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DBI mRNA is expressed in endocrine pancreas and its post-translational product DBI_{33-50} inhibits insulin release

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It has been previously demonstrated that DBI is present in endocrine pancreas and it is able to inhibit insulin release in isolated rat islets. Its mechanism of action has been investigated, demonstrating the possible involvement of cAMP and ATP-dependent K^+ channels. DBI_{33-50}, a post-translational product of DBI, is also able to inhibit insulin release, but its action has not been characterized. In the present study, we have investigated the presence of DBI mRNA in pancreas, islets and cultured β cells. The possible mechanism of action of DBI_{33-50} and the involvement of BZ/$GABA_A$ receptors has been studied.

Keywords: DBI; Islets of Langerhans; insulinoma; gene expression; MBR; $GABA_A$ receptor; insulin secretion

Introduction

DBI (Diazepam Binding Inhibitor) is a 9 kD peptide first isolated from rat brain for its ability to displace Benzodiazepines (BZs) from the binding site associated to the $GABA_A$ receptor ('central' BZ binding site) and from the 'peripheral' BZ receptor (PBR), also named 'mitochondrial' BZ receptor (MBR) (Guidotti *et al.*, 1983; Weizman & Gavish, 1993). Therefore DBI is considered as an endogenous modulator of $GABA_A$ receptor function through its interaction with the two classes of BZ binding sites (Costa *et al.*, 1983). In the Central Nervous System (CNS) DBI is the precursor of two major biologically active peptides: DBI_{33-50}, named ODN (Ferrero *et al.*, 1986), and DBI_{17-50} named TTN (Slobodyansky *et al.*, 1989). DBI_{33-50} and DBI_{17-50} have different physico-chemical properties and biological activities. In fact, DBI_{33-50} binds preferentially to the BZ binding site associated to the $GABA_A$ receptor, while DBI_{17-50} binds preferentially to the PBR (Berkovich *et al.*, 1989).

DBI and its processing products are also present in several peripheral tissues, such as gastro-intestinal tract, adrenals, testis, liver, kidney, as demonstrated by immunohistochemical (Bovolin *et al.*, 1990) and *in situ* hybridization experiments (Alho *et al.*, 1988). DBI exerts various peripheral actions, in particular at the level of adrenal cortical (Papadopoulos *et al.*, 1991) and Leydig cells (Garnier *et al.*, 1993), where it induces steroidogenesis, by stimulating the conversion of cholesterol to pregnenolone. Moreover, DBI is also able to bind and induce the synthesis of the medium-chain acyl-CoA esters, therefore it has been termed Acyl-CoA-binding protein (ACBP) by Mogensen *et al.* (1987) who isolated DBI/ACPB from bovine liver. Finally, it has also been demonstrated that porcine DBI, purified from upper intestine, inhibits glucose-induced insulin release from isolated and perfused rat pancreas (Chen *et al.*, 1988). More recently, using rat brain DBI and synthetic DBI-derived peptides, it has been shown that DBI and DBI_{33-50}, but not DBI_{17-50}, inhibit glucose-induced insulin release in isolated rat islets (Borboni *et al.*, 1991). This observation raised the hypothesis that a PBR is not involved in the negative modulation of insulin release exerted by DBI and DBI_{33-50}.

The aim of the present study was to determine the presence of DBI mRNA in endocrine pancreas and to investigate the ability of its biologically active product DBI_{33-50} to modulate insulin release induced by glucose and other secretagogues in cultured β cells. The possible type of receptor involved in this peripheral action of DBI_{33-50} has also been studied by means of specific ligands for the 'central' or 'peripheral' type BZ receptors.

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Results

PCR amplification products from pancreas, islets or RIN 1046-38 cells correspond to a unique band of the expected molecular weight (182 bp) when analysed on agarose gel. Based on the intensity of the ethidium bromide stained bands under U.V., it clearly appears that DBI mRNA is relatively more abundant in islets and RIN 1046-38 cells as compared to whole pancreas (Figure 1).

