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Inhibitory effect of ODN, a naturally occurring processing product of diazepam binding inhibitor, on secretagogues-induced insulin secretion

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Abstract

Diazepam binding inhibitor (DBI_{1-86}) is a peptide that is present in large amounts in the intestine and pancreas and which inhibits glucose-stimulated insulin release from both perfused pancreas and isolated islets in low nanomolar concentrations. Here, DBI_{33-50} (also known as ODN, octadecaneuropeptide), one of the naturally occurring processing products of DBI_{1-86}, and certain synthetic modified derivatives, have been shown to inhibit glucose and glibenclamide-stimulated insulin secretion from isolated rat islets and glibenclamide-stimulated insulin secretion from hamster-insulinoma (HIT-T15) β-cell line. DBI_{17-50} (TTN; triakontatetraneuropeptide), another prominent processing product of DBI, had no effect. The 50% inhibitory concentration (IC_{50}) for the effect of ODN on insulin secretion induced by 8.3 or 16.7 mM glucose was approximately the same: 5 to 6 nM. Moreover, ODN inhibited insulin release induced by 0.01 or 1 μM glibenclamide with a similar IC_{50} (8 to 10 nM) in both isolated pancreatic islets and in HIT-T15 β-cells. At concentration up to 1 μM, ODN had no effect on insulin secretion induced by PACAP (pituitary adenylate cyclase polypeptide), BAYK 8644 (methyl-(1,4-dihydro-2,6-dimethyl-3-nitro-4,2-trifluoromethylphenyl)pyridine-5-carboxylate), and only marginally it affected IBMX-(isobutylmethylxanthine) induced insulin secretion. This indicates that ODN does not act directly on ATP-regulated K^+ channels, voltage dependent Ca^{2+} channels or cAMP production. In contrast, ODN inhibited insulin secretion induced by sodium nitroprussiate in a manner that is independent from the presence of extracellular Ca^{2+}. These results suggest that ODN or ODN-like peptide fragments of DBI, may inhibit glucose or glibenclamide-induced insulin secretion via a signaling pathway that regulate the cytoplasmic free Ca^{2+} concentration.

Keywords: Octadecaneuropeptide; Insulin secretion; Pancreatic islet; HIT-T15 β-cell

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1. Introduction

Secretion of insulin from pancreatic β cells is controlled by nutrients in the circulation, classical neurotransmitters released by the autonomic nervous system, and peptides. Such regulatory peptides include glucagon-like peptide-$1_{(7-37)}$ [1] and glucose-dependent insulinogenic peptide [2], which are derived from the intestine, as well as peptides secreted from cells or nerve fibers of the pancreas [3]. Thus, cholecystokinin [3,4], vasoactive intestinal polypeptide [3,4], and pituitary adenylate cyclase polypeptide (PACAP) [5] have been localized to nerve fibers in the pancreas and stimulate insulin release; whereas somatostatin, galanin, neuropeptide Y [3,4], and diazepam binding inhibitor (DBI) [4,7–11], which have been localized in the parenchyma or nerve fibers of the endocrine pancreas and the intestine, decrease glucose-induced insulin secretion.

Among the peptides that inhibit glucose-induced insulin secretion, DBI appears of particular interest. Immunohistochemical studies with antisera to DBI have shown that DBI is present in considerable amounts in the endocrine pancreas (α cells in rat, δ cells in human and pig), as well as in epithelial cells of the exocrine pancreas and intestine [7,8,10]. At low concentrations ($\leq 10^{-8}$ M), DBI inhibits glucose-induced insulin secretion from perfused rat pancreas and isolated islets [8–11]; this effect is considered selective because, unlike galanin, DBI does not inhibit glucose-stimulated release of somatostatin from δ cells or glucagon from α cells [9,10]. Thus, the potency and efficacy with which DBI inhibits insulin release in response to glucose, together with its organ and cellular localization, suggest that DBI may function as a paracrine hormone of the entero-insular axis and play an important role in the regulation of insulin secretion.

DBI_{1-86} is a 9 kDa polypeptide that was first isolated from rat, bovine and human brain by monitoring its ability to displace diazepam from binding sites on brain synaptic membranes [12]. The DBI cDNA has been cloned and sequenced, and the structure of the DBI gene, including the promoter, has been elucidated [13,14]. Although the primary structure of DBI has been conserved among various animal species, the processing of DBI shows species-specific differences. In rat brain, DBI is naturally processed to peptides similar to DBI_{33-50} (ODN; octadecaneuropeptide) and DBI_{17-50} (TTN; triakontatetraneuropeptide) [12]. ODN and TTN exhibit different biological properties. For example, whereas ODN preferentially displaces diazepam from binding sites associated with γ-aminobutyric acid type A ($GABA_A$) receptors on neurons, TTN preferentially displaces diazepam from the mitochondrial DBI receptor complex (MDRC) present in several endocrine cell types and glial cells. Thus, DBI and TTN potently stimulate steroidogenesis in adrenal cortical, Leydig, and glial cells by binding to the MDRC and thereby facilitating cholesterol transport from the outer to the inner mitochondrial membrane [12]. In rat pancreatic islets DBI is processed to peptide fragments that react with an antiserum to ODN and exhibit a retention time on reversed phase high-performance liquid chromatography (HPLC) similar to that of ODN [11]. Moreover, ODN has been shown to inhibit the release of insulin induced by 16.7 mM glucose in isolated rat pancreatic islets [11]. Because the mechanism by which ODN and DBI attenuate secretagogues-induced release of insulin is still poorely understood at the molecular level, we have now analyzed the structure-activity relation of ODN and related peptides for the inhibition of glucose-induced insulin release and also examined the effects of these peptides on insulin release induced by different secretagogues in both isolated pancreatic islets and in hamster HIT-T15 insulinoma β-cell line.

