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Neuron-Like Phenotypic Changes in Pancreatic β-Cells Induced by NGF, FGF, and dbcAMP

Román Vidaltamayo, Ma. Carmen Sánchez-Soto, Tamara Rosenbaum, Teresa Martínez-Merlos, and Marcia Hiriart

Department of Bioenergetics, Institute of Cellular Physiology, Universidad Nacional Autónoma de México, México

We studied the effects of nerve growth factor (NGF), fibroblast growth factor (FGF), and dibutyryl-cAMP (dbcAMP) on rat pancreatic β-cell morphology and of NGF and dbcAMP on insulin secretion. After 2 wk in culture, nearly 3% of β-cells extended neurite-like processes spontaneously; when cells were treated with NGF, almost 30% of them extended processes. In the presence of dbcAMP, almost all β-cells flattened, and the extension of neurite-like processes was more pronounced in fetal than in adult cells. The most prominent effect, regardless of age, was observed in cells treated with NGF and dbcAMP together, since the percentage of neurite-like bearing β-cells increased to 50%. β-cells cultured under these conditions maintained their immunoreactivity to insulin and nearly all β-cells and their neurite-like processes were also positive to GABA, tubulin, tau protein, and N-CAM. FGF increased the percentage of adult β-cells bearing neurite-like processes to 13%, and FGF and dbcAMP applied together to 40%. β-cells treated with NGF and dbcAMP for 5 to 7 d preserved their capability to secrete the hormone in response to different extracellular glucose concentrations. Insulin secretion of dbcAMP-treated β-cells was 2.5-fold higher than in control cells. NGF-treated cells were able to discriminate between different glucose concentrations, a property lost in control cells with time in culture.

Key Words: NGF; cAMP; FGF; pancreatic β-cells; insulin secretion; tau protein.

Introduction

Nerve growth factor (NGF) is of critical importance in the development and maintenance of sensory and sympathetic neurons. Other cell types that respond to NGF include chromaffin adrenal cells (reviewed by Levi-Montalcini, 1987), and the tumoral cell lines PC12, derived from a rat pheochromocytoma (Greene and Tischler, 1976) as well as different insulin-secreting cell lines (Polak et al., 1993; Scharfmann et al., 1993; Tazi et al., 1995).

Phenotypical changes promoted by NGF include the extension of neurite-like processes; these changes are enhanced by a combined treatment with NGF and cAMP in normal and tumoral cells derived from the adrenal medulla (Schubert and Whitlock, 1977; Heidemann et al., 1985; Pacheco Cano et al., 1990).

Other important trophic factors are those derived from fibroblasts. Fibroblast growth factors (FGF) are capable of mimicking many actions of NGF in hippocampal neurons and in cerebral cortical neurons, as well as in the PC12 cell-line (Rydel and Greene, 1987).

It has been described that mice β-cells are able to extend neurite-like processes spontaneously. These processes contain neurite-specific intermediate filament proteins (Teitelman, 1990). However, the effects of NGF, FGF, and cAMP have not been fully characterized in normal pancreatic β-cells.

Recent reports show that cell lines RINm5F, INS-1, as well as rat β-cells, express high- and low-affinity nerve growth factor receptors, trk-A and $p75^{ngfr}$, respectively (Scharfmann et al., 1993; Kanaka-Ganteinbein et al., 1995a), and that RINm5F cells respond to NGF extending neuron-like processes (Polak et al., 1993).

Although β-cells express NGF receptors, it has not been studied if NGF induces neuron-like processes or physiological changes in normal β-cells, nor if the sensitivity to the factor depends on the age of cells.

In the present study, we investigated the effects of NGF and dbcAMP on the phenotype of rat adult and fetal pancreatic β-cells in primary culture, and of FGF on adult β-cells. We also evaluated the initial effects of NGF and dbcAMP on β-cell physiology, focusing on the insulin secretory response of treated cells to different glucose concentrations.

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Author to whom all correspondence and reprint requests should be addressed: Dr. Marcia Hiriart, Department of Bioenergetics, Institute of Cellular Physiology, U.N.A.M., Mexico D.F., 04510 Mexico.