Figure 2 shows that 0.1 mM and 2.8 mM glucose stimulate insulin release in RIN 1046-38 cells respectively by 1.25-fold and 2.06-fold compared to basal insulin release in the absence of glucose (24.14 ng/ml/h and 39.86 ng/ml/h respectively with 0.1 mM and 2.8 mM glucose vs 19.32 ng/ml/h in the absence of glucose). The extent of glucose stimulation is comparable to the stimulation obtained by Clark *et al.* (1990) with cells at the same passage. The same figure demonstrates that DBI_{33-50} inhibits dose-dependently glucose-induced insulin release in RIN 1046-38 cells. The effect is also glucose-dependent since it is more effective at high glucose concentrations. In fact, DBI_{33-50} does not affect insulin release in the absence of glucose, while it reduces significantly the release induced by 0.1 mM glucose ($-11.74 \pm 5.16\%$ at 10 nM and $-21.53 \pm 6.34\%$ at 100 nM) and by 2.8 mM glucose ($-19.59 \pm 2.20\%$ at 10 nM and $-27.86\% \pm 1.43\%$ at 100 nM).

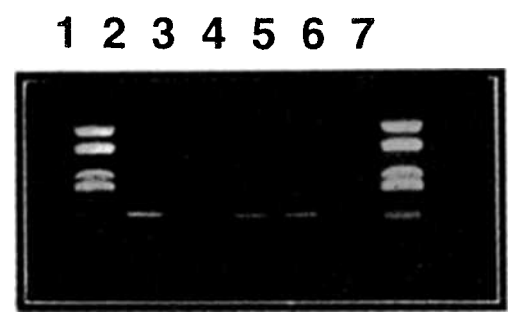

Figure 1 Agarose gel electrophoresis of PCR amplification products of DBI from different tissue/cells preparations. Lane 1: pUC18 marker; lane 2: brain; lane 3: pancreas; lane 4: islets; lane 5: RIN 1046-38 cells; lane 6: water; lane 7: pUC18 marker

Isobutylmethylxantine (IBMX) stimulates insulin release induced by 0.1 mM glucose by 45.08 ± 3.11% and DBI_{33-50} inhibits dose-dependently the action of IBMX, blunting insulin response by 8 ± 6.29%, 15.21 ± 4.45% and 41.22 ± 12.10% respectively at 1 nM, 10 nM and 100 nM (Figure 3).

DBI_{33-50} is uneffective on arginine-induced insulin release (data not shown).

Glibenclamide stimulates insulin release both in the absence (+55.63 ± 2.81%; Figure 4A) and in the presence of glucose (+33.80 ± 2.60% and 15.85 ± 1.89% respectively in the presence of 0.1 mM and 2.8 mM glucose; Figure 4B and 4C). It is noteworthy that the effect of glibenclamide is not potentiated by glucose, as already observed by Clark *et al.* (1990) in the same cell line. DBI_{33-50} inhibits dose-dependently glibenclamide-induced insulin release both in the absence of glucose (−5.07 ± 1.90%, −17.00 ± 4.02% and −21.32 ± 3.10% respectively at 1 nM, 10 nM and 100 nM; Figure 4A), as well as in the presence of 0.1 mM glucose (−8.12 ± 1.43%, −14.06 ± 2.82% and −15.70 ± 2.24% respectively at 1 nM, 10 nM and 100 nM; Figure 4B) and in the presence of 2.8 mM glucose (−11.86 ± 3.91%, −14.23 ± 3.10% and −16.75 ± 3.64%, respectively at 1 nM and 100 nM; Figure 4C).

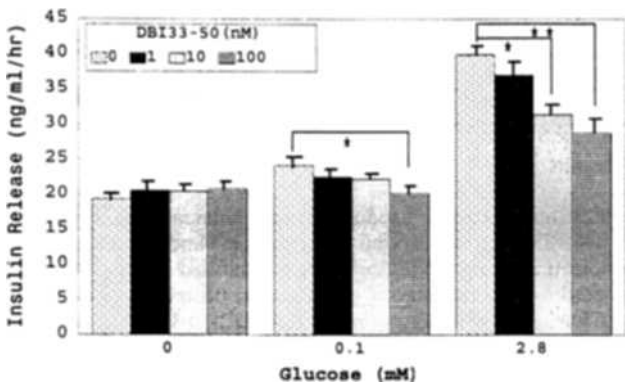

Figure 2 Dose-response effect of DBI_{33-50} on glucose-induced insulin release from RIN 1046-38 cells. Cells were incubated for 10 min in the absence or in the presence of DBI_{33-50} (1 nM, 10 nM, 100 nM) in glucose-free buffer (pre-incubation period). Thereafter, the incubation was continued for 1 h, in the absence or in the presence of DBI_{33-50} (1 nM, 10 nM, 100 nM), without or with glucose (0.1 mM or 2.8 mM) in the designated wells. Results are means ± SEM ($n = 7$). * = $P<0.05$; ** = $P<0.01$

