), fura-2 acetoxymethylester (fura-2AM; Molecular Probes, Eugene, OR, USA), 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 PDBu and BAPTA/AM.

Rat insulinoma derived β cells

Rat insulin-secreting cell line INS-1E sensitive to glucose stimulation [25], kind gift from Dr. Claes B. Wolheim (University Medical Center, Geneva, Switzerland), were used for determination of the effect of BAPTA/AM on intracellular calcium changes. Cells were cultured in humidified atmosphere containing 5% CO_2 in complete medium composed of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 1 mmol/l sodium pyruvate, 50 $\mu\text{mol/l}$ 2-mercaptoethanol, 2 mmol/l glutamine, 25 mmol/l HEPES, 100 U/ml penicillin, and 100 $\mu\text{g/l}$ streptomycin.

Isolation of islets of Langerhans

Islets were isolated from adult male Wistar rats according to the method by Lacy and Kostianovsky [26]. 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

A) Basal medium. Krebs-Ringer Hepes buffer (KRH) (118 mmol/l NaCl, 4 mmol/l KCl, 1.2 mmol/l MgSO_4 , 1.2 mmol/l KH_2PO_4 , 5 mmol/l NaHCO_3 , 25 mmol/l HEPES, 3 mmol/l D-glucose, 2.5 mmol/l CaCl_2 , 0.1% BSA, pH 7.4. This medium was used for preincubation and non-stimulated incubation periods.

B) Stimulating media. *Hyposmotic medium:* 30% hyposmotic medium (202 mOsm/kg H_2O) was prepared by appropriate dilution of basal medium with deionized water. *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. *Depolarization medium:* Plasma membrane-depolarizing medium contained 50 mmol/l KCl, whereas NaCl concentration was reduced to 72 mmol/l to maintain constant osmolarity. Other components were the same as in the basal medium. Media without calcium ($-\text{Ca}^{2+}$) contained 1 mmol/l EGTA.

All media were gassed with 5% CO_2 /95% O_2 atmosphere for 30 min and pH was 7.4.

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 followed by the incubation for two or four subsequent 30 min periods in basal or stimulating media in final volume of 200 µl. In specified experiments 1 or 3 µmol/l BIM (an inhibitor of PKC), 1 µmol/l PDBu (an activator of PKC), 10 µmol/l BAPTA/AM (a cellular Ca²⁺ scavenger) or 1 µmol/l noradrenaline were present throughout the experiment. For solvent control, medium with 0.1% DMSO was used in the experiments with PDBu and BAPTA/AM. At the end of each incubation period medium was saved for insulin radioimmunoassay.

Insulin extraction from pancreatic islets

At the end of each experiment, 250 µl 0.1 mol/l ascorbic acid was added to the pancreatic islets and sonicated. The samples were then centrifuged at 12 000 rpm (4°C) for 10 minutes. After addition of 50% methanol to the sediment and vortexing, samples were centrifuged at the same conditions. The supernatants from the first and second centrifugations were pooled and lyophilized. The lyophilizates were kept at -20°C and reconstituted in the assay buffer on the day of RIA (described below). The insulin standards for the calibration curve were treated in the same way.

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. 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. Radioactive [¹²⁵I]-insulin was prepared in our Institute (IEE SAS, Bratislava) [27]. 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 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 pellets was measured by RIA multidetector counter JNG-402, URVJT, Prague).

Fluorescent [Ca²⁺]_i measurements

Intracellular Ca²⁺ of individual cells was monitored with a photometric system (TILL Photonics, USA). This method was described elsewhere [28]. Briefly, cells were loaded with 1 µM fura-2 acetoxymethylester (fura-2AM) for 30 min and subsequently washed for 30 min at room temperature. Then these cells were transferred to a recording chamber where recordings at pair wavelengths of 360 and 380 nm were collected every 200 ms. The fluorescence signals were acquired on an inverted microscope (Olympus IX70, Japan) using a dry ×40 objective (NA=0.55), attached with Photometric devices from TILL Photonics, USA. In all experiments, the signals were averaged over whole cell. As calibration of fluorescence into

absolute [Ca²⁺]_i values can be subject to artefactual inaccuracies [29], data are presented as ratio signals (F₁/F₀). The excitation light waves were adjusted by monochromator Polychrome IV (TILL Photonics, USA) operating with EPC9 amplifier and Pulse/XChart software (HEKA Electronics, Germany).