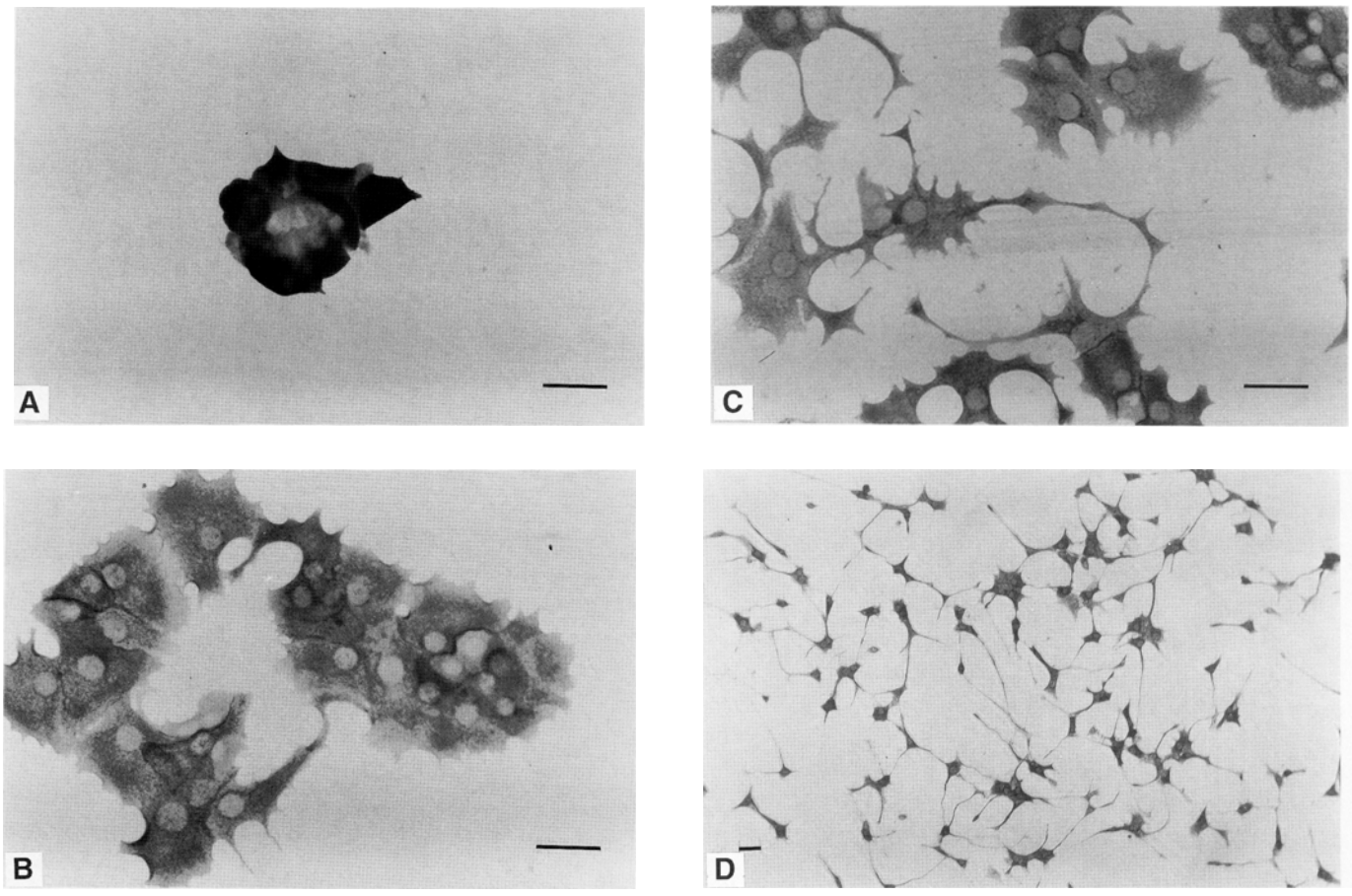

Fig. 1. Effect of NGF and cAMP on pancreatic β-cell morphology. Isolated adult islet cells were cultured for 2 wk, and processed for insulin immunocytochemistry. Culture conditions were as follows: **(A)** Control cells. **(B)** Cells treated with 5 m*M* dbcAMP. **(C, D)** Cells treated with 2.5S NGF (40 ng/mL) and 5 m*M* dbcAMP. Bar, 20 μm.

Results

NGF and dbcAMP Induce Phenotypical Changes in β-cells

After 2 wk in culture, control β-cells showed a characteristic round appearance and formed small clusters, as shown in Fig. 1A. A small percentage of control β-cells developed neurite-like processes spontaneously (Table 1).

The percentage of adult β-cells bearing neurite-like processes did not significantly increase when cells were exposed during 2 wk to the complete molecule of NGF (7S NGF). Instead, when cells were cultured with the biologically active NGF β-subunit (2.5S NGF) (Angeletti and Bradshaw, 1971), 26% of adult β-cells developed neurite-like processes, as shown in Table 1.

In contrast, 29% of fetal β-cells developed neurite-like processes when exposed to 7S NGF for the same period (Table 1).

It has been shown in other cell types that permeable analogues of cAMP can partially mimic the effects of NGF and act synergistically with NGF to promote neurite outgrowth (Schubert and Whitlock,1977; Heidemann, et al., 1985). As shown in Fig. 1B, most of the β-cells exposed in culture to 5 m*M* dbcAMP, flattened, acquired stellate-like forms, and tended to converge forming monolayers, not clumps. Under this condition, 18.8% of adult cells and 30.5% of fetal cells extended neurite-like processes (Table 1).