2. Materials and methods

2.1. Rat pancreatic islets

Male Sprague-Dawley rats (body mass, 180 to 200 g) were injected i.p. with pilocarpine (25 μg per kg of body mass) (Sigma, St. Louis, MO) and killed

1 h later by decapitation. Pancreatic islets were isolated as described by Lacy and Kostianosky [15] with minor modifications. Briefly, after digestion with collagenase type V (500 I.U./ml) (Sigma) for 5 min at 37°C, the pancreatic tissue was applied to a discontinuous Ficoll gradient (Sigma). After centrifugation, the islets were collected from the region of the gradient between 20.5 and 11% Ficoll, washed with phosphate-buffered saline, and suspended in a modified Krebs bicarbonate medium (KBRH) containing 118.4 mM NaCl, 4.96 mM KCl, 1.18 mM $MgSO_4$, 1.18 mM NaH_2PO_4, 2.52 mM $CaCl_2$, 10 mM Hepes (pH 7.4), and 0.5% bovine serum albumin, which has been equilibrated with 100% O_2. After incubation for 45 min at 37°C in the same buffer containing 2.8 mM glucose, the islets were collected with a Lang-Levy pipette under the guide of an inverted light microscope in order to exclude other contaminating cells.

2.2. Hamster insulinoma (HIT-T15) cells

The hamster insulinoma cell line HIT-T15 (American Cell Type Culture Collection, Rockville, MD) was used. Cells were cultured in Ham's F-12K medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% horse serum, 2 mM glutamine, 1% penicillin/streptomycin, and 5% fetal bovine serum bought from Gibco (Grand Islets, NY) and maintained at 37°C in an atmosphere of 5% carbon dioxide. The culture medium was changed two times weekly after the cells had been rinsed with Hanks' balanced salt solution (Gibco).

2.3. Insulin secretion experiments

All the experiments were carried out in KBRH, containing specified glucose or Ca^{2+} concentrations, at pH 7.4.

Isolated pancreatic islets

Three to five islets were incubated for 10 min at 37°C in KBRH medium containing 2.8 mM glucose in a final volume of 2 ml. After removal of 0.2 ml of the incubation medium for insulin determination, the islets were incubated for a further 60 min at 37°C in a final volume of 2 ml of KBRH buffer containing either 2.8 or 16.7 mM glucose, in the presence or absence of ODN, other DBI-related peptides, PACAP, or glibenclamide. At various time intervals, 0.1 to 0.2 ml of the incubation medium was collected for insulin determination. Finally, the insulin remaining in the islets was determined in 1 M acetic acid extracts after lyophilization. Insulin was than assayed by radioimmunoassay.

Although the insulin content of the islets and the amount of insulin released during the initial 10 min exposure to 2.8 mM glucose varied by 20 to 30% between incubations, the fold increase in insulin release induced by secretagogues during the subsequent 60-min incubation was consistently proportional (variability, $<5\%$) to the amount of insulin release during the initial 10-min period. Because the release of insulin in the presence of 2.8 mM glucose was not affected by ODN or its derivatives, the amount of insulin released into the medium during the initial 10-min incubation period was subtracted from that measured in the medium at the end of the incubation with the various secretagogues; this remaining amount of insulin was arbitrarily assigned the value of 100%. Thus, the 50% inhibitory concentration (IC_{50}) and the inhibitory efficacy of ODN and its derivatives with respect to the various secretagogues were then determined relative to the insulin released in response to the secretagogues alone. In each experiment, incubations were performed in triplicate, and the insulin in each sample was assayed in duplicate.

HIT-T15 β-cells

12 h before the experiment, cells were detached from their culture dishes using trypsin-EDTA (from Gibco) and than plated in 24-multiwell dishes to a final density of 5 to $6 \cdot 10^5$ cell/cm^2. At the moment of the experiment the cells were preincubated for 10 min at 37°C in 1 ml of KBRH with or without

ODN. After the preincubation the cells were incubated for further 60 min (release time) in the same condition used during the preincubation period in the presence or the absence of glucose, glibenclamide or other secretogogues, as indicated. At the end of the release time, the medium was collected and assayed by radioimmunoassay. Total insulin present in the cells was determined in acetic acid extract following lyophilization. The results are expressed as insulin secreted in ng/10^6 cells/h.

2.4. Insulin radioimmunoassay

Radioimmunoassays were performed with an antibody raised against porcine insulin [11] and porcine insulin as standards. The separation of antibody-bound ligand from free ligand was achieved with protein A (Sigma) [16]. ^{125}I-radiolabeled insulin (2200 Ci/mmol) was obtained from NEN, Boston. Insulin assay buffer contained 23 mM Na_2HPO_4, 8 mM NaH_2PO_4, 0.9% NaCl, 0.5% bovine albumin, and 0.1% NaN_3 at a pH adjusted to 7.4. Radioactivity measurements were done using a LKB gamma scintillation counter.

2.5. [^{3}H]Glibenclamide and [^{3}H]nitrendipine binding assay

[^{3}H]Glibenclamide (0.6 nM) and [^{3}H]nitrendipine (1 nM) were added in 1 ml KBRH to 35 mm dishes containing approx. $2 \cdot 10^6$ HIT-T15 cells. Cells were incubated with the radiolabelled ligand at 37°C for 45 min. The bound ligand was rapidly (15–20 s) separated from free ligand by washing three times with 2 ml of ice-cold 50 mM Tris-HCl buffer at pH 7.4. Cell were than digested with 500 μl of 1 M NaOH and an aliquot was transferred to plastic counting vials and radioactivity was counted by a liquid scintillation counter (efficiency 50%). Specific binding was defined as a difference between that in absence and that in presence of 10 μM of unlabeled ligand.

2.6. Statistical analysis

The IC_{50} values for ODN and its derivatives were calculated by probit analysis with the computer program of Tallarida and Murry [17]. Four to six concentrations of each agent were used to determine the IC_{50} value. Data are expressed as means ± S.E.M., and comparisons between groups were performed by analysis of variance with the Dunnet test. A P value of < 0.05, with n (number of experiments) values of ≥ 3, was considered significant.

2.7. Peptide synthesis

Peptides were synthesized by the Merrifield solid-phase technique with a Beckman 990 automated synthesizer [18]. Amino acids were coupled to a chloromethylated polystyrene divinyl-benzene resin (Peninsula Laboratories, San Carlos, CA). Boc-amino acids were of the L- and D-configuration, and were obtained, together with *N*-methylleucine from Bachem (Torrance, CA).