Data analysis

Results are shown as mean ± SE. Data were compared by Student's t-test and for multiple comparisons one way ANOVA for repeated measurements was used and if normality test failed Friedman Repeated Measures Analysis of Variance on Ranks was applied. Differences between groups were considered statistically significant when P values were less than 0.05.

Results

To specify a role of the Ca²⁺ in the glucose- and hyposmolarity-induced insulin secretion 10 µmol/l BAPTA/AM, a membrane permeable Ca²⁺ chelator, was added to the calcium free and 1 mmol/l EGTA containing medium. This treatment was proved to be very effective in reversibly decreasing of the intracellular Ca²⁺ during hyposmolar stimulation as shown by microfluorescent measurements on the INS-1E cell line (Fig. 1). Plasma membrane depolarizing KCl concentration in Ca²⁺ containing medium induced immediate increase of intracellular Ca²⁺. When Ca²⁺ free hypotonic medium was applied, transient decrease of intracellular Ca²⁺ was demonstrated (CaO HYPO). BAPTA added to this medium induced further deep reversible decrease of intracellular Ca²⁺ (Fig. 1).

Glucose in 20 mmol/l concentration was a powerful stimulator of insulin secretion from freshly isolated pancreatic islets in the presence of Ca²⁺ (Fig. 2A) but not in a calcium free medium either with or without BAPTA/AM. In contrast, clear effect of hyposmotic (30%) stimulation on insulin release was found in both calcium free and in the calcium containing medium (Fig. 2B). The stimulating effect was not hindered by the addition of 10 µmol/l BAPTA/AM. A decrease of insulin release in the presence of 20 mmol/l glucose compared to basal secretion in Ca²⁺ free media (Fig. 2A) was noted.

BIM, an inhibitor of PKC, was used to test a PKC role in cell swelling- and glucose-induced insulin secretion. Stimulation of insulin secretion by 20 mmol/l glucose was completely abolished by 1 µmol/l BIM (Fig. 3A). Hypotonic medium-induced insulin release was not inhibited by 1 µmol/l (not shown) or 3 µmol/l BIM either in the presence or the absence of Ca²⁺ (Fig. 3B) in the

Fig. 1. Changes of intracellular Ca^{2+} during various stimulations. Relative change of the level of intracellular Ca^{2+} is presented as ratio of fluorescence (F_1/F_0). INS-1E cells were incubated in basal medium containing Ca^{2+} , stimulating medium containing 50 mmol/l KCl (KCl) at the time indicated by arrow, after recovery in basal medium, hypotonic (30%) medium without Ca^{2+} containing 1 mmol/l EGTA was applied (CaO Hypo, marked by horizontal line), after another recovery period CaO Hypo medium containing 10 $\mu\text{mol/l}$ BAPTA/AM (BAPTA) was applied for given period (120 sec, marked by horizontal line) and thereafter replaced again with basal, Ca^{2+} containing medium. KCl (causing membrane depolarization used routinely as a non-specific control stimulus to induce hormone secretion) induced immediate increase of intracellular Ca^{2+} . When CaO HYPO medium was applied, a decrease of intracellular Ca^{2+} was demonstrated. When BAPTA was added to CaO HYPO medium further deep reversible decrease of intracellular Ca^{2+} was induced.