We observed the most dramatic changes in adult and fetal β-cells when they were cultured with both NGF (7S or 2.5S) and dbcAMP. In this case, most β-cells from both ages flattened and converged, forming large groups of cells in which peripheral β-cells developed long neurite-like processes (Fig. 1C, D). Nearly 50% of adult and fetal β-cells extended neurite-like processes (Table 1).

In fetal β-cells, we did not observe an increase in neurite-like process length. However, in adult β-cells, the processes were 50% longer when treated with 7S NGF and dbcAMP, and 117% longer when treated with 2.5S NGF and dbcAMP, as compared to control cells (Table 1). These results suggest different but synergistic effects for NGF and dbcAMP on β-cell morphology.

Also, to determine whether the modified insulin-positive cells were also synthesizing GABA, we processed cultures of islet cells for double immunocytochemical visualization of insulin and GABA antibodies. Almost all treated and untreated β-cells, as well as the neurite-like processes, were doubly labeled, as shown in Fig. 2.

Table 1
Morphological Changes of Pancreatic β-Cells Stimulated by NGF and dbcAMP

Group	% Neurite Bearing Cells	Neurite Length, μm
Adult Cells		
Control (9)[a]	3.7 ± 0.4	46.3 ± 1.4
dbcAMP (9)	18.8 ± 3.4[b]	61.2 ± 0.5[c]
NGF 7S (5)	5.2 ± 0.9	50.8 ± 1.4
NGF 2.5S (4)	26.15 ± 2.2[b]	57.9 ± 0.7
NGF 7S + dbcAMP (5)	50.2 ± 8.8[b]	69.2 ± 0.3[c]
NGF 2.5S + dbcAMP (4)	43 ± 3.5[b]	100.7 ± 1.2[b]
FGF (4)	12.6 ± 1.4[b]	61.3 ± 1.0[c]
FGF + dbcAMP (4)	40 ± 3.0[b]	81 ± 10.4[c]
Fetal Cells		
Control (4)	2.5 ± 1.5	36.7 ± 8.5
dbcAMP (4)	30.5 ± 7.7[c]	28 ± 0.4
NGF 7S (4)	29 ± 3.0[b]	50.8 ± 0.7
NGF 7S + dbcAMP (4)	51.5 ± 11.5[b]	42 ± 0.3

[a] The number in parentheses denotes the number of independent experiments assessed, each one per duplicate.
[b] $P < 0.01$, with respect to the control.
[c] $P < 0.05$, with respect to the control.

Microtubule Involvement in Neurite-Like Extension in β-Cells

The outgrowth and maintenance of neurite-like processes require the assembly of microtubules. Tau protein is a microtubule-associated protein (found primarily in neuronal tissue) that promotes microtubule assembly and stabilization (Olmsted, 1986; Matus, 1988).

We searched for β-tubulin (data not shown) and tau presence in insulin positive-β cells, by double staining with ICC and indirect IF. As shown in Fig. 3, in cells treated with NGF and dbcAMP, insulin and tau were present in the cell body and the neurite-like processes. However, both antigens were also present in control cells, but no quantitative experiments to detect if the concentration of these molecules was modified by the treatment were performed.

N-CAM Expression in β-Cells

The neural adhesion molecule (N-CAM) is a cell surface glycoprotein expressed in a variety of cell types, including neurons and glia, that has been implicated in the cell-to-cell recognition process during development. NGF has been described to induce a four- to fivefold increase in relative levels of N-CAM in PC12 cells (Prentice et al., 1987). We assessed the expression of N-CAM in β-cells under different treatments. In Fig. 4, we show cells treated with NGF and dbcAMP double labeled for insulin and N-CAM. It is clear that all insulin-positive cells also contain N-CAM (in both the cell body and the neurite-like processes); however, control cells also contained N-CAM, and we did not evaluate to see if the relative levels of N-CAM were modified by NGF.