Peptides were purified by gel filtration on Sephadex G-10 (Pharmacia, Piscataway, NJ), followed by HPLC on a semipreparative reversed phase C_{18} μ-Bondapak column (0.78 cm × 30 cm) (Waters, Milford, MA). The synthetic material was eluted with a 2 h gradient from 10 to 60% acetonitrile in 0.1% trifluoroacetic acid. The purity of the peptides was confirmed by thin layer chromatography using two different developing system: (a) n-butanol/acetic acid/water (4:1:1); (b) n-butanol/acetic acid/water/pyridine (15:3:12:10) and by reversed phase HPLC. The amino acid composition of peptides was determined after hydrolysis in seal tubes in 6 M HCl at 110°C for 24 h by conventional ion-exchange with a Beckman 6000 amino acid analyzer. The amino acid sequence of peptides was determined with a gas phase sequenator; amino acids were identified by HPLC alter phenylthiohydantoin derivatization [18].

3. Results

3.1. ODN inhibition of high glucose- and glibenclamide-induced insulin secretion

To study the inhibitory potency and efficacy of ODN and its congeners on secretagogues-induced insulin secretion we have used two model systems: (a) the rat pancreatic islets and (b) the HIT-T15 β-cells.

Rat pancreatic islets

The insulin content of the isolated rat pancreatic islets was between 15 and 25 ng per islet. During the initial 10-min incubation in buffer containing 2.8 mM glucose, approx. 2 to 3% of the islet insulin content (0.30 to 0.75 ng per islet) was released into the medium. However, during the subsequent incubation in the same buffer for a further 60 min, the amount of insulin released increased by only 10 to 20% (Fig. 1A). In contrast, when, after the initial 10-min incubation in 2.8 mM glucose, the glucose concentration was increased to 16.7 mM, the amount of insulin secreted in the medium during 60 min increased by 3- to 5-fold (Fig. 1A) and accounted for up to 20% of the islet insulin content. The sum of the islet insulin content and the insulin secreted into the medium was comparable for islets treated with 2.8 mM glucose (25 ± 3.5 ng per islet; $n = 8$) and those treated with 16.7 mM glucose (26 ± 2.8 ng per islet; $n = 8$), indicating that under the experimental conditions used 16.7 mM glucose did induce a detectable increase in insulin secretion but not a detectable increase in insulin biosynthesis.

In the presence of 2.8 mM glucose, ODN, at concentrations up to 1 μM, did not affect islet insulin content or the amount of insulin released into the medium (Fig. 1A). However, ODN abolished insulin secretion induced by 16.7 mM glucose (Fig. 1A) with an IC_{50} of 6 nM (Tables 1 and 2). Using 8.3 mM glucose as stimulant, although the amount of insulin secreted was smaller (approx. 9 ng/4 islet/h), the potency of ODN was similar (IC_{50} approx. 7 nM; Table 1).

Insulin secretion in response to glibenclamide began more promptly than that in response to 16.7 mM glucose (Fig. 1B). Insulin secretion induced by 1 μM glibenclamide (1 μM is a maximally effective concentration of this drug) continued to increase throughout the 60-min incubation but at 60 min it

Table 1

Effect of various ODN concentrations on insulin secretion stimulated by glucose or glibenclamide in isolated rat pancreatic islets and in hamster insulinoma cells (HIT-T15 β-cells)

ODN (nM)	Inhibition of secretagogue-induced insulin secretion (%)				
	Isolated rats		Pancreatic islets[a]	Hamster insulinoma cells (HIT-T15 β-cells)	
	Glucose (8.3 mM)	Glucose (16.7 mM)	Glibenclamide (1 μM)	Glibenclamide (0.01 μM)	Glibenclamide (1 μM)
1	9.0 ± 0.50	5.0 ± 0.80	2.3 ± 0.82	5.0 ± 0.30	3.5 ± 0.33
5	38 ± 4.2*	30 ± 2.8*	33 ± 7.2*	39 ± 6.0*	35 ± 2.5*
10	70 ± 6.5*	68 ± 8*	50 ± 9.5*	62 ± 8.2*	58 ± 3.2*
100	90 ± 5.8*	83 ± 7.6*	71 ± 9.8*	90 ± 8.0*	92 ± 6.4*

[a] Values are expressed as a percentage of insulin release in presence of secretagogue alone, after subtracting insulin released during the initial 10-min incubation in 2.8 mM glucose.

Each value is the mean ± S.E.M. of three to five experiments. * $P < 0.01$ compared to control.

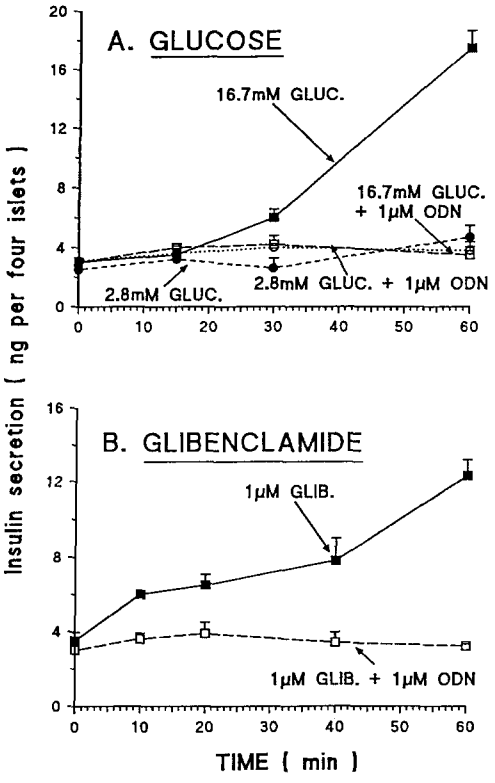

Fig. 1. Effect of ODN on insulin secretion elicited by 16.7 mM glucose (A) or glibenclamide (B). Islets were incubated for 10 min at 37°C in 2 ml of KBRH buffer containing 2.8 mM glucose. At the end of this period (time 0) 0.2 ml of the medium was collected for determination of insulin, and 0.2 ml of secretagogue (10 × in incubation buffer) in absence (closed square) or in presence (open square) of ODN (1 μM) was added. At various times after the addition of the secretagogue, 0.1 ml of the incubation medium was collected for insulin determination. After 60 min, the islets were separated from the medium by centrifugation at 900 *g* and insulin present in both the supernatant and the islets was assayed. Values are means ± S.E.M.

was slightly lower than that obtained with 16.7 mM glucose (Fig. 1B).

ODN (1 μM) inhibited glibenclamide-induced insulin secretion (Fig. 1) and the IC_{50} for this effect was approx. 10 nM (Table 1).

