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Pancreatic β cells synthesize and secrete nerve growth factor

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ABSTRACT **Differentiation and function of pancreatic β cells are regulated by a variety of hormones and growth factors, including nerve growth factor (NGF). Whether this is an endocrine or autocrine/paracrine role for NGF is not known. We demonstrate that NGF is produced and secreted by adult rat pancreatic β cells. NGF secretion is increased in response to elevated glucose or potassium, but decreased in response to dibutyryl cAMP. Moreover, steady-state levels of NGF mRNA are down-regulated by dibutyryl cAMP, which is opposite to the effect of cAMP on insulin release. NGF-stimulated changes in morphology and function are mediated by high-affinity Trk A receptors in other mammalian cells. Trk A receptors are present in β cells and steady-state levels of Trk A mRNA are modulated by NGF and dibutyryl cAMP. Taken together, these findings suggest endocrine and autocrine roles for pancreatic β-cell NGF, which, in turn, could be related to the pathogenesis of diabetes mellitus where serum NGF levels are diminished.**

Nerve growth factor (NGF) has been implicated in the survival and differentiation of neuronal and non-neuronal systems (1). In particular, it induces morphological and physiological changes in pancreatic β cells, including an increase in voltage-dependent sodium current density (2) and the extension of neurite-like processes. This latter effect is potentiated by dibutyryl cAMP (dbcAMP) (3). Furthermore, pancreatic β cells treated with NGF in culture (5 days) secrete more insulin in response to stimulation with 20.6 mM glucose than with 5.6 mM glucose. In contrast, with increasing time in culture, insulin secretion of control cells tends to be similar in both glucose concentrations (3). The effects of NGF on β cells are mediated through the high-affinity receptor Trk A (4), which has been shown to exist in pancreatic β cells (5, 6).

Together, these data suggest that NGF is an important factor in the maintenance of the endocrine function of β cells *in vitro*. However, pancreatic NGF has not been detected. A possible source is the pancreatic β cell itself, since it has been observed that endogenous NGF levels in diabetic animals (7) and NGF serum levels in type II diabetic patients (8) are decreased. Nevertheless, despite efforts to detect NGF expression in fetal β cells or in β cell lines by Northern blot analysis (9), to date, neither synthesis nor secretion of NGF by β cells has been demonstrated.

In the present study, we demonstrate NGF synthesis and secretion by single adult rat pancreatic β cells. Furthermore, since regulation of the levels of Trk A receptors can modulate responsiveness to NGF (10, 11), we also examined whether NGF and/or dbcAMP modulate β cell Trk A mRNA levels.

EXPERIMENTAL PROCEDURES

Cell Culture. Animal care was performed according to the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* (National Institute of Health Publication No. 85-23 revised, 1985). Pancreatic β cells were obtained from young adult male Wistar rats (200–250 g) as described previously (3). Isolated cells from rat pancreatic islets were seeded for 24 h in bacteriological Petri dishes to promote fibroblast attachment. After this period, supernatants with islet cells were recollected and cultured under control conditions in cell culture Petri dishes (Corning) with RPMI 1640 medium (with 11.6 mM glucose) supplemented with 5% fetal bovine serum, 100 μg/ml streptomycin, 100 units/ml penicillin, and 0.25 μg/ml Fungizone. Experimental cells were cultured in the presence of either dbcAMP (5 mM), NGF (50 ng/ml), or NGF + dbcAMP (50 ng/ml, 5 mM).

PC12 cells were maintained in RPMI 1640 medium supplemented with 10% horse serum and 2.5% fetal bovine serum (GIBCO), 100 μg/ml streptomycin, 100 units/ml penicillin, and 0.25 μg/ml Fungizone.