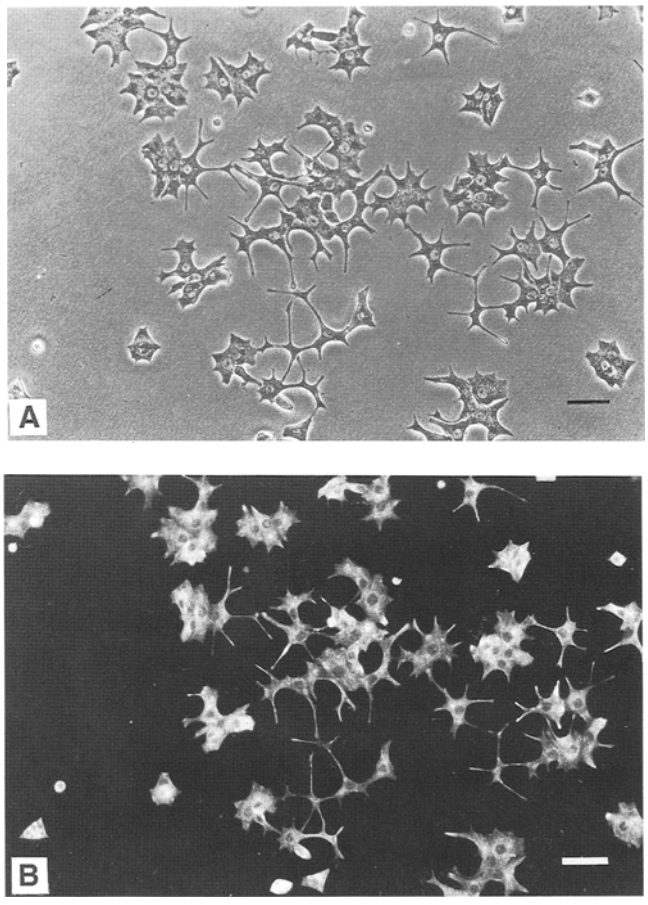

Fig. 2. Presence of GABA in adult β-cells. β-cells cultured for 2 wk with 2.5S NGF and dbcAMP. **(A)** Immunocytochemistry for insulin. **(B)** Immunofluorescence for GABA at a light wavelength of 450–490 nm. Bar, 20 μm.

FGF Also Induces Morphological Changes in Adult β-Cells

As stated, after 2 wk in culture, 3% of adult β-cells developed neurite-like processes spontaneously under control conditions, and when cells were treated with dbcAMP, the percentage raised to 19% (Table 1).

In the presence of FGF, 13% of cells developed neurite-like processes, and when dbcAMP was also included with FGF, 40% of the cells were modified (Table 1). Neurite-like processes were 30% longer in cells treated with FGF, and 75% longer when treated with FGF and dbcAMP, as compared to control cells (Table 1), suggesting again that dbcAMP has a synergistic effect with FGF on β-cell morphology.

Insulin Secretion by Single Adult β-Cells Treated with NGF and dbcAMP

In order to determine if the initial phenotypical changes were associated to functional modifications, insulin secretion of isolated β-cells exposed to the different NGF and dbcAMP conditions was measured with a reverse hemolytic plaque assay (Neill and Frawley, 1983). We decided to use β-cells between d 5 and 7 in culture because phenotypic

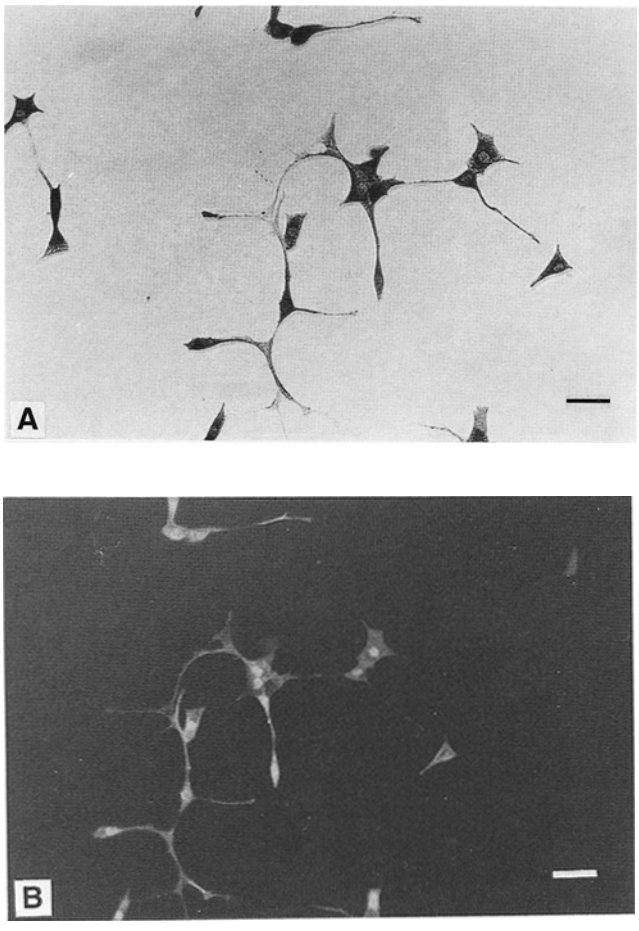

Fig. 3. Tau protein is present in β-cells. Adult islet cells were cultured for 2 wk in the presence of 2.5S NGF and dbcAMP. **(A)** Immunocytochemistry for insulin. Bar, 25 μm. **(B)** Immunofluorescence for tau protein. Bar, 20 μm.