Fig. 2. Effect of Ca^{2+} free medium and 10 $\mu\text{mol/l}$ BAPTA/AM on stimulated insulin secretion from pancreatic islets. Islets (5 per well) were incubated for four subsequent 30 min periods in medium containing alternately basal and stimulating medium (A: 20 mmol/l glucose, B: 30% hypotonicity). Media without Ca^{2+} contained 1 mmol/l EGTA and effect of solvent (DMSO) and BAPTA/AM (BAPTA) present in both basal and stimulating media was tested. Data are shown as mean $\pm$ SE, $n=10$. Part A: The stimulating effect of 20 mmol/l glucose ($***P<0.001$) was prevented in Ca^{2+} free media. A decrease of insulin release in presence of 20 mmol/l glucose in Ca^{2+} free medium was confirmed by Friedman Repeated Measures Analysis of Variance on Ranks in all experimental groups e.g. without DMSO ($*P<0.05$), with DMSO ($***P<0.001$) and with BAPTA dissolved in DMSO ($*P<0.05$). Part B: The stimulating effect of 30% hypotonic medium (hypo) was not diminished in Ca^{2+} free media with or without BAPTA. One way ANOVA for repeated measurement revealed significant effect of hypotonicity at each condition ($***P<0.001$).

medium. The presence of BIM in the medium did not affect insulin content when analyzed after incubation in Ca^{2+} free condition (127.0 ± 7.6 ng insulin/ islet, + BIM

125.9 ± 11.3 ng insulin/ islet) or with Ca^{2+} (128.9 ± 7.5 ng insulin/ islet, + BIM 132.8 ± 11.9 ng insulin/ islet, $n=10$ in all groups).

Fig. 3. A: Effect of 1 $\mu\text{mol/l}$ BIM (an inhibitor of PKC) on glucose-induced insulin secretion from pancreatic islets. Islets (5 per well) were incubated for two subsequent 30 min periods in alternately basal and stimulating medium. Data are shown as mean $\pm$ SE, $n=6$. The stimulating effect of 20 mM D-glucose ($P<0.01$) was prevented by BIM. B: Effect of 3 $\mu\text{mol/l}$ BIM on cell swelling-induced (30% hypotonic medium, hypo) insulin secretion from pancreatic islets. Islets (5 per well) were incubated for two subsequent 30 min periods in medium containing alternately basal and stimulating medium. Media without Ca^{2+} contained 1 mmol/l EGTA. Data are shown as mean $\pm$ SE, $n=15$. The stimulating effect of hypotonic medium ($*P<0.005$) was not prevented by BIM.

The presence of a PKC activator, 1 $\mu\text{mol/l}$ PDBu, in a Ca^{2+} containing basal medium stimulated insulin secretion (Fig. 4); the change after simultaneous hyposmotic stimulation did not reach statistical significance. PDBu had no effect on basal or cell swelling-induced insulin secretion in the absence of Ca^{2+} (Fig. 4).

When both stimuli – the glucose and hyposmolarity were applied together, their effect on insulin release was additive (Fig. 5). Noradrenaline abolished glucose-induced insulin secretion but not that induced by hypotonic medium (Fig. 6). KCl stimulation at the end of experiment proved that tissue was in good condition after both types of stimulation. Noradrenaline was unable to inhibit hypotonic medium-induced insulin secretion either in the presence or in the absence of Ca^{2+} in the medium (Fig. 7). When combined stimulation was applied, only part induced by the glucose was inhibited (Fig. 8).

Discussion

Hypotonic medium induces cell swelling and release of various hormones [7]. 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 dilution is made with aqueous 5% mannitol to maintain isosmolarity on both sides of the plasma membrane [30], or by biologically inactive L-glucose [16, 31], no secretion is induced. Similarly the insulin response did not occur if the reduction in NaCl was compensated by the addition of an equivalent osmolar amount of sorbitol or choline chloride [2, 6].

Several signaling pathways involved in the insulin secretion were described. The conventional glucose-induced insulin secretion is Ca^{2+} dependent. Although the

Fig. 4. Effect of 1 μ mol/l PDBu (a PKC activator) on swelling-induced (30% hypotonic medium, hypo) insulin secretion in presence (+Ca²⁺) or absence (-Ca²⁺) of Ca²⁺. Islets (5 per well) were incubated for two subsequent 30 min periods in medium containing alternately basal and stimulating medium. Data are shown as mean $\pm$ SE, n=10. The stimulating effect of hypotonic medium without Ca²⁺ (*P<0.05) was not changed. The PDBu increased basal secretion (#P<0.001) compared to basal media in control and DMSO groups with Ca²⁺. PDBu solvent (DMSO) did not affect the results.