HIT-T15-β cells

The insulin content of this tumor β cell line was between 3.8 and 5.2 ng/10^6 cells. During 60 min incubation in buffer containing 2.8 mM glucose, 6 to 8% of the total insulin content was released in the medium. Incubation with increasing concentrations of glucose (from 8.3 to 33.4 mM) produced only a modest increment (5 to 6% over control level) in the amount of insulin secreted. Due to the glucose incompetence of this cell line further experiments with glucose were not conducted.

In contrast incubation of the cells with glibenclamide produced a dose related increase of insulin secretion that reached a maximum (approx. 2.5-fold over the control release measured in presence of 2.8 mM glucose) for doses of glibenclamide included between 10^{-7} and 10^{-6} M (Fig. 2). The extent of glibenclamide-induced insulin secretion from HIT-T15 β cells was similar to that obtained in rat pancreatic islets (see Fig. 1B), it increased with time up to 60 min and then leveled off between 60 and 90 min of incubation. In HIT-T15 β-cells, ODN inhibited insulin secretion induced by 1 μM glibenclamide with

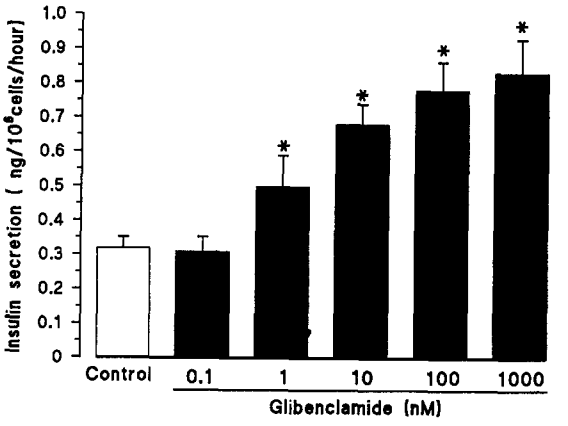

Fig. 2. Glibenclamide elicits a dose-related increase of insulin secretion in HIT-T15 β-cells. Cells were incubated for 60 min in KRBH buffer containing 2.8 mM glucose and variable amount of glibenclamide as indicated. Each bar is the mean ± S.E.M.; $n = 6$; $^*P < 0.01$ when compared with control cells.

an IC_{50} of approx. 8 nM (Table 1). The inhibitory potency of ODN was independent from the concentration of glibenclamide because ODN inhibited insulin secretion elicited by 0.01 μM glibenclamide with a potency ($IC_{50} = 7.8 \pm 1.5$ nM) similar to that observed when 1 μM glibenclamide was used (Table 1). These data suggest that ODN is not a competitive glibenclamide antagonist at its binding sites. Indeed [^{3}H]glibenclamide binding to the HIT-T15 β cells (60 ± 0.12 fmol/mg protein; $n = 3$) was not displaced by ODN up to concentrations of 1 μM.

3.2. *Structure-activity inhibition of high glucose- or glibenclamide-induced secretion of insulin by ODN peptide fragments*

To investigate the structural features of ODN required for negative modulation of insulin secretion elicited by 16.7 mM glucose, in rat pancreatic islets or by glibenclamide in HIT-T15 β-cells we synthesized peptides corresponding to the amino acid sequences present in DBI_{1-86} or ODN and we substituted some of the natural amino acids of ODN with the corresponding enantiomers or *N*-methyl derivatives.

Modification of the NH_2-terminal region of ODN resulted in a reduction in inhibitory activity with both secretagogues (Table 2). Thus, removal of the NH_2-terminal glutamine (DBI_{34-50}) or elimination of the NH_2-terminal nine residues (DBI_{42-50}) of ODN markedly reduced inhibitory potency. Modification of the COOH-terminal region of ODN also resulted in a loss of inhibitory potency. The amide derivative of ODN was one order of magnitude less potent than ODN; DBI_{33-52}, which has the same sequence of ODN with an extra glycine and lysine at the COOH-terminus, DBI_{33-42}, DBI_{38-43}, and TTN (DBI_{17-50}), were all ineffective inhibitors of glucose-induced insulin secretion up to 1 μM concentration.

Structural analogs of ODN in which Asp^{48}, both Asp^{42} and Asp^{48}, or Asp^{38}, Asp^{42}, and Asp^{48} all were replaced by D-aspartate were approximately equipotent to ODN with IC_{50} values between 5 and 7 nM. ODN derivatives in which Leu^{49} or both Leu^{46}

Table 2

Effect of ODN and related synthetic peptides on glucose and glibenclamide-stimulated insulin secretion in isolated rat pancreatic islets and in hamster insulinoma cells (HIT-T15 β-cells), respectively

Peptide	Amino-acid sequence	Glucose-induced insulin release in isolated islets		Glibenclamide-induced insulin release in HIT-T15 β-cells	
		IC_{50} (nM)	inhibition % at 10 nM	IC_{50} (nM)	inhibition % at 10 nM
ODN (DBI_{33-50})	QATVGDVNTDRPGLLDLK	6	68 ± 8.0	9	85 ± 6.0
DBI_{34-50}	ATVGDVNTDRPGLLDLK	100	32 ± 5.0	–	–
DBI_{42-50}	DRPGLLDLK	> 100	4 ± 0.6	> 100	2 ± 0.3
ODN-NH_2	QATVGDVNTDRPGLLDLKGK-NH_2	100	31 ± 8.0	–	–
DBI_{33-52}	QATVGDVNTDRPGLLDLKGK	600	2 ± 0.5	–	–
DBI_{33-43}	QATVGDVNTD	> 100	22 ± 9.0	–	–
DBI_{38-43}	DVNTDR	> 100	17 ± 8.0	–	–
TTN (DBI_{17-50})	TQPTDEEMLFIYSHFKQATVG DVNTDRPGLLDLK	700	24 ± 3.0	> 100	5 ± 0.7

Value for the inhibition of insulin secretion in the presence of glucose (16.7 mM) and glibenclamide (1 μM) are means $\pm$ S.E.M. of 4 to 6 experiments. The IC_{50} values were calculated according to Tallarida and Murray [17] (as indicated in Materials and methods).

and Leu[49] were replaced with *N*-methylleucine, or in which Arg[43] and Leu[47] were both replaced by the corresponding D-amino acids, all inhibited glucose-stimulated insulin secretion from rat pancreatic islets or glibenclamide-induced insulin release in HIT-T15 β-cells with IC_{50} values of ~ 10 nM.