Immunocytochemistry. Freshly isolated single cells or cells cultured for 11 days were processed (3) and incubated overnight with rabbit anti-mouse NGF antibody (Sigma) and guinea pig anti-porcine insulin antibody (Incstar, Stillwater, MN), as recommended by the supplier's technical bulletins. Finally, a second CY5-conjugated (excitation = 650 nm, emission = 670 nm) goat anti-rabbit IgG antibody was added in the case of NGF detection and a fluorescein isothiocyanate-conjugated (excitation = 494 nm, emission = 520 nm) goat anti-guinea pig IgG antibody in the case of insulin detection. Cells were observed under confocal microscopy using the red channel for CY5 (NGF) and the green channel for fluorescein isothiocyanate (insulin).

PC12 cells were stained by the avidin–biotin complex method (3) using a rabbit anti-Trk A antibody (Santa Cruz Biotechnology) as recommended by the supplier, followed by an incubation with biotin-labeled goat anti-rabbit IgG antibody and development with avidin-peroxidase complex and 3,3′-diaminobenzidine as final substrate, to form a brown reaction product.

Cytoplasmic RNA Isolation and Reverse Transcriptase (RT)–PCR for NGF and Trk A Detection. Cytoplasmic RNA was extracted from cells cultured for 5 days under the different experimental conditions described above. The TRIzol reagent (catalogue no. 15596-026; GIBCO/BRL) was used as instructed in the technical bulletin. Cells were lysed directly in a culture dish by adding 1 ml of TRIzol reagents per 1×10^6 cells (12). Cytoplasmic RNA (200 ng) was reverse transcribed following the supplier's recommended protocol (Perkin–Elmer catalogue no. N808-0143).

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Abbreviations: NGF, nerve growth factor; dbcAMP, dibutyryl cAMP; RT, reverse transcriptase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CM, conditioned medium.

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RT–PCR were carried out with 200 ng of total RNA for NGF mRNA detection in β cells. The PC12 pheochromocytoma and the C6 glioma cell lines were used as negative and positive controls, respectively. The reaction was also performed with freshly isolated β cells. A parallel reaction was carried out in the same mRNA sample using the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) housekeeping gene for quantitative purposes.

All oligonucleotide primers were synthesized and used to prime the amplification of the cDNA template. Based on the published sequences of mouse NGF (13), Trk A (14), and GAPDH (15), we chose the 5′-GGCATGCTGGACCCAAGCTC-3′ sequence for the sense (5′) primer and the 5′-GCGCTTGCTCCGGTGAGTCC-3′ for the antisense (3′) primer for NGF mRNA detection, the 5′-GCAAAGCCGTGGAACAG-3′ sequence for the sense (5′) primer and the 5′-CCCATGGCGCATGTACT-3′ for the antisense (3′) primer for Trk A mRNA detection, and the 5′-GCCCCCATGTTTGTGAT-3′ sequence for the sense (5′) primer and the 5′-GCCCCAGCATCAAAGGT-3′ sequence for the antisense (3′) primer in the case of GAPDH mRNA amplification. Twenty-five cycles of amplification were performed with an annealing temperature of 59°C for NGF and 57°C for Trk A and GAPDH.

Reaction products were sequenced and proved to have a 100% identity with the sequence reported for the *NGF*, *Trk A*, and *GAPDH* genes (13–15). The amplified material was visualized by ethidium bromide staining, following 1% agarose gel electrophoresis. Resolved PCR bands were transferred to a Hybond-N nylon membrane. Membranes were cross-linked by exposure to UV light.

To confirm the specificity of the RT–PCR, internal antisense (3′) oligonucleotides were designed with the following sequence: 5′-ATCTAGACTTCCAGGCCC-3′ for NGF, 5′-GGAGGGCAGAAAGGAAG-3′ for Trk A, and 5′-GTGGATCTGACATGCCG-3′ for GAPDH. Hybridizations were carried out with the internal oligonucleotides labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Band intensities were determined with the use of a PhosphorImager.