Fig. 5. Additive effect of glucose and hypotonicity (hypo). Islets (5 per well) were incubated for two subsequent 30 min periods in medium containing alternately basal and stimulating medium (Ca²⁺ was present). Data are shown as mean $\pm$ SE, n=8. The stimulating effect of glucose and hypotonic medium (*P<0.005) was additive.

independence of cell swelling-induced insulin release from the extracellular Ca²⁺ has been repeatedly demonstrated [2, 3, 6], a role of Ca²⁺ recruited from intracellular stores remained unclear. Cell swelling was shown to increase intracellular Ca²⁺ in Ca²⁺ containing but not in Ca²⁺ free extracellular medium [7]. Here we showed even transient decrease of the intracellular Ca²⁺ of INS-1E cells in Ca²⁺-deprived medium after hypotonic stimulation. To eliminate the possibility of the intracellular Ca²⁺ involvement in the cell swelling-induced insulin secretion, we used a powerful membrane permeable Ca²⁺ chelator BAPTA/AM in combination with the calcium free medium. This treatment is very powerful [32] as confirmed by Ca²⁺ intracellular monitoring on INS-1E cell line in present experiments. As expected high KCl causing membrane depolarization used as a non-specific control stimulus of hormone

secretion induced immediate increase of intracellular Ca²⁺ in Ca²⁺ containing medium.

As expected, 20 mmol/l glucose-stimulated insulin secretion from pancreatic islets incubated in the presence of Ca²⁺ but not in Ca²⁺-deprived conditions. In contrast, the hypotonic stimulation of insulin release was pronounced in a calcium free medium and the addition of BAPTA/AM to the medium did not have any inhibitory effect. It is therefore concluded, that cell swelling-induced insulin secretion is independent of both extracellular and intracellular Ca²⁺ and that a signaling pathway involved in this process bypasses Ca²⁺. An inhibiting effect of 20 mmol glucose on insulin secretion in Ca²⁺ free media noted in this experiment was unexpected finding, this phenomenon undergoes further analysis in our laboratory.

The glucose stimulation of insulin release in the

Fig. 6. Effect of 1 μ mol/l noradrenaline (NA) on insulin secretion from pancreatic islets. Islets (5 per well) were incubated for four subsequent 30 min periods in medium containing alternately basal and stimulating medium (Ca^{2+} was present). Data are shown as mean $\pm$ SE, $n=5$. In contrast to 20 mM glucose the stimulating effect of hypotonic (hypo) medium ($*P<0.05$) was not hindered. KCl stimulation at the end of experiment proved that tissue was in good condition after both types of stimulation.

Fig. 7. Comparison of the effect of 1 μ mol/l noradrenaline (NA) on cell swelling-induced (30% hypotonic medium) insulin secretion from pancreatic islets in Ca^{2+} free (30% hypo + NA - Ca^{2+}) or Ca^{2+} containing (30% hypo + NA + Ca^{2+}) medium. Islets (5 per well) were incubated for two subsequent 30 min periods in medium containing alternately basal and stimulating medium. Data are shown as mean $\pm$ SE, $n=5$. The stimulating effect of hypotonic medium ($*P<0.005$) was not different in Ca^{2+} free medium.

absence of extracellular Ca^{2+} requires specific conditions: simultaneous activation of PKC, PKA as well as glucose metabolism in the *in vitro* system [22]. PKC is also involved in cell response to volume changes [33, 34] and in carbachol-stimulated insulin release [35]. It was therefore of much interest to test an involvement of PKC in the cell swelling-induced insulin secretion. The presence of BIM (potent, selective inhibitors of all PKCs) in the medium abolished glucose-induced insulin secretion from the islets in our experiments thus showing that glucose-stimulated insulin release in the presence of Ca^{2+} requires PKC activation. The involvement of PKC in glucose-stimulated insulin release has been recently reported [36, 37]. The PKC activator PDBu (activates all isoforms except α PKCs, Ca^{2+} independent) stimulated insulin secretion in basal Ca^{2+} containing medium but had no

effect in the absence of Ca^{2+} . We conclude that only Ca^{2+} dependent PKC isoforms are involved in signaling inducing insulin secretion. In both the presence and the absence of Ca^{2+} in the medium, non-specific PKC inhibitor BIM did not inhibit cell swelling-induced insulin secretion. Similarly, PDBu had no effect on the cell swelling-induced insulin secretion in the absence of Ca^{2+} . These results indicated that in contrast to glucose stimulation, PKCs are not involved in the hypotonic medium-induced insulin secretion.