3.3. Signaling pathways mediating the effect of ODN on secretagogues-induced insulin secretion

These studies were aimed to elucidate at what level, in the cascade of events leading to secretion of insulin, ODN produced its effects.

Effect via influx of extracellular Ca^{2+}

The glibenclamide-induced insulin secretion from HIT-T15 β-cells was dependent on extracellular Ca^{2+} (Fig. 3). Thus, we have explored whether ODN attenuated glibenclamide-induced insulin secretion by preventing the opening of voltage dependent Ca^{2+} channels conseguent to depolarization induced by closure of the ATP-regulated K^+ channels. However, ODN in concentrations that blocked glibenclamide induced insulin secretion failed to inhibit the binding of nitrendipine on these cells (0.60 ± 0.050 pmol/mg protein in control and 0.58 ± 0.048 pmol/mg protein in cells treated with 1 μM ODN; $n = 3$) and more important, in concentration of 1 μM ODN failed to block insulin secretion elicitd by the voltage dependent Ca^{2+} channel activator BAYK 8644 ((methyl-(1,4-dihydro-2,6-dimethyl-3-nitro-4,2-trifluoromethylphenyl)pyridine-5-carboxylate)) (Fig. 4).

Effect via regulation of intracellular Ca^{2+}

As shown in Fig. 3, 1 mM sodium nitroprussiate elicited an approx. 2-fold increase of insulin secretion over the control level in absence or presence of extracellular Ca^{2+}. Incubation of the culture with 1 μM ODN resulted in inhibition of sodium nitroprussiate-elicited insulin secretion from HIT-T15 β-cells in presence and absence of Ca^{2+} (Fig. 3A and B).

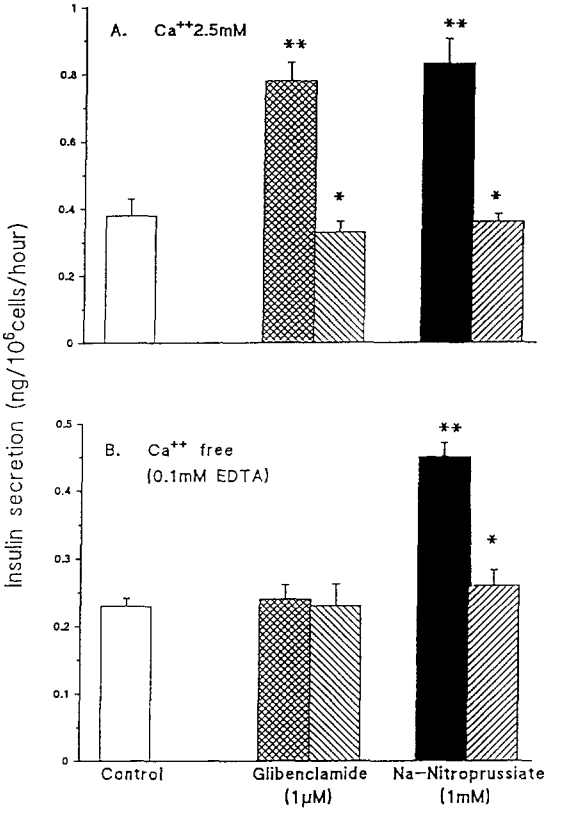

Fig. 3. Role of extracellular Ca^{2+} in glibenclamide-and sodium nitroprussiate-induced insulin secretion from HIT-T15 β-cells; effect of ODN. □, control cells; ⊠, glibenclamide 1 μM; ■, sodium nitroprussiate 1 μM; \\, glibenclamide + ODN 1 μM; //, sodium nitroprussiate + ODN 1 μM. All samples were incubated for 60 min in KRBH buffer containing 2.8 mM glucose and the indicated concentration of secretagogue. Note that ODN (1 μM) inhibits sodium nitroprussiate induced release of insulin in presence and absence of extracellular Ca^{2+}. Each bar is the mean ± S.E.M. of 6 experiments. $^{**}P < 0.01$ when compared with the respective control. $^{*}P < 0.01$ when cells treated with glibenclamide or sodium nitroprussiate without ODN were compared with cells treated with glibenclamide and sodium nitroprussiate with ODN.

Effect via accumulation of cAMP

PACAP is a potent stimulant of adenylate cyclase that has been shown to increase insulin secretion

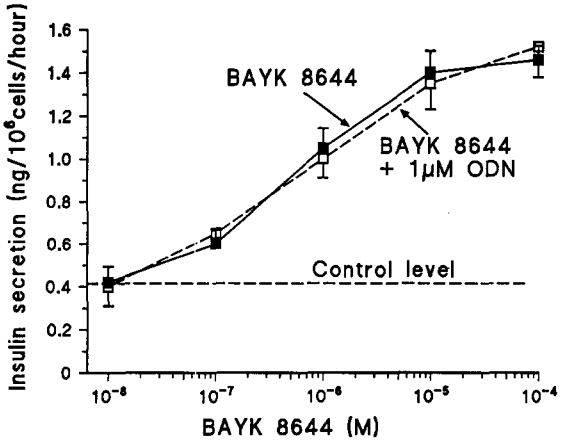

Fig. 4. ODN fails to inhibit secretion elicited by BAYK 8644. Experimental condition as described in Fig. 2. Each point is the mean ± S.E.M. of three experiments.

from pancreatic β cells [6]. As shown in Fig. 5A PACAP stimulated insulin secretion from pancreatic islets with a maximal efficacy (2-fold) at 1 nM concentration. This secretion approached a plateau after 10 min of incubation. ODN (1 μM) did not inhibits insulin secretion elicited by PACAP (Fig. 5A).

Incubation of islets for 10, 20, 40, 60 min with ODN before exposure to PACAP, or addition of ODN after PACAP application, also had no effect on PACAP-induced insulin secretion. The 50% effective stimulation of insulin secretion by PACAP is 0.5 nM; ODN also had no effect on insulin secretion induced by PACAP at the concentration of 0.5 nM (control = 3.8 ± 0.02 ng/four islets/h, 0.5 nM PACAP 8.0 ± 0.6 ng/four islets/h; 0.5 nM PACAP + 1 μM ODN 1 μM = 7.8 ± 0.7 ng/four islets/h; $n = 3$).

IBMX (isobutylmethylxanthine) by blocking phosphodiesterases, increased cAMP content [9] and released insulin from HIT-T15 β-cells (Fig. 5B). However ODN produced only a marginal inhibitory effect on IBMX-induced insulin secretion (Fig. 5B).