Bioassay with PC12 Cells. Islet cells were precultured for 24 h under control conditions to ensure proper attachment to culture dishes. After this time, cells were cultured with a chemically defined medium supplemented with 20 mM glucose for 4 h. The chemically defined medium consisted of RPMI 1640 supplemented with insulin–transferrin–sodium selenite solution (1:100; GIBCO), putrescine (100 μM), and progesterone (20 nM). PC12 cells were subcultured for 24 h in the supernatants from the β cell-conditioned medium (CM). At the end of this incubation, PC12 cells were stained with rabbit anti-Trk A antibody as described above and the percentage of neurite-bearing cells was counted. A neurite-bearing cell was defined as a cell with one or more processes, at least 10 μm long. The length of the processes was measured with the aid of the JAVA video analysis software (Jandel Scientific 1.4, Corte Madera, CA) by projecting the image of the cell on a monitor attached to a video camera and a Nikon Axiophot inverted microscope. To determine specificity of NGF effects, a rabbit anti-mouse NGF-neutralizing antibody (Sigma) was added to the CM.

Detection of NGF Secretion by Pancreatic β Cells. Secretion of NGF by pancreatic β cells was determined with a two-site ELISA assay NGF Emax Immunoassay System (catalogue no. G3550; Promega). Experiments were performed as instructed by the technical bulletin using the supernatants of β cells incubated for 4 h in HBSS (Sigma) with 5, 11, or 20 mM glucose as well as in 11 mM glucose with 40 mM KCl or 5 mM dbcAMP.

RESULTS

Presence of NGF Protein in Rat Pancreatic β Cells. The expression of NGF by pancreatic β cells was demonstrated using immunocytochemistry for protein detection and RT–PCR for detection of mRNA. The latter technique was used because of the limited amount of cytoplasmic RNA that can be obtained from primary cultures. To obtain cytoplasmic RNA from pure islet cells, without fibroblasts a differential seeding technique was performed (as described in *Experimental Procedures*).

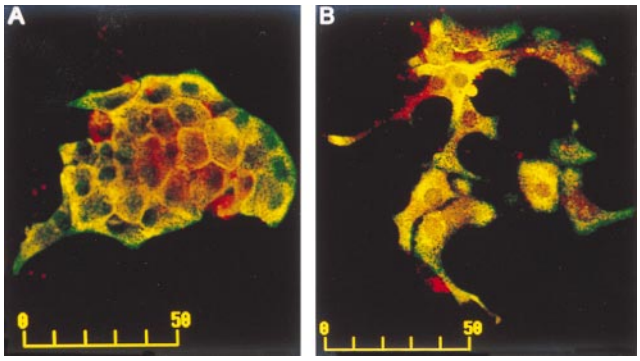

FIG. 1. Presence of NGF in adult β cells. β cells cultured for 11 days with NGF (50 ng/ml) and dbcAMP (5 mM). (*A*) Cells cultured under control conditions for 11 days. (*B*) Cells cultured for 11 days in the presence of NGF + dbcAMP. Bar, 50 μm.

Because pancreatic islets contain several cell types, a double labeling technique was used to determine whether β cells express NGF: a CY5 probe (red fluorescence) for NGF and fluorescein isothiocyanate probe (green fluorescence) for insulin. The experiments of Fig. 1 were performed after 11 days of cell culture, when effects of NGF and dbcAMP on β cell morphology were evident (3). The yellow staining, a consequence of colocalized green and red dyes, represents cells positive for both insulin and NGF. All control (Fig. 1*A*) and treated (Fig. 1*B*) β cells are NGF positive (red or yellow); of these, approximately 3% of the cells are completely negative