Stimulation by 20 mmol glucose or 30% hyposmolarity induces similar increase of insulin secretion in our system (Figs. 2-5). The hypotonic medium had an additive effect on the glucose-induced insulin secretion (Fig. 5) suggesting possible involvement of different pools of secretory granules. This was not confirmed in

Fig. 8. Effect of 1 μ M noradrenaline (NA) on insulin secretion from pancreatic islets induced by glucose or combined stimulation (glucose + hypotonic medium, hypo). Islets (5 per well) were incubated for two subsequent 30min periods alternately in basal and stimulating medium. Ca²⁺ was present in all media. Data are shown as mean $\pm$ SE, n=10. The stimulating effect of combined stimulation was only partially inhibited (*P<0.05).

experiments with PDBu (Fig. 4) where simultaneous stimulation with hypotonic medium did not induce statistically significant increase. It is likely that in this experiment stimulation induced by PDBu might have been already maximal. If this was the reason, it indicates utilization of the same pool of secretory vesicles.

Noradrenaline is physiological inhibitor of glucose-induced insulin secretion [1]. An alternative signaling pathway leading to insulin secretion independently from Ca²⁺ can be induced by black-widow spider neurotoxin α -latrotoxin [23]. However, this and other types of Ca²⁺ independent stimulation of insulin secretion are inhibited by catecholamines [21-24]. Here we have clearly demonstrated failure of noradrenaline to inhibit cell swelling-induced insulin secretion either in the presence or the absence of Ca²⁺ in the medium (Fig. 6-7). When noradrenaline was applied during combined stimulation by glucose and hypotonic medium, its inhibitory effect was only partial (Fig. 8). Apparently only the secretion induced by glucose but not that induced by the cell swelling was inhibited. This indicates that different signaling pathways were used in parallel for glucose and hypotonicity during combined stimulation in Ca²⁺ containing medium and that swelling affects insulin secretory pathway at a more distal step of the cascade.

It has already been shown that signaling pathway involved in the cell swelling-induced insulin secretion does not include extracellular Ca²⁺ [2, 6, 7], stretch activated channels [5], Cl⁻ channels [38], Na⁺ and K⁺ channels, microtubules or microfilaments, cAMP, IP₃ or prostaglandins (for review see [7]). Our present experiments demonstrate the independence of swelling-induced insulin release from the intracellular Ca²⁺ and PKC as well as its resistance to noradrenaline inhibition.

The basic mechanism of cell swelling-induced hormones secretion is rather general with only limited specificity for hormones involved in water and salt regulation [5]. Moreover it acts at a distal end of the signaling pathway. We have hypothesized that cell swelling triggered exocytosis of the insulin secretion involves a direct biophysical effect of the osmotic gradient on secretory vesicles [39]. The swelling of secretory vesicles has been implicated in exocytosis [40] and its role in secretory process is receiving support by the recent finding that vesicle swelling potentiates its fusion with the plasma membrane, and is required for expulsion of intravesicular contents [41, 42].

Two distinct mechanisms of hyposmotic shock stimulated insulin secretion have been reported to be utilized in the presence or the absence of extracellular Ca²⁺ respectively in betaHC9 cells [6]. We did not see this dichotomy in freshly isolated rat pancreatic islets in present experiments. We believe that finding of Straub et al [6] is specific for at least some cell line(s) similarly as special requirements of extracellular Ca²⁺ in swelling induced hormone release was reported for pituitary tumor cell lines [43].