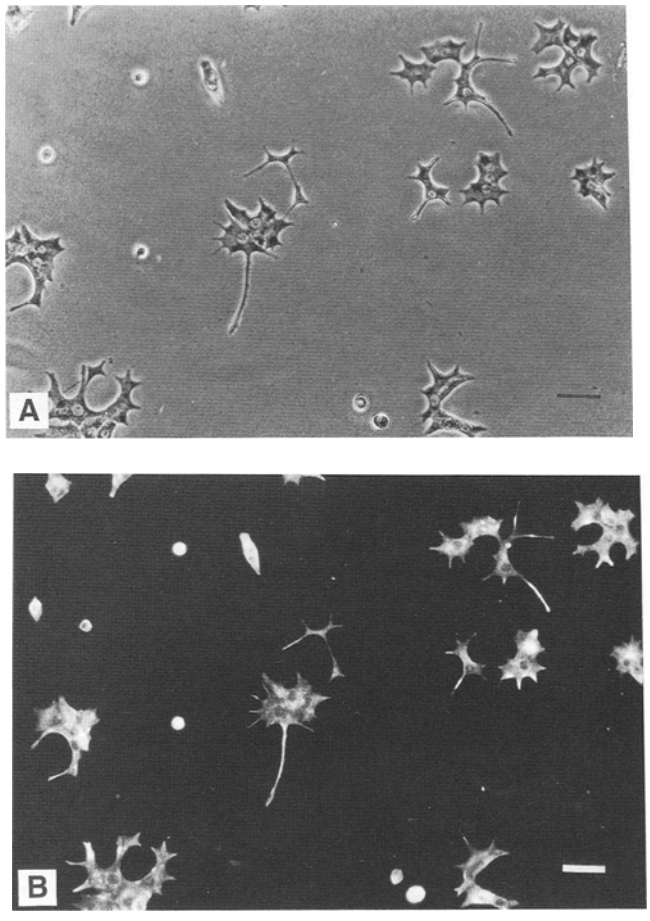

Fig. 4. N-CAM presence in β-cells. Adult islet cells were cultured for 2 wk in the presence of 2.5SNGF and dbcAMP. **(A)** Immunocytochemistry for insulin. **(B)** Immunofluorescence for N-CAM. Bar, 20 μm.

changes are mild at this time, and it is possible to detach cells from Petri dishes and assay them for insulin secretion, thereby avoiding cell damage.

The effect of NGF and dbcAMP on total insulin secretion index of adult β-cells after 5–7 d in culture is shown in Fig. 5. Control cells secreted insulin in response to glucose stimulation; however, their capability to discriminate between different concentrations of glucose declined, as their response to 20.6 m*M* glucose was not significantly different from the basal secretion (in 5.6 m*M* glucose).

Insulin secretion by NGF-treated cells was not significantly different from that of control cells; however, NGF-treated cells were more sensitive to glucose concentration changes, as the insulin secretion index in 20.6 m*M* glucose was 79% higher than in 5.6 m*M* glucose.

The response to glucose of dbcAMP cultured β-cells was remarkable, even though the compound was not present during the assay. The response of the dbcAMP-treated β-cells to 5.6 m*M* and 20 m*M* glucose was in both cases nearly threefold higher than in control cells. Moreover, the insulin secretion index in dbcAMP-treated cells increased 39% in high glucose to basal glucose, respectively.

Finally, total insulin secretion of β-cells cultured with NGF and dbcAMP was very similar to that of cells cultured with dbcAMP only. The cellular mechanism that explains the increase in insulin secretion by dbcAMP involves the amplification of hormone secretion by individual cells, and the increase in the percentage of plaque-forming cells, in both glucose concentrations (Table 2).

Discussion

Pancreatic β-cells manifest an extraordinary morphological plasticity in vitro. Teitelman (1990) observed that 10–20% of β-cells from adult CD-1 mice developed neuritic processes spontaneously and demonstrated that the cytoplasmic elongations were positive to neurofilament antibodies. In our experiments, only 3% of rat β-cells developed neurite-like processes spontaneously; the differences between the two studies could be owing to species variation and/or the percentage of fetal bovine serum used in the culture media (10 vs 1% in our experiments). It is important to note that rat pancreatic islet cells survive better in the presence of 10% fetal bovine serum (FBS) in the culture