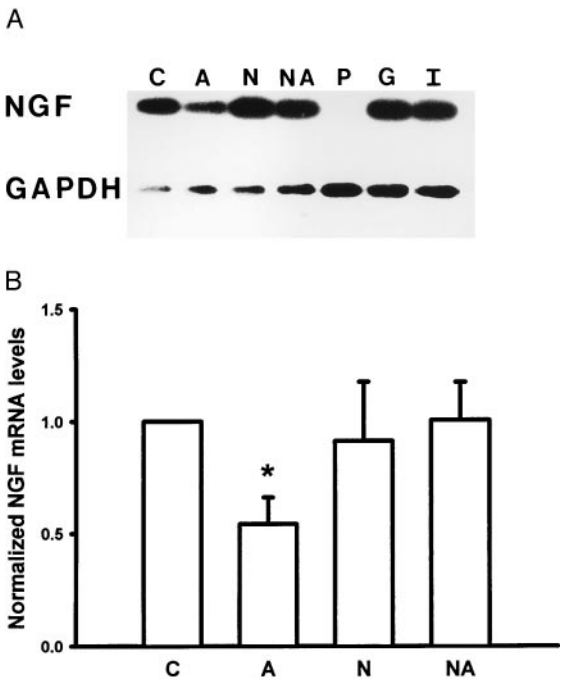

FIG. 2. NGF mRNA levels are dependent on dbcAMP. (*A*) RT–PCR reaction products from rat pancreatic β cells cultured under different experimental conditions. (*B*) dbcAMP induced a 46% decrease in NGF mRNA levels ($n = 6$; $^*P < 0.01$, Student's *t* test) with respect to control, whereas treatment with NGF and NGF + dbcAMP had no effect. C, control; A, dbcAMP; *N* = NGF; N + A, NGF + dbcAMP; P, PC12 cells; G, C6 glioma; I, freshly isolated islet cells.

for insulin (red only). The localization of NGF and insulin staining in the cells was not always in the same areas. This could indicate that NGF and insulin are localized in different secretory granules.

Effects of NGF and/or dbcAMP on NGF Steady-State mRNA Levels. mRNA for NGF was present in fresh and cultured isolated pancreatic β cells (Fig. 2), implying that constitutive expression *in vivo* continues during *in vitro* culture. Moreover, the presence of NGF protein and its mRNA was also found in the rat insulinoma cell line RINm5F (data not shown).

We determined the effect of culturing islet cells for 5 days in the presence of NGF and/or dbcAMP on the steady-state levels of NGF mRNA. We found that although NGF alone has no effect, the presence of a saturating concentration of 5 mM dbcAMP in the culture induces a 46% decrease in the levels of mRNA for NGF. However, when cells were cultured with both compounds, this latter decrease in NGF mRNA was not observed (Fig. 2).

Pancreatic β Cells Secrete Biologically Active NGF. The above data demonstrate that β cells contain NGF mRNA and protein. However, they do not indicate whether β cells secrete biologically active NGF. To explore this possibility, we used PC12 responsiveness to NGF as a bioassay because these cells are well known to extend neurites in response to this factor (16). In the bioassay, neurite outgrowth was assessed in PC12 cells grown for 24 h in the presence of a CM from β cells stimulated with 20 mM glucose. Neurite outgrowth in PC12 cells cultured in the β cell CM was fourfold higher than in control cells. The addition of a NGF-neutralizing antibody blocked this effect (Fig. 3). This result indicates that pancreatic β cells secrete a biologically active NGF-like molecule.

NGF Secretion by Pancreatic β Cells in Response to Increasing Extracellular Glucose Concentrations. Previous data obtained with the reverse hemolytic plaque assay technique show that insulin secretion in single cells increases nearly three times in 20 mM glucose with respect to 5 mM glucose (17). To identify and measure NGF secretion in single β cells, we used an ELISA.

Pancreatic β cells stimulated with 20 mM glucose secrete 50–180 pg/ml NGF, two to three times more than cells incubated with 5 mM glucose (Fig. 4). Moreover, KCl (40 mM) + 11 mM glucose induces a threefold increase in NGF secretion with respect to cells incubated with 5 mM glucose. On the other hand, the addition of dbcAMP + 11 mM glucose induces a 67% decrease in NGF secretion with respect to the group treated only with 11 mM glucose.