The physiological significance of cell swelling in insulin control is not yet clear. Besides relative anisomolarity, cell swelling and insulin secretion can be also induced by other mechanisms including permeant presence in isosmolar extracellular fluid [3, 4, 7]. Minor osmotic changes occurring in daily life might participate in regulation of hormone secretion. Moreover, hypertonicity of extracellular fluid could suppress secretion [44, 45] of insulin in dehydrated organism and thus aggravate pathophysiological changes in type 2 diabetes. When tissue becomes ischemic there is a shift of the cells to anaerobic glycolysis and production of metabolites which can increase the intracellular osmolarity by 70 and 130 mOsmol/l after 15 and 60 minutes, respectively, thus increasing transmembrane osmotic pressure differences and producing cell swelling [46]. Uncontrolled release of the hormone could have pathophysiological implications.

Possibility to induce or inhibit insulin secretion by drugs affecting cell volume regulating mechanisms may represent powerful tool for research, diagnostic and even therapy in the future.

In conclusion, we demonstrated that glucose and cell swelling induce insulin secretion through separate signal transduction pathways. In contrast to glucose, hypotonic medium-induced stimulation of insulin release is independent from both extracellular and intracellular Ca^{2+} , does not involve PKC, and is not inhibited by noradrenaline. These data indicate a novel signaling pathway for stimulation of insulin secretion exploited by cell swelling.

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References

- 1 Straub SG, Sharp GW: Glucose-stimulated signaling pathways in biphasic insulin secretion. *Diabetes Metab Res Rev* 2002;18:451-463.
- 2 Blackard WG, Kikuchi M, Rabinovitch A, Renold AE: An effect of hyposmolarity on insulin release in vitro. *Am J Physiol* 1975;228:706-713.
- 3 Benicky J, Nikodemova M, Scsukova S, Zorad S, Strbak V: Four-week ethanol drinking increases both thyrotropin-releasing hormone (TRH) release and content in rat pancreatic islets. *Life Sci* 2000;66:629-639.
- 4 Najvirtova M, Baqi L, Kuceroval J, Strbak V: Cell swelling induced secretion of TRH by posterior pituitary, hypothalamic paraventricular nucleus and pancreatic islets: effect of L-canavanine. *Cell Mol Neurobiol* 2002;22:35-46.
- 5 Najvirtova M, Greer SE, Greer MA, Baqi L, Benicky J, Strbak V: Cell volume induced hormone secretion: studies on signal transduction and specificity. *Cell Physiol Biochem* 2003;13:113-122.
- 6 Straub SG, Daniel S, Sharp GW: Hyposmotic shock stimulates insulin secretion by two distinct mechanisms. Studies with the betaHC9 cell. *Am J Physiol Endocrinol Metab* 2002;282:E1070-E1076.
- 7 Strbak V, Greer MA: Regulation of hormone secretion by acute cell volume changes: Ca^{2+} -independent hormone secretion. *Cell Physiol Biochem* 2000;10:393-402.