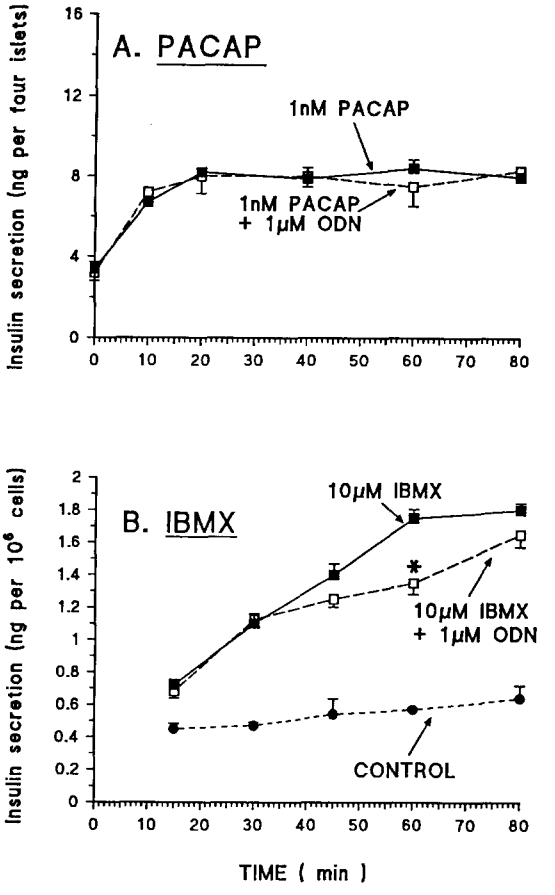

Fig. 5. ODN fails to inhibits insulin secretion elicited by PACAP (A) in rat pancreatic islets and by IBMX (B) in hamster insulinoma cells (HIT-T15 β-cells). (A) Islets incubated in KRBH buffer containing 2.8 mM glucose were treated with 1 nM PACAP. ODN (1 μM) was added together with PACAP. The condition of the experiment are identical to that described in Fig. 1. (B) HIT-T15 β-cells incubated in KRBH buffer containing 2.8 mM glucose were treated with 10 μM IBMX. The conditions of the experiment are identical to that described in Fig. 2. Note that the first measurement of insulin release was made 15 min after the beginning of the incubation. Each point is the mean ± S.E.M. of three (A) to five (B) experiments. *$P < 0.05$ when IBMX + ODN is compared with IBMX alone.

4. Discussion

The present results in rat pancreatic islets of Langheran and in hamster HIT-T15 β insulinoma cells demonstrate that ODN (DBI_{33-50}), a naturally occurring peptide fragment of rat DBI, inhibits glucose- and glibenclamide-induced insulin secretion with a potency one or two order of magnitude higher than that of DBI_{34-50}, DBI_{42-50}, DBI_{33-52}, DBI_{33-42}, or DBI_{38-43}, suggesting specific structural requirements for the interaction of this family of peptides with receptors located on β cells. The inhibitory potency of ODN is comparable to that described for DBI_{1-86} [8–11] suggesting that ODN exhibits the same biological activity as the parent molecule with regard to regulation of glucose- and glibenclamide-stimulated insulin secretion.

Because the potency and specificity of ODN on glibenclamide-elicited insulin secretion observed in isolated hamster β-cells (HIT-T15 β cells) is virtually identical to that observed in pancreatic islets, it is tempting to speculate that ODN is a paracrine hormone that once released from α or δ, cells [10] directly modulates secretion of insulin from β cells and does not act indirectly via receptors located on α or δ cells or on nerve terminals in which transmitters or neuropeptides capable of modulating insulin secretion are located.

The potency and efficacy of ODN appear to depend on the integrity of both the NH_2- and COOH-termini of the peptide. Thus, removal of the NH_2-terminal glutamine (DBI_{34-50}) or the NH_2-terminal nine amino acid residues (DBI_{42-50}) markedly reduced the inhibitory potency. Addition of glycine and lysine to the COOH-terminus (DBI_{33-52}) or amidation of the carboxy group of the terminal lysine (ODN-NH_2) also significantly reduced the potency of ODN.

In contrast, substitution of one, two, or all three aspartate residues in the central region of the ODN sequence with D-aspartate, or substitution of Leu^{46} and Leu^{49} with *N*-methylleucine, produced only minor changes in the potency of ODN.

The mechanism behind the modulatory effect of ODN on the secretory process of insulin is still unclear. Both DBI and ODN act at benzodiazepine binding sites associated with the α subunit of the $GABA_A$ receptors in brain [12]. GABA is present in high concentration in pancreatic β cells and it is released during glucose-induced depolarization [19]. Although the role of GABA on insulin secretion remain controvesial, it has been reported that GABA may facilitate glucose-induced insulin release [20], acting at $GABA_A$ receptors located on α, δ [19] or β cells [21]. Thus, the possibility must be considered that the inhibitory effect of ODN is mediated via a negative modulatory action at $GABA_A$ receptors. However, clonazepam, a specific positive allosteric modulator at $GABA_A$ receptors in the central nervous system, has no effect on glucose-induced secretion of insulin from rat pancreatic islets in vitro [22]. Thus, it seems unlikely that the inhibition of glucose-induced insulin release by ODN would be mediated by down-regulation of the action of GABA at $GABA_A$ receptors, given that, in the same preparation, anxiolytic benzodiazepines have no effect on insulin secretion. On the other hand both PK 11195 and 4′-chlorodiazepam, drugs that exhibit high affinity for the benzodiazepine biding site located on MDRC, have been shown to inhibit glucose-induced insulin secretion from isolated rat islets in vitro [22]. Thus, the inhibitory action of ODN on insulin release may be mediated via interaction with the MDRC. However, this hypothesis is not supported by the observation that TTN, the DBI processing product that exhibits the highest affinity for the MDRC [12,18], was markedly less potent than ODN in inhibiting glucose or glibenclamdie-induced insulin secretion (see Table 2).

It has been postulated that the action of ODN or DBI on the stimulation of insulin secretion by glucose can be mediated via an attenuation of the inhibition of ATP-regulated K^+ channels. The amount of insulin released following the closing of ATP sensitive K^+ channels by glucose is dose related and requires the activation of (i) the low-affinity, high-

capacity glucose transporter 2 (GT2), (ii) the high-K_m (Michaelis constant) glucokinase that catalyzes the conversion of glucose to glucose-6-phosphate, and (iii) anaerobic glycolysis, which generates ATP [23–27].