Effects of NGF and/or dbcAMP on Trk A mRNA Steady-State Levels. The effects of NGF are mediated by Trk A receptors, which are present in pancreatic β cells (6). Because pancreatic β cells synthesize and secrete NGF and the treatment of β cells with NGF and/or dbcAMP induces the extension of neurite-like processes, we investigated the effects of these molecules on the levels of Trk A mRNA.

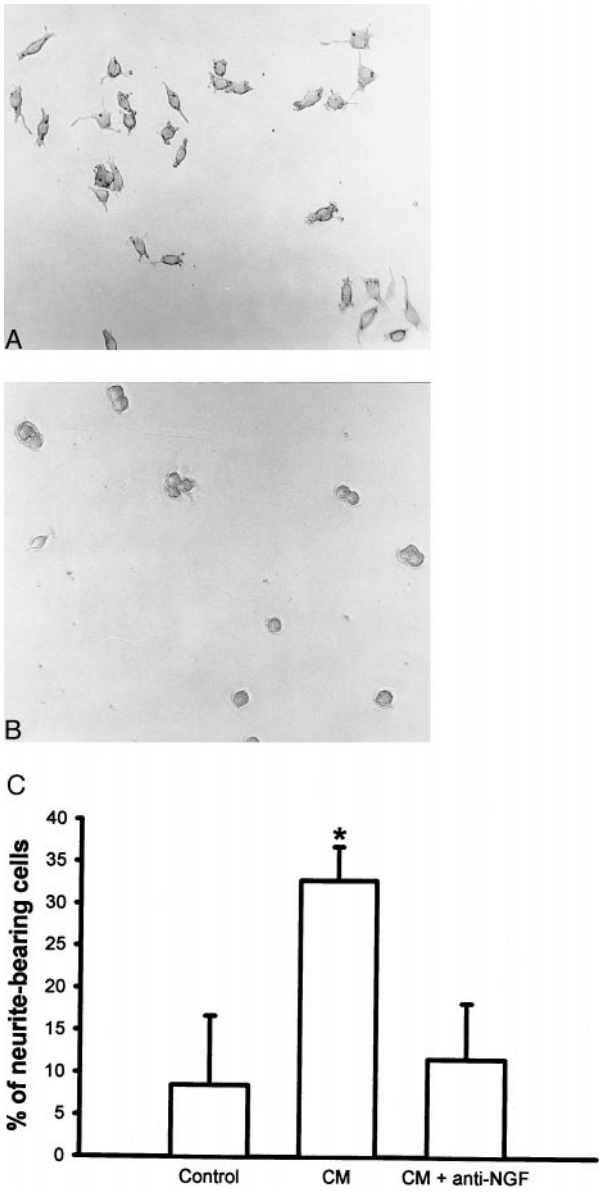

FIG. 3. Pancreatic β cells secrete a molecule with NGF-like biological activity. (*A*) PC12 cells cultured in the presence of the CM extended neuritic processes. (*B*) When an NGF-neutralizing antibody was added to the CM, extension of neurites by PC12 was abolished to control levels because cells did not extend neuritic processes and eventually (48 h) detached from the plate. (*C*) The addition of the CM to PC12 cells induced a fourfold increase in the percentage of neurite-bearing cells ($n = 4$; $^*P < 0.01$, ANOVA, followed by two-tailed Student's t test). Control, chemically defined medium (20 mM glucose); CM, conditioned medium (20 mM glucose); CM + anti-NGF, conditioned medium (20 mM glucose) + anti-NGF antibody.