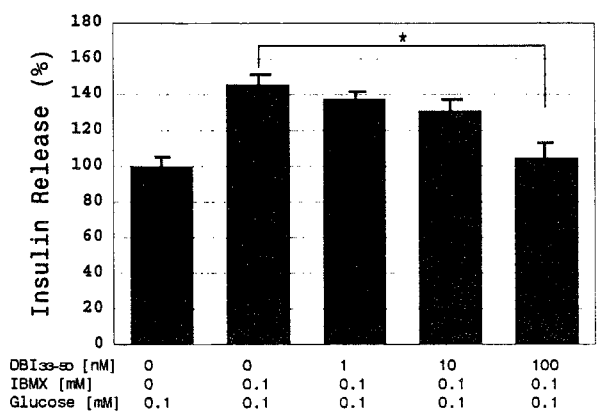

Figure 3 Dose-response effect of DBI_{33-50} on IBMX-induced insulin release from RIN 1046-38 cells. Cells were incubated for 10 min in the absence or in the presence of DBI_{33-50} (1 nM, 10 nM, 100 nM) in glucose-free buffer without IBMX (pre-incubation period). Thereafter, the incubation was continued for 1 h, in the absence or in the presence of DBI_{33-50} (1 nM, 10 nM, 100 nM), with glucose (0.1 mM) and IBMX (0.1 mM) in the designated wells. Results are means ± SEM ($n = 5$) and are expressed as percent of control, where the control is the insulin release induced by 0.1 mM glucose in the absence of IBMX and DBI_{33-50} (control absolute value = 28.34 ± 2.05 ng/ml/h). * = $P<0.05$; ** = $P<0.01$