However, ODN, potently (IC_{50} 5–6 nM) and with similar efficacy inhibits insulin secretion elicited by different doses (8.3 and 16.7 mM) of glucose and in doses as high as 1 μM fails to modify the release of insulin in the presence of 2.8 mM glucose. This is taken to indicate that ODN is not competitive with glucose in the above mentioned glucose-induced cascade of glycolytic events. Because in addition to glucose ODN with high potency also inhibits secretion of insulin elicited by glibenclamide, we considered it possible that ODN acts on a site located in the domain of the ATP-regulated K^+ channel. However, two observations are against this hypothesis: (1) the effect of ODN on glibenclamide-induced insulin secretion did not appear competitive in nature and (2) ODN up to 1 μM concentration failed to displace the binding of [^{3}H]glibenclamide from its recognition sites located on HIT-T15 β cells. Thus, ODN may inhibit insulin secretion induced by glucose or glibenclamide, by preventing the closing of ATP-regulated K^+ channels, binding to a site different from that where the glibenclamide acts or by affecting a more distal component of the signaling pathway, the L-type voltage sensitive Ca^{2+} channels, for example [28]. Hence, we have addressed the question of whether ODN influences glucose or glibenclamide-induced insulin secretion binding to voltage dependent Ca^{2+} channels. However, this does not appear to be the case because ODN, up to concentration of 1 μM, failed to inhibit the binding of nitrendipine to its binding sites on HIT-T15 β cells or to modify the BAKY-8644 induced-insulin secretion.

Indirect evidence suggest that DBI inhibits cAMP-induced secretion of insulin from pancreatic islets [9]. Since cAMP may act as intracellular messenger in the release of insulin induced by glucose and glibenclamide [29] we also studied if ODN could suppress insulin release induced, in isolated pancreatic islets or in HIT-T15 β-cells, by PACAP, a polypeptide which potently increases cAMP content and releases insulin [6] or by IBMX, a blocker of phosphodietearase that also increases the islets cAMP content and releases insulin [9]. ODN failed to antagonize the insulin secretion elicited by PACAP in isolated islets and, only marginally, reduced the insulin secreation elicited by IBMX in HIT-T15 β-cells. Because DBI, in concentration of 0.1 μM, strongly suppressed insulin secretion elicited by IBMX in rat pancreatic islets [9], our data may suggest that: (a) the insulin secretion eliceted by IBMX in isolated islets is not mediated via an increase of cAMP content in β-cells, or (b) that the effect of ODN on insulin secreation, unlike that of DBI, is unrelated to changes in cAMP content; however, an accurate dose-responce study using IBMX or PACAP in presence or absence of ODN has not jet been performed, therefore a better understanding regarding the possible role of ODN on glucose- or glibenclamide-induced insulin secretion requires further investigation.

Another second messenger, presumably involved in the cascade of events leading to insulin secretion after glucose or glibenclamide stimulation, is cyclic-adenosine-diphosphate-ribose (cADPR) [30,31] which mobilizes intracellular Ca^{2+} from a ryanodine sensitive (IP_3 insensitive) Ca^{2+} stores [30,31].

In order to explore whether ODN inhibits glucose- and glibenclamide-elicited insulin secretion modulating the cADPR content and/or activity, we have used sodium nitroprussiate, which is a nitric oxide (NO) donor. Nitric oxide has been shown to mediate insulin secretion from pancreatic β-cells eliceted by high glucose and tolbutamide [33], presumably by enhancing the production of cADPR [32] and therefore by regulating the release of Ca^{2+} from ryanodine sensitive stores [30]. ODN inhibited insulin secreation elicited by 1 mM sodium nitriprussiate in pancreatic β-cells regardless of the extracellular Ca^{2+} concentration.

Taken together these data suggest that ODN may

attenuate glucose or glibenclamide-induced insulin secretion affecting intracellular Ca^{2+} signaling. Initial measurement of free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) with fura II, single β insulinoma cells, indicates that ODN attenuates the acculuation of $[Ca^{2+}]_i$ elicited by glibenclamide and by sodium nitroprussiate. The effect of the later drug on $[Ca^{2+}]_i$ occurs in absence of extracellular Ca^{2+} and it is abolished by ODN in cells incubated in Ca^{2+} free medium. Since the modulation of the cAMP pathway does not appear to contribute substantially to the action of ODN on glucose- and glibenclamide-induced insulin secreation and $[Ca^{2+}]_i$ rise in pancreatic β-cells, perhaps others mechanisms that regulate intracellular Ca^{2+} stores such as IP_3 or cyclic ADP-ribose can be regulated by OND.

References

[1] Holtz, G.G., Kuhtreiber, W.M., Habener, J.F., Pancreatic beta-cells are rendered glucose competent by the insulinotropic hormone glucagon-like peptide-1 (7.37), Nature, 361 (1993) 362–365.

[2] Tseng, C.C., Jarboe, L.A., Landau, S.B., Williams, E.K., Wolfe, M.M., Glucose-dependent insulinotropic peptide: Structure of the precursor and tissue specific expression in rat, Proc. Natl. Acad. Sci. USA, 90 (1993) 1992–1996.

[3] Ahren, B., Ostenson, C.G. and Efendic, S., Other islet hormones. In E. Samols (Ed.), The Endocrine Pancreas, Raven Press, New York, pp. 153–173, 1991.

[4] Mutt, V., Hormonal Peptides. In Fidia Research Foundation Neuroscience Award Lecutre, Vol. 3, Raven Press, New York, 1989, pp. 125–218.

[5] Arimura, A., Pituitary adenylate cyclase activating polypeptide (PACAP): discovery and current status of research, Regul. Pept., 37 (1992) 287–303.

[6] Kawai, K., Ohse, C., Watamabe, Y., Suzuki, S., Yamashita, K., Ohashi, S., Pituitary adenylate cyclase activiating polypeptide stimulates insulin release from the isolated perfused rat pancrea, Life Sci., 51 (1992) 795–806.

[7] Bovolin, P., Schlichting, J., Miyata, C., Ferrarese, C., Guidotti, A., Alho, H., Distribution and characterization of diazepam binding inhibitor (DBI) in peripheral tissues of rat, Regul. Pept., 29 (1990) 267–281.