- 8 Wehner F, Olsen H, Tinel H, Kinne-Saffran E, Kinne RKH: Cell volume regulation: osmolytes, osmolyte transport, and signal transduction. *Rev Physiol Biochem Pharmacol* 2003;148:1-80.
- 9 Junankar PR, Karjalainen A, Kirk K: Osmotic swelling activates two pathways for K^{+} efflux in a rat hepatoma cell line. *Cell Physiol Biochem* 2004;14:143-154.
- 10 Greer MA, Greer SE, Maruta S: Hyposmolar stimulation of secretion of thyrotropin, prolactin, and luteinizing hormone does not require extracellular calcium and is not inhibited by colchicine, cytochalasin B, ouabain, or tetrodotoxin. *Proc Soc Exp Biol Med* 1990;193:203-209.
- 11 Lund PE, Berts A, Hellman B: Stimulation of insulin release by isosmolar addition of permeant molecules. *Mol Cell Biochem* 1992;109:77-81.
- 12 Benicky J, Greer MA, Strbak V: Hyposmolar medium and ethanol in isosmotic solution induce the release of thyrotropin-releasing hormone (TRH) by isolated rat pancreatic islets. *Life Sci* 1997;60:865-872.
- 13 Greenwald JE, Apkon M, Hruska KA, Needleman P: Stretch-induced atriopeptin secretion in the isolated rat myocyte and its negative modulation by calcium. *J Clin Invest* 1989;83:1061-1065.
- 14 Skott O: Calcium and osmotic stimulation in renin release from isolated rat glomeruli. *Pflugers Arch* 1986;406:485-491.
- 15 Inukai T, Wang X, Greer SE, Greer MA: Cell swelling induced by medium hyposmolarity or isosmolar urea stimulates gonadotropin-releasing hormone secretion from perfused rat median eminence. *Brain Res* 1992;599:161-164.
- 16 Nikodemova M, Greer MA, Strbak V: Hypo-osmolarity stimulates and high sodium concentration inhibits thyrotropin-releasing hormone secretion from rat hypothalamus. *Neuroscience* 1999; 88:1299-1306.
- 17 Ammala C, Eliasson L, Bokvist K, Berggren PO, Honkanen RE, Sjöholm A, Rorsman P: Activation of protein kinases and inhibition of protein phosphatases play a central role in the regulation of exocytosis in mouse pancreatic beta cells. *Proc Natl Acad Sci U S A* 1994;91:4343-4347.
- 18 Mendez CF, Leibiger IB, Leibiger B, Hoy M, Gromada J, Berggren PO, Bertorello AM: Rapid association of protein kinase C-epsilon with insulin granules is essential for insulin exocytosis. *J Biol Chem* 2003;278:44753-44757.
- 19 Kaneto H, Suzuma K, Sharma A, Bonner-Weir S, King GL, Weir GC: Involvement of protein kinase C beta 2 in c-myc induction by high glucose in pancreatic beta-cells. *J Biol Chem* 2002;277:3680-3685.
- 20 Tian YM, Urquidí V, Ashcroft SJ: Protein kinase C in beta-cells: expression of multiple isoforms and involvement in cholinergic stimulation of insulin secretion. *Mol Cell Endocrinol* 1996;119:185-193.
- 21 Sharp GW: Mechanisms of inhibition of insulin release. *Am J Physiol Cell Physiol* 1996;271:C1781-C1799.
- 22 Komatsu M, Shermehorn T, Aizawa T, Sharp GW: Glucose stimulation of insulin release in the absence of extracellular Ca^{2+} and in absence of any increase in intracellular Ca^{2+} in rat pancreatic islets. *Proc Nat Acad Sci USA* 1995;92:10728-10732.