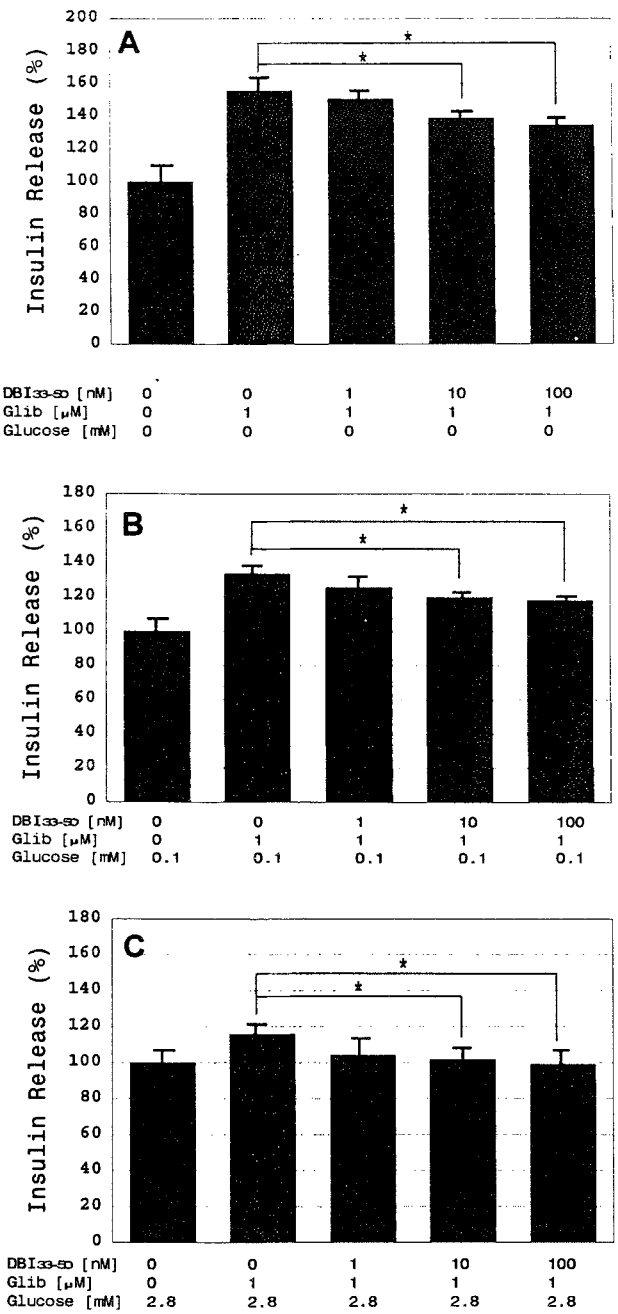

Figure 4 Dose-response effect of DBI_{33-50} on glibenclamide-induced insulin release from RIN 1046-38 cells. Cells were incubated for 10 min in the absence or in the presence of DBI_{33-50} (1 nM, 10 nM, 100 nM) in glucose-free buffer without glibenclamide (pre-incubation period). Thereafter, the incubation was continued for 1 h in the absence or in the presence of DBI_{33-50} (1 nM, 10 nM, 100 nM), without glucose (A) or with glucose (B: 0.1 mM, C: 2.8 mM) and with glibenclamide (1 μM) in the designated wells. Results are means ± SEM ($n = 6$) and are expressed as percent of control, where the control is the insulin release in the absence of DBI_{33-50} and glibenclamide, without glucose (A), or with glucose 0.1 mM (B) and 2.8 mM (C). (Control absolute value: A = 23.59 ± 1.86; B = 28.18 ± 2.15; C = 44.52 ± 3.22 ng/ml/h). * = $P<0.05$; ** = $P<0.01$

The action of 10 nM DBI_{33-50} on insulin release induced by 2.8 mM glucose is partially antagonized by 4' chlorodiazepam (RO 5-4864), but neither by flumazenil (RO 15-1788) or by the isoquinoline carboxamide (PK 111-95). The effect of DBI_{33-50} is reduced by 12.56% ± 6.19% with the addition of 10 μM RO 5-4864 (Figure 5).

Discussion

Several peptides, deriving both from brain and gastrointestinal tract, are involved in the control of endocrine pancreatic secretion. There are peptides stimulating insulin release, such as glucose-dependent insulinogenic polypeptide (GI) (Wahl *et al.*, 1992), glucagon-like peptide-1 (GLP-1-) (Thorens & Waeber, 1993), vasoactive intestinal peptide (VIP) (Wahl *et al.*, 1993), colecistokinin (CCK) (Karlsson & Ahren, 1992) and peptides inhibiting insulin release, such as somatostatin (Hsu *et al.*, 1991), galanin (Ahren & Lindskog, 1992) and neuropeptide Y (NPY) (Skoglund *et al.*, 1993). The latter group of peptides may have relevance in the pathogenesis of non-insulin-dependent diabetes mellitus or in the states of impaired insulin response.

DBI can be included among the peptides of brain or intestinal origin, with inhibitory activity on insulin release. Nevertheless, the role of DBI in the modulation of insulin release has not been completely clarified, and the action of its post-translational product DBI_{33-50} has not been characterized. Immunohistochemical studies revealed DBI immunoreactivity (DBI-IR) in α cells of rat pancreas and in δ cells of porcine (Hoog *et al.*, 1989) and human pancreas (Johansson *et al.*, 1991), suggesting a paracrine action of DBI. *In situ* hybridization experiments have not been carried out in pancreas, therefore it is not known whether the peptide is produced in a specific pancreatic cell type. Further investigations on the possible mechanism of action, showed that porcine DBI inhibits IBMX- and glibenclamide-induced insulin release and arginine-induced glucagon release (Ostenson *et al.*, 1990). Moreover only indirect evidences have been provided on the type of receptor involved in the action of DBI on insulin release (Borboni *et al.*, 1991).