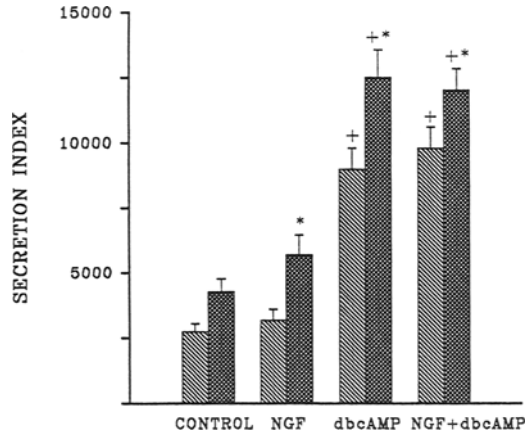

Fig. 5. Effect of 7S NGF and dbcAMP on insulin secretion by isolated adult β cells. Islet cells were cultured for 5 d under the different above-mentioned conditions and were harvested and challenged with 5.6 mM (▨) and 20.6 mM (▩) glucose concentrations for 1 h. Insulin secretion of isolated β-cells was measured with the RHPA. Each bar represents the mean ± SEM of 5 separate experiments. *Denotes the significance level with respect to control cells ($P < 0.01$). +Denotes significance level of 20.6 m*M* glucose with respect to 5.6 m*M* glucose in the respective group ($P < 0.01$).

Table 2
Insulin Secretion by Single β Cells Cultured with NGF and dbcAMP

Treatment	Glucose, m*M*	% of plaque-forming cells	Plaque area, μ*M*	*n*
Control	5.6	62 ± 3.7	4415 ± 499	5
	20.6	71 ± 5.2	6015 ± 696	5
NGF	5.6	63 ± 1.5	5039 ± 663	4
	20.6	74 ± 3.2[a]	7691 ± 1031	4
dbcAMP	5.6	75 ± 2.9[b]	11977 ± 1095[b]	5
	20.6	83 ± 2.3[b]	15075 ± 1278[b]	5
NGF+	5.6	79 ± 1.5[b]	12413 ± 1014[b]	5
dbcAMP	20.6	85 ± 1.5[b]	14139 ± 981[b]	5

[a] Denotes significance level with respect to 5.6 m*M* glucose of the same group of β-cells, $P < 0.05$.

[b] Denotes the significance level with respect to control cells, $P < 0.01$.

medium; however, we used only 1% FBS to minimize serum factors that could modify the neurite-like processes outgrowth by β-cells (Ziller, et al., 1983).

After two weeks in culture, the effects of NGF on β-cell morphology were clear and the differential responses of adult and fetal β-cells to the trophic factor are interesting.

In adult β-cells, 7S NGF could only stimulate neurite-like processes outgrowth in the presence of dbcAMP; however, 2.5S NGF alone was sufficient to induce the phenotypic changes described above.

In contrast, fetal β-cells did respond to 7S NGF indicating that β-cell responsiveness to trophic factors changes with the developmental age of the cell. Differences in β-cell plasticity depending on age could be due, in part, to a low expression of NGF receptors in adult cells, which could be overcome using the active β-subunit of NGF.

Similar to what has been observed in other cell types (Gunning et al.,1981), dbcAMP partially mimics the effects of the trophic factors on β-cell morphology and acts synergistically with them to induce process outgrowth; since β-cells were cultured with NGF or FGF and dbcAMP together, nearly 50% and 40%, respectively, extended neurite- like processes.

This synergic action between trophic factors and dbcAMP is also observed on the increase of the neurite-like process length. In adult β-cells cultured with 7S NGF and dbcAMP, the processes were 50% longer than those present in control cultures. Moreover, when cells were exposed to 2.5S NGF and dbcAMP the processes were 117% longer. Comparably, we observed a 75% increase in the process length with FGF and dbcAMP.

On the other hand, the length of the neurite-like processes in fetal β-cells tended to increase with NGF and with dbcAMP, but it was not statistically different from control cells that infrequently developed neurite-like processes. Surprisingly, the mean length of the processes in fetal cells was near one-half the mean length reached by adult cells. This difference could be owing to the developmental stage, since immature cells generate less stable microtubules, as it has been observed in the developing brain (Nunez, 1986; Matus, 1988).

This synergy between dbcAMP and NGF or FGF on neurite-like extension by β-cells could be owing to an increase in intracellular cAMP; this could indicate the activation of cAMP-dependent protein-kinases (PKA) or other signal transduction mechanisms. However, the question of whether PKAs play a crucial role in the actions of these trophic factors remains controversial (Ginty et al., 1991; Tan et al., 1994).

DbcAMP could induce cell adhesion and spreading, and therefore facilitate the effects of the trophic factors, as well as increase the stability of the neurite-like microtubules, as it has been observed in PC12 cells (Heidemann et al., 1985).

Morphological changes are determined by microtubule assembly and stabilization, and an increase in the levels of tubulin and several microtubule associated proteins (MAPs), which has been observed following long-term NGF treatment (Drubin et al.,1985). One of these MAPs, tau protein, has been generally associated to neurons (reviewed by Nunez, 1986), where it is known to induce tubulin assembly and to stabilize microtubules (Drubin and Kirschner, 1986). We observed that most of the adult insulin-positive cells and their neurites are also positive to β-tubulin and tau protein antibodies. However, we were not able to determine if the concentration of these molecules was higher in treated cells with respect to the control cells, which also expressed them. It is likely for NGF and

dbcAMP to induce the synthesis of tubulin, tau, and probably other MAPs that participate in the development of the processes.