- 23 Lang J, Ushkaryov, Grasso A, Wollheim CB: Ca^{2+} -independent insulin exocytosis induced by α -latrotoxin requires latrophilin, a G protein-coupled receptor. *The EMBO Journal* 1998;17:648-657.
- 24 Cheng H, Straub SG, Sharp GWG: Protein acylation in the inhibition of insulin secretion by norepinephrine, somatostatin, galanin, and PGE_2 . *Am J Physiol Endocrinol Metab* 2003;285:E287-E294.
- 25 Merglen A, Theander S, Rubi B, Chaffard G, Wollheim CB, Maechler P: Glucose sensitivity and metabolism-secretion coupling studied during two-year continuous culture in INS-1E insulinoma cells. *Endocrinology* 2004;145:667-678.
- 26 Lacy PE, Kostianovsky M: Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes* 1967;16:35-39.
- 27 Zorad S, Svabova E, Klimes I, Macho L: Comparison of radiochemical purity and tissue binding of labeled insulin prepared by lactoperoxidase and chloramine T iodination. *Endocr Exper* 1985;19:267-275.
- 28 Zaika OL, Pochynyuk OM, Kostyuk PG, Yavorskaya EN, Lukyanetz EA: Acetylcholine-induced calcium signalling in adrenaline- and noradrenaline-containing adrenal chromaffin cells. *Arch Biochem Biophys* 2004;424:23-32.
- 29 Duchon MR: Monitoring neuronal activity, A practical approach; Stamford JA (Eds.) Oxford University Press, Oxford, 1992, pp.231-260.
- 30 Greer MA, Greer SE, Opsahl Z, McCafferty L, Maruta S: Hyposmolar stimulation of in vitro pituitary secretion of luteinizing hormone: a potential clue to the secretory process. *Endocrinology* 1983;113:1531-1533.
- 31 J Benicky' and V Strbak: Glucose stimulates and insulin inhibits release of pancreatic TRH *in vitro*. *Eur J Endocrinol* 2000;142:60-65.
- 32 Boudreault F, Grygorczyk R: Cell swelling-induced ATP release is tightly dependent on intracellular calcium elevations. *J Physiol* 2004;561:499-513.
- 33 Jakab M, Furst J, Gschwentner M, Botta G, Garavaglia ML, Bazzini C, Rodighiero S, Meyer G, Eichmueller S, Woll E, Chwatal S, Ritter M, Paulmichl M: Mechanisms sensing and modulating signals arising from cell swelling. *Cell Physiol Biochem* 2002;12:235-258.
- 34 Gong W, Xu H, Shimizu T, Morishima S, Tanabe S, Tachibe T, Uchida S, Sasaki S, Okada Y: CIC-3-independent, PKC-dependent activity of volume-sensitive Cl channel in mouse ventricular cardiomyocytes. *Cell Physiol Biochem* 2004;14:213-224.
- 35 Tang SH, Sharp GW: Atypical protein kinase C isozyme ζ mediates carbachol-stimulated insulin secretion in RINm5F cells. *Diabetes* 1998;47:905-912.
- 36 Hashimoto N, Kido Y, Uchida T, Matsuda T, Suzuki K, Inoue H, Matsumoto M, Ogawa W, Maeda S, Fujihara H, Ueta Y, Uchiyama Y, Akimoto K, Ohno S, Noda T, Kasuga M: PKC λ regulates glucose-induced insulin secretion through modulation of gene expression in pancreatic beta cells. *J Clin Invest* 2005;115:138-145.
- 37 Yang Y, Gillis KD: A highly Ca^{2+} -sensitive pool of granules is regulated by glucose and protein kinases in insulin-secreting INS-1 cells. *J Gen Physiol* 2004;124:641-651.
- 38 Kinard TA, Goforth PB, Tao Q, Abood ME, Teague J, Satin LS: Chloride channels regulate HIT cell volume but cannot fully account for swelling-induced insulin secretion. *Diabetes* 2001;50:992-1003.
- 39 Strbak V, Benicky J, Greer SE, Bacova Z, Najvirtova M, Greer MA: Cell swelling-induced peptide hormone secretion, in: *Cell Volume and Signal Transduction*, Lauf PK, Adragna NC (eds), Advances in Experimental Medicine and Biology, New York, Springer Science + Business Media, 2004, vol 559, pp.325-330.
- 40 Finkelstein A, Zimmerberg J, Cohen FS: Osmotic swelling of vesicles: Its role in the fusion of vesicles with planar phospholipid bilayer membranes and its possible role in exocytosis. *Ann Rev Physiol* 1986;48:163-174.
- 41 Kelly ML, Cho WJ, Jeremic A, Abu-Hamdah R, Jena BP: Vesicle swelling regulates content expulsion during secretion. *Cell Biol Int* 2004;28:709-716.
- 42 Tsuboi T, Kikuta T, Sakurai T, Terakawa S: Water secretion associated with exocytosis in endocrine cells revealed by micro forcemetry and evanescent wave microscopy. *Biophys J* 2002;83:172-183.
- 43 Sato N, Murakami M, Wang X, Greer MA: The contrasting role of calcium influx in secretion induced by cell swelling can differentiate normal and tumor-derived rat pituitary cells. *Endocrinology* 1991;129:2541-2546.
- 44 Sato N, Wang X, Greer MA: Medium hyperosmolarity depresses TRH-induced Ca^{2+} influx and prolactin secretion in GH4C1 cells. *Mol Cell Endocrinol* 1991;77:193-198.
- 45 Wang X, Sato N, Greer MA: Medium hyperosmolarity inhibits prolactin secretion induced by depolarizing K^+ in GH4C1 cells by blocking Ca^{2+} influx. *Mol Cell Endocrinol* 1992;83:79-84.
- 46 Wright AR, Rees SA: Targeting ischaemia - cell swelling and drug efficacy. *Trends Pharmacol Sci* 1997;18:224-228.