In the present study we have demonstrated that DBI mRNA is present in endocrine pancreas and in particular it appears to be relatively more represented in islets and RIN

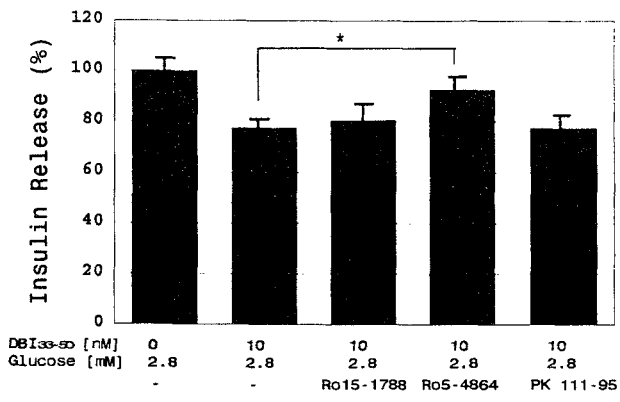

Figure 5 Effect of flumazenil (RO15-1788), 4' chlorodiazepam (RO 5-4864) or isoquinoline carboxamide (PK 111-95) on the inhibition of insulin release by DBI_{33-50}. Cells were incubated for 10 min in the absence or in the presence of the various compounds (10 μM) in glucose-free buffer without DBI_{33-50} (pre-incubation period). Thereafter, the incubation was continued for 1 h, in the absence or in the presence of the various compounds, with DBI_{33-50} (10 nM) and glucose (2.8 mM), in the designated wells. Results are means ± SEM ($n = 5$) where the control is the insulin release induced by 2.8 mM glucose alone (Control absolute value = 41.88 ± 3.08 ng/ml/h). * = $P < 0.05$; ** = $P < 0.01$

1046-38 cells, as compared to whole pancreas. The reduced DBI mRNA expression in pancreas might be due to a dilution effect, since a mixed cell population is present in this tissue, such as adipocytes, fibroblasts, exocrine cells and endocrine cells. Since the latter cell type is relatively less represented, it is possible to suggest that DBI mRNA is mainly derived from the endocrine component of pancreatic tissue. The presence of DBI mRNA in β cells seems to be in contrast with previous experiments showing DBI-IR in islets cells other than β cells, thus it would imply that the peptide is synthesized in β cells and then transported to the other cell types. Nevertheless, the presence of DBI mRNA on purified α or β cells has not been investigated in this study. Furthermore, it should be emphasized that RIN 1046-38 are transformed β cells deriving from a rat insulinoma obtained by irradiation; it is thus possible that they may express genes in an abnormal manner (Clark *et al.*, 1990).

DBI_{33-50} is a post-translational product of DBI, with potent biological activity in the CNS (Ferrero *et al.*, 1986). The present data demonstrate that it is also effective peripherally, being able to affect insulin release induced by glucose, IBMX and glibenclamide in cultured pancreatic β cells. IBMX increases the intracellular cAMP level by inhibiting phosphodiesterase activity (Grill & Cerasi, 1978; Grill, 1980), while glibenclamide and glucose act at a common site in the stimulus-secretion coupling of β cells by reducing the activity of the ATP-dependent K^+ channels, which in turn determines the opening of voltage-dependent Ca^{2+} channels initiating insulin release (Ashcroft & Ashcroft, 1992). Our data are consistent with the hypothesis that the mechanism of action of DBI involves modulation of adenylate cyclase-cAMP system and/or activation of ATP-dependent K^+ channels.

Because previous experiments demonstrated that DBI fails to affect somatostatin release, it was proposed that its inhibitory action on insulin secretion is not mediated by somatostatin (Chen *et al.*, 1988). Our study supports the hypothesis that DBI may act directly on the β cells, since DBI_{33-50} is effective on RIN 1046–38 cells.

Most of the known neurobiological effects of DBI and DBI-derived peptides are mediated either through its interaction with BZ binding site associated to the $GABA_A$ receptor, or with PBR (Costa & Guidotti, 1991).