Insulin secretion is not modified by culturing the cells for 5 d in the presence of NGF, compared to control cells. However, whereas control cells partially lost their glucose sensitivity, NGF-treated cells secreted more insulin in response to a high glucose concentration than control cells.

We have previously observed that after 1 or 2 d in culture in 10% FBS supplemented RPMI-1640, single β-cells secreted threefold more insulin in response to 15.6 m*M* glucose than to 5.6 m*M* glucose (Hiriart and Ramirez-Medeles, 1991). In this model, the secretory response to high glucose concentrations decreases with time in culture (Hiriart, 1988). Moreover, when β-cells are cultured in the presence of only 1% FBS, this decline is more pronounced and can be observed earlier in culture. Our data suggest that NGF treatment could sustain the capability of single β-cells to discriminate between different extracellular glucose concentrations.

On the other hand, the effect of culturing β-cells with dbcAMP on insulin secretion was remarkable, since even when dbcAMP was not present during the hormone assay, β-cells treated with this molecule dramatically increased their response to different glucose concentrations. This could be explained by the fact that cAMP is one of the second messengers on insulin secretion (Hedeskov, 1980) and because tubulin comprises part of the exocytotic machinery of the cells, and it has been shown that tubulin synthesis in β-cells is stimulated by this factor (Pipeleers et al.,1976).

It is not known if β cells are exposed to NGF in vivo during embryonic development or in the adult state. A recent report demonstrated the expression of Trk-A in islet cells and the secretion of NGF by nonendocrine adjacent cells in an in vitro model of developing fetal pancreatic islet (Kanaka-Gantenbein et al., 1995b). Moreover, Trk-A is also present in adult rat pancreas (Kanaka-Gantenbein et al., 1995a), and our data indicate that NGF could be an important trophic factor for adult β-cells.

It is interesting to note that the heterogeneity among β-cells, which has been shown in different studies, prevails in the response of the cells to trophic factors, as only 40–50% of cultured cells exhibit phenotypical changes when cultured with FGF or NGF and dbcAMP. It is then conceivable that differences in cell plasticity may correlate to the existence of β-cell subpopulations, as it has been shown for insulin secretion (Hiriart and Ramirez-Medeles, 1991, 1993).

Materials and Methods

Reagents were obtained from the following sources: collagenase type IV from Worthington (Freehold, NJ); guinea-pig insulin antisera for RHPA and rabbit GABA (glutaraldehyde conjugate) antisera, from Biodesign International (Kennebunkport, ME); guinea-pig insulin histochemical antisera from Incstar (Stillwater; MI); NGF from submaxillary glands 7S-NGF and 2.5S-NGF; FGF from bovine pituitary glands; monoclonal anti-τ (tau); monoclonal anti-β tubulin; monoclonal anti-N-CAM (neural cell adhesion molecule); bovine serum albumin (BSA) (fraction V); chromium chloride; staphylococcal protein A; HEPES; poly-L-lysine hydrobromide (mol wt > 380,000); RPMI-1640 salts; Spinner-Eagle's salts; 3-3' diaminobenzidine tetrahydrochloride; tissue culture dishes (Corning, Cat. No. 25000-35) from Sigma (St. Louis, MO); fetal bovine serum (FBS); guinea-pig complement; Hanks' balanced salt solution (HBSS); penicillin-streptomycin solutions from GIBCO (Grand Island, NY); Biotin/Avidin Vectastain ABC kit; and Texas red avidin D from Vector Laboratories (Burlingame, CA).

Animals and Pancreatic Islet Cell Cultures

Animal care was performed according to the *NIH guide for the care and use of laboratory animals* (National Institute of Health Publication No. 85-23, revised, 1985).

Young adult male Wistar rats (200–250 g), or pregnant rats in the 18th d of gestation, were obtained from the local animal facility, maintained in a 14-h light (06:00–20:00 h), 10-h dark cycle, and allowed free access to standard laboratory rat chow and tap water.

Adult and fetal pancreatic islet cells were isolated as described previously (Hiriart and Ramirez-Medeles, 1991), with minor modifications. Briefly, pancreatic islets were separated from the acinar tissue by collagenase digestion and a Ficoll gradient centrifugation, clean islets were then hand-picked. Dissociation of the cells was achieved by incubating the cells in a shaker bath, for 10 min, at 37°C in calcium-free Spinner s solution, with 15.6 m*M* glucose, 0.5% BSA, and 0.01% trypsin, followed by mechanical disruption. Isolated islet cells were cultured in RPMI-1640, $CaCl_2$ (600 μ*M*) was added to give a final concentration of 1.2 m*M*, and supplemented with 1% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL fungizone, on standard tissue culture dishes. Experimental cells were cultured with either 7S NGF (50 ng/mL) or 2.5S NGF (40 ng/mL), dbcAMP 5 m*M*, or both NGF and dbcAMP. Cultures were maintained at 37°C, in a humidified incubator (5% CO_2 in 95% air) for 2 wk, changing the medium every other day.