[8] Chen, Z., Agerberth, B., Gell, K., Andersson, M., Mutt, V., Ostenson, C.G., Efendic, S., Barros-Soderling, J., Persson, B., Jornvall, H., Isolation and characterization of porcine diazepam binding inhibitor, a polypeptide not only of cerebral occurence but also common in the intestinal tissues and with effects on regulation of insulin release, Eur. J. Biochem., 174 (1988) 239–245.

[9] Ostenson, C.G., Ahren, B., Karlsson, J., Sandberg, E., Efendic, S., Effects of porcine diazepam binding inhibitor (DBI) and glucagon secretion in vitro from rat endocrine pancreas, Regul. Pept., 29 (1990) 143–151.

[10] Ostenson, C.G., Ahren, B., Johansson, O., Karlsson, S., Hilliges, M., Efendic, S., Diazepam binding inhibitor and the endocrine pancreas, Neuropharmacology, 30 (1991) 1391–1398.

[11] Borboni, P., Condorelli, I., De Stefanis, P., Sesti, G., Lauro, R., Modulation of insulin secretion by diazepam binding inhibitor and its processing products, Neuropharmacology, 30 (1991) 1399–1403.

[12] Costa, E., Guidotti, A., Diazepam binding inhibitor (DBI): A peptide with multiple biological actions, Life Sci., 49 (1991) 325–344.

[13] Mandrup, S., Hummel, R., Ravn, S., Jensen, G,. Andreasen, P.H., Gregersen, N., Knudsen, J., Kristiansen, K., Acyl-CoA-binding protein/diazepam binding inhibitor gene and pseudogenes, J. Mol. Biol., 228 (1992) 1011–1022.

[14] Kolmer, M., Alho, H., Costa, E., Pani, L., Cloning and tissue-specific functional characterization of the promoter of the rat diazepam binding inhibitor, a peptide with multiple biological actions, Proc. Natl. Acad. Sci. USA, 90 (1993) 8439–8443.

[15] Lacy, P.E., Kostianovsky, M., Method for the isolation of intact islets of langerhands from the rat pancreas, Diabetes, 16 (1967) 35–39.

[16] Ferrarese, C., Vaccarino, F.M., Alho, H., Mellstrom, B., Costa, E., Guidotti, A., Subcellular localization and neuronal release of diazepam binding inhibitor, J. Neurochem., 48 (1987) 1093–1102.

[17] Tallarida, R.J., Murray. R.B., Manual of pharmacological calculations with computer programs, Springer-Verlag, New York, 1987.

[18] Berkovich, A., McPhie, P., Campagnone, M., Guidotti, A., Hensley, P., A natural processing product of rat diazepam binding inhibitor, triakontatetraneuropeptide (diazepam binding inhibitor 17–50) contains and α helix, which allows discrimination between benzodiazepine binding site subtypes, Mol. Pharmacol., 37 (1990) 164–172.

[19] Rorsman, P., Berggren, P., Bokvist, K., Ericson, H., Mohler, H., Ostenson, C.G., Smith, P., Glucose inhibition of glucacon secretion involves activation of $GABA_A$ receptor chloride channel, Nature, 341 (1989) 233–236.

[20] Cavagnini, F., Pinto, M., Dubin, A., Invitti, C., Cappelletti, G., Polli, E.E., Effects of gamma-aminobutyric acid

(GABA) and muscimol on endocrine pancreatic function in man, Metabolism, 31 (1982) 73–77.
[21] Borboni, P., Porzio, O., Fusco, A., Sesti, G., Lauro, R., Marlier, L., Molecular and cellular characterization of the $GABA_A$ receptor in the rat pancreas, Mol. Cell. Endocrinol., in press.
[22] Petit, P., Manteghetti, M., Berdeu, D., Ribes, G., Loubatieres-Mariani, M.M., Effects of a peripheral-type benzodiazpine on glucose-induced insulin secretion, Eur. J. Pharmacol., 21 (1992) 359–363.
[23] Meglasson, M.D., Matschinsky, F.M., Pancreatic islet glucose metabolism and regulation of insulin secretion, Diabetes/Metab. Rev., 2 (1986) 163–214.
[24] Epstein, P.N., Boschero, A.C., Atwater, I., Xingang, C., Overbeek, P.A., Expression of yeast hexokinase in pancreatic β cells of transgenic mice reduces blood glucose, enhances insulin secretion and decreases diabetes, Proc. Natl. Acad. Sci. USA, 89 (1992) 12038–12042.
[25] German, M.S., Glucose sensing in pancreatic islet beta cell: The key role of glucokinase and the glycolytic intermediates, Proc. Natl. Acad. Sci. USA, 90 (1992) 1781–1785.
[26] Ashcroft, F.M., Rorsman, P., Electrophysiology of the pancreatic beta-cell, Prog. Biophys. Mol. Biol., 54 (1991) 87–143.
[27] Rajan, A.S., Aguilar-Bryan, L., Nelson, D.A., Yaney, G.C., Hsu, W.H., Kunze, D.L., Boyd, A.E., Ion channels and insulin secretion, Diab. Care, 13 (1990) 340–363.
[28] Arkammar, P., Nilsson, T., Rorsman, P., Berggen, P.O., Inhibition of ATP-regulated K^+ channels preceeds depolarization-induced increase in cytoplasmic free Ca_{2+} concentration in pancreatic β-cells, J. Biol. Chem., 262 (1987) 5448–5454.
[29] Grill, V., Cerasi, E., Interacting effects of sulfonylureas and glucose on cAMP metabolism and insulin release in pancreatic islets of the rat, J. Clin. Invest., 61 (1978) 1346–1354.
[30] Takasawa, S., Nata, K., Yonekura, H., Okamoto, H., Cyclic ADP-ribose in insulin secreation from pancreatic β-cells, Science, 259 (1993) 370–373.
[31] Berridge, M.J., A tale of two messengers, Nature, 365 (1993) 388–389.
[32] Gallione, A., Cyclic ADP-ribose: A new way to control calcium, Science, 259 (1993) 325–326.
[33] Schmidt, H.H.W., Warner, T.D., Ishii, K., Sheng, H., Murad, F., Insulin secretion from pancreatic β-cells caused by L-arginine-derived nitric oxide, Science, 255 (1992) 721–723.