The so called 'central' BZ site is located in the allosteric modulatory site of the $GABA_A$ receptor, a ligand-gated Cl^- channel (Schoefield *et al.*, 1987; Wisden & Seeburg, 1992). There are three types of ligands for such a modulatory site: anxiolitic BZs (diazepam) acting as positive allosteric modulators, thus increasing the frequency of Cl^- currents; anxiogenic derivatives of β-carboline-3-carboxylate, acting as negative allosteric modulators, therefore decreasing the frequency of Cl^- currents; flumazenil (RO 15-1788), acting as a pure antagonist of the modulatory site, since it is devoid of intrinsic activity but antagonizes the action of both positive and negative allosteric modulators (Olsen & Tobin, 1990). Moreover, a novel modulatory site associated to the $GABA_A$ receptor has been described at which 4'-chlorodiazepam (RO 5-4864), a proconvulsant BZ, binds with high affinity (Gee, 1987; Gee *et al.*, 1988). The so called PBR, (or MBR), is associated with a protein in the outer mitochondrial membrane of both central (glial cells) and peripheral (adrenal cortical and Leydig cells) steroidogenic cells (Papadopoulos *et al.*, 1990, 1992; Krueger & Papadopoulos, 1990). Both RO 5-4864 and the isoquinoline carboxamide (PK 111-95) are used to probe this receptor in different peripheral tissues (Kinally *et al.*, 1993).

It has been reported that DBI_{17-50} is not able to affect insulin release, suggesting that a PBR is not involved in the pancreatic action of DBI (Borboni *et al.*, 1991). The present data confirm that DBI_{33-50} does not act through a PBR, since PK 111-95 is not able to inhibit its action. On the contrary, the effect of DBI_{33-50} can be antagonized by RO 5-4864 but not by RO 15-1788, thus suggesting that DBI_{33-50} acts via the

BZ allosteric modulatory site coupled to the $GABA_A$ receptor, and more likely, via the RO 5-4864 site. Studies with transfection of cDNAs encoding various $GABA_A$ receptor subunits in a kidney tumor cell line, showed that RO 5-4864 down-regulates $GABA_A$ receptor activity in a manner that requires γ2 subunits but is independent from the presence of α subunits (Puia *et al.*, 1989). The presence of $GABA_A$ receptors has been demonstrated in islet cells by immunohistochemical studies (Rorsman *et al.*, 1989); moreover, molecular and cellular characterization of the $GABA_A$ receptor in endocrine pancreas provided evidence for expression of specific receptor subunits, including γ2 subunit, both in α and β cells and in RIN 1046-38 cells. (Borboni *et al.*, 1994).

Based on these observations, it is possible to suggest that DBI_{33-50} acts at the level of β cells on the allosteric modulatory site of putative pancreatic $GABA_A$ receptors, that are equipped with γ2 subunit.

In conclusion, we demonstrate that DBI mRNA is present in islets and transformed β cells and that the post-translational product DBI_{33-50} is able to modulate negatively insulin release. Although its effect is weak, DBI could be of biological importance in combination with other hormones in certain conditions. In particular DBI, as well as other brain- or intestinal-derived peptides, is hypersecreted during stress, therefore it could reach high circulating concentrations, that are effective on insulin release.

The possible mechanism of action of DBI_{33-50} may involve cAMP metabolism and ATP-dependent K^+ channels activity. It is tempting to suggest that DBI interacts with $GABA_A$ receptors located on β cells.

Materials and Methods

Tissue preparation

Sprague-Dawley male rats (200–250 g) were sacrificed by decapitation. Pancreases were excised and carefully dissected from the surrounding fat and connective tissue and immediately frozen for subsequent RNA extraction.

For islets isolation, newborn rats (7–10 days) were sacrificed by decapitation, pancreases were excised and processed as previously described, with minor modifications (Hellerstron *et al.*, 1979). Islets were resuspended in RPMI 1640 medium containing 10% FCS, 2 mM glutamine, 50 I.U. Penicillin and 50 μg/ml Streptomycin (Flow), plated in 35 mm Petri dishes and incubated at 37°C in an atmosphere of 95% humidified air/5% CO_2. After 6 h most of fibroblasts, present as contaminating cells, were attached to the bottom of the dishes; the supernatant, containing islets and exocrine tissue, was replated in new dishes. After 24 h, the islets were attached to the bottom, therefore the supernatant with the floating exocrine tissue was discarded, and fresh medium was added to the cultures for additional 24 h. This procedure allowed to obtain almost pure islets cultures.

Cell cultures

RIN 1046-38 cells were cultured in M199 medium supplemented with 10% FCS, 2 mM glutamine, 50 I.U. Penicillin and 50 μg/ml Streptomycin (Flow), at 37°C in an atmosphere of 95% humidified air/5% CO_2. For insulin release experiments cells were used at passage 15–25, at which the glucose responsiveness is maintained (Clark *et al.*, 1990).