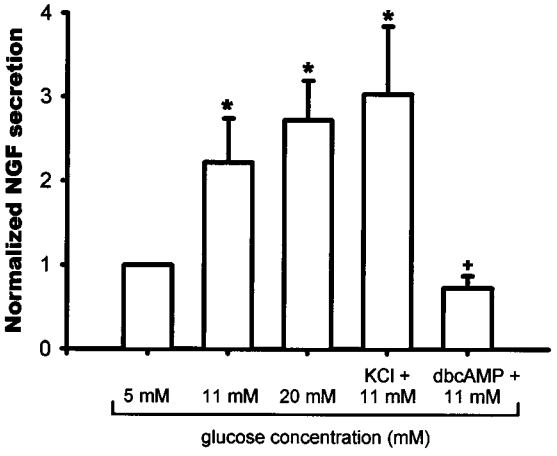

FIG. 4. Pancreatic β cells secrete NGF in response to different glucose concentrations. Cells stimulated with 20 mM glucose secrete 50–180 pg/ml NGF. Cells stimulated with 5 mM glucose secrete 20–60 pg/ml NGF. NGF secretion in KCl (40 mM) + 11 mM glucose was significantly different from secretion in 5 mM glucose, but not from secretion in 20 mM glucose (50–220 pg/ml vs. 50–180 pg/ml). NGF secretion is diminished by 67% when cells are cultured with dbcAMP (5 mM) + 11 mM glucose with respect to 11 mM glucose alone. An $*$ denotes $P < 0.05$ with respect to 5 mM glucose; + denotes $P < 0.05$ with respect to 11 mM glucose (Student's t test, $n = 4$).

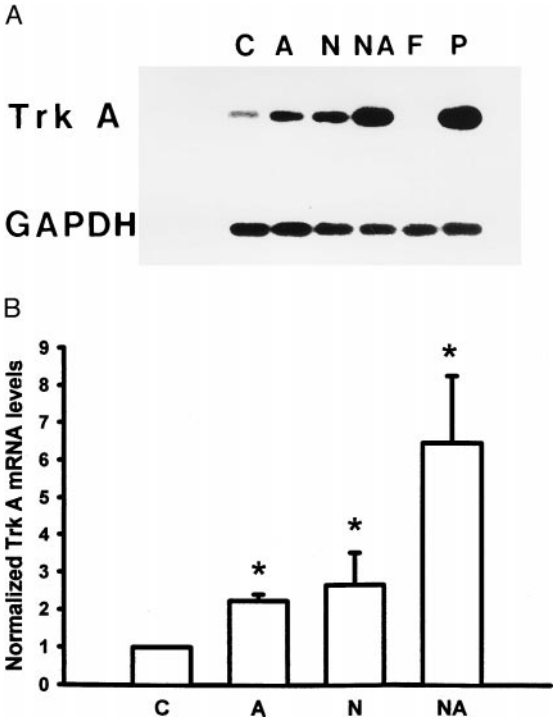

FIG. 5. dbcAMP and/or NGF up-regulate Trk A mRNA levels. (*A*) RT–PCR reaction products for Trk A detection under different experimental conditions. (*B*) dbcAMP and NGF each induced a twofold increase in Trk A mRNA levels ($P < 0.05$) with respect to control, whereas treatment with NGF + dbcAMP induced a sixfold increase with respect to control ($n = 6$; $P < 0.05$, Student's *t* test). C, control; A, dbcAMP (5 mM); *N*, NGF (50 ng/ml); NA, NGF + dbcAMP; F, fibroblasts; P, PC12 cells.

The RT–PCR technique was used to determine whether NGF and/or dbcAMP had any effect on the levels of Trk A mRNA. As shown in Fig. 5, treatment of β cells with NGF or dbcAMP induced an increase in Trk A mRNA levels. The most potent enhancement on Trk A mRNA levels was observed when cells were treated with NGF and dbcAMP together. In this case, the increase in Trk A mRNA levels was sixfold with respect to control levels.

DISCUSSION

In the present study, we have demonstrated for the first time that pancreatic β cells synthesize and secrete NGF. Moreover, biologically active pancreatic NGF is secreted in response to changes in extracellular glucose concentrations similar to the dependence of insulin secretion on external glucose. This parallel increase in the secretion of both molecules suggests that glucose-induced depolarization leads to a calcium influx and finally to exocytosis and secretion. High potassium, a known secretagogue of insulin (18) that depolarizes β cells, also leads to an increase in NGF secretion.