Immunohistochemistry

After 12 d in culture, β-cells were identified with immunocytochemistry (ICC) against insulin. Briefly, cells were fixed overnight, at 4°C, in a solution of 4% paraformaldehyde in 0.1*M* sodium phosphate buffer (pH 7.4), washed three times in 0.1*M* tris buffer solution (pH 7.4), perforated for 30 min with 0.3% of Triton X-100 in tris buffer, and incubated for 48 h at 4°C, with the primary antiserum. Dilution of antisera was as follows: insulin 1:4000, tau protein 1:1000, β-tubulin 1:500, GABA 1:2000, and N-CAM 1:100.

Usually, the first ICC was performed to detect insulin with a secondary peroxidase-antiperoxidase antibody and 3,3'-diaminobenzidine as final substrate, to form a brown reaction product. After this procedure, double antigenic staining to detect either tau, β-tubulin, N-CAM, or GABA was accomplished, using a secondary biotinylated antibody and Texas red-labeled streptavidin. Cells were observed under a Nikon fluorescent microscope at a wavelength of 450–490 nm. We avoided spurious labeling of cells with the second antibody by using antibodies raised in different species.

Morphological changes were evaluated measuring two parameters: (1) the percentage of neurite-bearing cells and (2) the average length of neurite-like processes. A neurite-bearing cell is defined as a cell with one or more processes, at least two times longer than the perinuclear diameter. We measured the length of neurite-like processes by projecting the image of the cell on a monitor attached to a video camera and a Nikon Axiophot inverted microscope, with the aid of the JAVA video analysis software (Jandel Scientific, Version 1.40, Corte Madera, CA).

During culture, cells tended to form clusters, the neurites were measured only in isolated β-cells, or in cells from the periphery of a cluster that could be clearly distinguished from the rest. At least 150 cells were measured per culture dish and all experiments were done by duplicate. At any given condition, a minimum of four separate cultures were evaluated.

Reverse Hemolytic Plaque Assay (RHPA)

Insulin secretion from individual beta cells was analyzed, after 5–7 d in culture under different experimental conditions, with a reverse hemolytic plaque assay (RHPA) (Neill and Frawley, 1983), as previously described (Hiriart and Matteson, 1988; Hiriart and Ramirez-Medeles, 1991).

We chose d 5–7 in culture to evaluate the initial secretory changes of the cells under the different treatments because phenotypical changes were mild at this time. In the RHPA, it is necessary to detach cells from the Petri dishes to mix them with the red blood cells and seed them together on the Cunningham chambers. Using cells in the first week of culture thus obviated mechanical injury of β-cells with long neuritic processes, that are observed in the cultures on subsequent days.

Briefly, islet cells were detached from culture dishes by incubating them for 10 min in calcium-free Spinner s solution, with 15.6 m*M* glucose, 0.5% BSA; equal volume of cells were mixed with protein A-coated sheep red blood cells, introduced into Cunningham chambers treated with poly-L-lysine to promote cell attachment, and incubated for 45 min. Then the chambers were rinsed and filled with Hanks balanced salt solution, which contained 5.6 or 20.6 m*M* glucose, and incubated for an hour in the presence of insulin antiserum. The monolayer was further incubated for 30 min with guinea-pig complement. Insulin released during the incubation time with the insulin antiserum was revealed by the presence of hemolytic plaques around the secretory cells, which result from the complement-mediated lysis of red blood cells bearing insulin-anti-insulin complexes bound to protein A.

We measured the size of plaques with the same equipment used to measure neurite-like processes length (*see* Immunohistochemistry section), and plaque size was expressed as area. At least 60 cells were measured per experimental condition. We also counted the number of cells that formed plaques, and these results were expressed as percentage of insulin-secreting cells; at least 100 cells were counted per experimental condition. All the experiments were carried out in duplicate.

The overall secretory activity of β-cells under a given experimental condition was expressed as a secretion index, calculated by multiplying the average plaque area by the percentage of plaque-forming cells (Smith et al., 1986; Hiriart and Ramirez-Medeles, 1991).

Statistical Analysis

Significant differences between data were evaluated by analysis of variance (ANOVA), followed by Fisher's multiple range test or by two-tailed Student's *t*-test for unpaired data, using the Number Cruncher Statistical System (NCSS, 4.2, Dr. Jerry L. Hintze, Kaysville, UT, 1983). All results are expressed as mean ± SEM.

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