RNA preparation

Pancreatic RNA was obtained after overnight ultracentrifugation through a cesium chloride cushion (Chomczynsky & Sacchi, 1987). Total RNAs from islets and RIN 1046-38 cells were prepared using RNAzol B method. Islets were used on the third day of culture, RIN 1046-38 cells were used at confluence. The RNA pellets were resuspended in diethylpyrocarbonate- (DEPC-) treated water and stored at −70°C until use.

Table 1 Primer sequences

us primer 5'-CAG GCT GAT TTT GAC AAA GCC GCT-3'
ds primer 5'-TTT CAG CTT GTT CCA CGA GTC CCA-3'
Sequence amplified from: 124 to 306 bp

RT-PCR

1 μg of total RNA from pancreas, islets and RIN 1046-38 cells were reverse transcribed by 200 U of Maloney-Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega) using 2.5 mM random hexamers (Pharmacia) in the presence of 100 μM deoxynucleotide triphosphate (Pharmacia) and 15 U RNAsin (Promega), in a final volume of 20 μl. The reaction mixture was incubated for 1 h at 37°C, then heat denatured for 5 min at 95°C. The obtained cDNAs were PCR amplified in a thermal cycler (Perkin Elmer Cetus) using specific primers designed in order to obtain a 182 base pair (bp) cDNA product (Table 1). The amplification mixture contained 2.5 U Taq polymerase (Promega), 1.5 mM $MgCl_2$ and 300 nM specific primers pair in a final volume of 100 μl (Table 1). The mixture was overlaid with 50 μl of mineral oil and amplified for 30 cycles. Each amplification cycle consisted of a 45 s denaturation step at 94°C, 1 min annealing step at 60°C and 1 min elongation step at 72°C with a final elongation step of 5 min at 72°C. DEPC-treated water samples and brain RNA samples were processed in parallel as controls. The amplification products were analysed on 1.8% (w/v) agarose gel (Nu Sieve 3:1, FMC) in 0.5× Tris-Borate-EDTA (TBE) buffer (Sigma). The relative abundance of the amplification product was estimated by the intensity of the ethidium bromide stained bands under UV. Each experiment was repeated twice, using two different tissue or culture preparations.

Insulin release

Cells were plated at 10^5/ml in 24 well plates in 0.5 ml of culture medium. After 48 h, the medium was removed and the cells were washed twice, at 37°C for 30 min, with a glucose-free buffer containing 114 mM NaCl, 25.5 mM $NaHCO_3$, 10 mM HEPES, 2.5 mM $CaCl_2$, 4.7 mM KCl, 1.21 mM KH_2PO_4, 1.16 mM $MgSO_4$, 0.1% BSA (pH 7.2). Thereafter, cells were incubated for 1 h in the same buffer in the absence or in the presence of DBI_{33-50} (synthesized and kindly provided by Dr A. Berkovich, FGIN, Washington DC, USA) at concentrations ranging from 1 nM to 100 nM with increasing glucose concentrations (from 0 to 2.8 mM) and with different insulin secretagogues: 20 mM arginine, 0.1 mM isobutylmethylxantine (IBMX) or 1 μM glibenclamide. In other sets of experiments, flumazenil (RO 15-1788), 4'chlorodiazepam (RO 5-4864) or the isoqinoline carboxamide (PK 111-95) were added in excess (10 μM) to DBI_{33-50} (10 nM), in the presence of 2.8 mM glucose, in order to determine the type of receptor involved in its inhibitory action on insulin release. At the end of the incubation period, aliquots of the supernatant were collected and stored at −20°C for subsequent insulin radioimmunoassay. Data are presented as the average ± SEM and statistical analysis was carried out by Student's *t* test.

Insulin radioimmunoassay

Insulin was determined by a dextran-charcoal method as previously described, using an anti-insulin antibody raised in guinea pig, porcine insulin standard (Sigma) and ^{125}I-Insulin from New England Nuclear (Boston, MA) (Herbert *et al.*, 1965).

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B.P. dedicates the paper to the memory of Prof. Roberto De Pirro.

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