It has been shown that insulin granules are complex and contain products from nearly 50 independent genes besides insulin and peptide C (19). Although our immunocytochemical results suggest that insulin and NGF are not stored in the same compartment, they do not preclude simultaneous secretion of NGF and insulin. However, not all insulin secretagogues have the same effect on NGF release. Our data show that dbcAMP, which increases insulin secretion (20), inhibits NGF secretion induced by 11 mM glucose.

The fact that β cells are both secreting and responding to NGF implicates an autocrine and/or paracrine role for NGF in the pancreas. Such a feedback scenario must include the regulation of the receptor component. For example, it has been suggested that up-regulation of Trk A receptors in insulinoma INS-1 and forebrain cholinergic neurons can increase sensitivity to NGF (11, 10). Treatment of β cells with NGF or dbcAMP induced an increase in Trk A mRNA levels. The most potent enhancement on Trk A mRNA levels was observed when cells were treated with NGF and dbcAMP together. This result correlates with our previous finding (3) of an additive effect of NGF and dbcAMP on β cell morphology.

The increased expression of Trk A mRNA by dbcAMP-treated cells suggests that a higher number of membrane receptors for NGF may exist in these cells. This would render cells more sensitive to NGF actions due to a higher probability of interaction between the receptors and the available NGF. Prolonged NGF exposure induces an increase of mRNA for Trk A in PC12 cells (21) and in cholinergic neurons (11), where NGF may play a critical role in *Trk A* gene expression during development (22). It has also been shown that growth hormone induces an increase in NGF-binding sites in the insulinoma cell line INS-1 (11). These studies also suggest that the increase in high-affinity NGF-binding sites may render cells more sensitive to the actions of NGF.

On the other hand, we observed the presence of mRNA for NGF in freshly isolated islet cells and in cells cultured for 5 days. Experiments performed by other research groups have not been able to demonstrate the presence of NGF in pancreatic fetal β cells in primary cultures nor in insulin-secreting cell lines (9). This is probably because of the use of techniques not sensitive enough to detect low levels of mRNA; however, we do not know whether fetal β cells synthesize NGF.

We also describe that the presence of a saturating concentration of 5 mM dbcAMP in the culture induces a 46% decrease in the levels of mRNA for NGF. Although NGF itself does not modulate NGF mRNA levels, it apparently protects against the aforementioned down-regulation by dbcAMP. Finally, these down-regulatory effects of dbcAMP on NGF secretion and NGF mRNA steady-state levels could be overcome by the increase on Trk A receptors on the membrane surface of β cells exposed to NGF, permitting β cells to respond even if the concentration of the trophic factor is low.

The sustained sensitivity of β cells to NGF could be necessary for the maintenance of β cell function. NGF treatment enhances glucose-stimulated insulin secretion in fetal rat β cells (23) and in the INS-1 cell line (24), and NGF treatment prevents the loss of sensitivity to external glucose concentration changes in β cells cultured *in vitro* (3). The action mechanism for NGF could be through the mitogen-activated protein kinase pathway (25). It has been described that glucose-induced insulin secretion in INS-1 cells involves this pathway and that NGF can potentiate this effect (24). Moreover, NGF could be necessary for the maintenance of β cell electrical activity, since it induces a 30% increase in sodium current density of adult rat β cells after 5 days in culture (2).

Furthermore, NGF has been implicated in islet morphogenesis and ontogeny because the use of an inhibitor of Trk A activity, K252a, prevents islet maturation (9). These data suggest that NGF may play a relevant role in normal function of pancreatic islet.

NGF, as well as insulin, is secreted in response to increasing glucose concentrations, suggesting both an autocrine and endocrine role for NGF *in vivo*. Finally, it is possible that a deficient secretion of NGF by pancreatic β cells contributes to the decreased serum levels of NGF in patients with diabetic neuropathies (8), and may, along with decreased insulin levels, be causally related to the development of neuropathology.

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