Nerve Growth Factor Increases Insulin Secretion and Barium Current in Pancreatic β-Cells

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We analyzed the effect of a brief exposure to nerve growth factor (NGF) on insulin secretion and macroscopic barium currents of single adult rat pancreatic β-cells. After a 1-h exposure to NGF (50 ng/ml), single β-cells show a 2.5-fold increase in the insulin secretion index in 5.6 mmol/l glucose and a nearly twofold increase in 15.6 mmol/l glucose compared with control cells. We have recently demonstrated that pancreatic β-cells synthesize and secrete NGF. We analyzed the effect of endogenous NGF on insulin secretion by incubating islet cells in the presence of an anti-NGF monoclonal antibody for 1 h in different glucose concentrations. Although the basal insulin secretion index (5.6 mmol/l glucose) is not affected, glucose-stimulated insulin secretion (15.6 mmol/l glucose) is decreased by 41% in the presence of the antibody. This effect is mediated by the activation of the NGF receptor TrkA because the specific inhibitor of Trk phosphorylation K252a also blocks NGF-induced increase in insulin secretion, both in the presence and absence of exogenous NGF. Using the whole-cell variation of the patch-clamp technique, we found that cells exposed to NGF for 5 min exhibit a 32% increase in the average barium current density. These results suggest that the effects of NGF on insulin secretion are partially mediated by an increase in calcium current through Ca channels. These results further suggest that NGF plays an important autoregulatory role in pancreatic β-cell function. Two targets of short-term NGF-modulation are insulin secretion and calcium-channel activity. *Diabetes* 50:1755–1762, 2001

Nerve growth factor (NGF) is a neurotrophic factor that promotes neurite outgrowth during development and provides trophic support for sensory, sympathetic, and certain cholinergic neurons (1,2). NGF also has trophic actions in cells of the endocrine and immune systems, i.e., it may participate in acute inflammatory responses (3) and in the stress response to regulate functions of anterior pituitary cells (4).

This growth factor is capable of modulating β-cell plasticity because it promotes neurite-like outgrowth in fetal and adult pancreatic β-cells from primary cultures (5) and in RINm5F and INS-1 insulinoma cells (6). In the later cell line, NGF also enhances glucose-stimulated insulin secretion (7). NGF has also been implicated in pancreatic islet morphogenesis and ontogeny (8). In addition, NGF modifies β-cell physiology. Adult rat β-cells cultured for 5 days with NGF secrete more insulin in response to stimulation with 20.6 mmol/l glucose than with 5.6 mmol/l glucose. This property is lost in control cells cultured without NGF (5). Therefore, NGF is responsible for this physiological modification in single β-cells.

The presence of the high-affinity receptor for NGF, TrkA, has been described in insulinoma cell lines as well as in fetal and adult β-cells (9,10). In addition, TrkA expression is modulated by NGF (11). We have recently demonstrated that adult β-cells synthesize and secrete NGF in response to increasing extracellular glucose concentrations as well as depolarizing agents, such as 40 mmol/l KCl (11). The fact that β-cells secrete and respond to NGF implicates an autocrine role for NGF in the pancreas.

The activity of ATP-sensitive K^+ channels and voltage-sensitive Na, K, and Ca channels regulates insulin secretion (12). In particular, the activity of voltage-dependent calcium channels is essential for this process because blockade of these channels inhibits insulin secretion (13). The presence of T- and L-type calcium channels has been described in the membrane of adult primary cultured β-cells (14,15). It is generally agreed that high-voltage–activated calcium channels, particularly L-type channels, play a dominant role in glucose-induced insulin secretion.

In the present study, we analyzed the effect of a short exposure to NGF on insulin secretion and macroscopic barium currents of single adult rat pancreatic β-cells. We also analyzed the effects of endogenous NGF on insulin secretion and demonstrated that the effects of NGF on insulin secretion are mediated by the activation of TrkA high-affinity NGF receptor.

RESEARCH DESIGN AND METHODS

Reagents were obtained from the following sources: collagenase type IV from Worthington (Freehold, NJ); guinea-pig insulin antisera for reverse hemolytic plaque assay (RHPA) from Biogenesis (Sandown, NH); 2.5S NGF, bovine serum albumin, chromium chloride, staphylococcal protein A, HEPES, poly-L-lysine, trypsin, Spinner-Eagle's salts, tissue culture dishes (Corning, Cat. No 25000-35) and all salts for electrophysiological recordings from Sigma (St. Louis, MO); fetal bovine serum from Equitech-BIO (Ingram, TX); guinea-pig complement, Hank's balanced salt solution, RPMI-1640 salts, and penicillin-streptomycin-amphotericin B solution from Life Technologies (Grand Island, NY); tetrodoxin from Calbiochem (La Jolla, CA); nifedipine and K252a from Alamone (Jerusalem, Israel), mouse monoclonal anti-NGF from Boehringer Mannheim (Mannheim, Germany), and mouse anticytochrome oxidase monoclonal from Molecular Probes (Eugene, OR).

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Received for publication 26 June 2000 and accepted in revised form 10 April 2001.

IBa, barium current; LP, large placque-forming; NGF, nerve growth factor; RHPA, reverse hemolytic plaque assay; SP, small placque-forming.

Pancreatic β-cell culture. Animal care was performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publ. no. 85–23, revised 1985). Young adult male Wistar rats (200–250 g) were obtained from the local animal facility, maintained in a 14-h light (0600–2000)/10-h dark cycle, and allowed free access to standard laboratory rat diet and tap water. Animals were anesthetized with intraperitoneal sodium pentobarbital (40 mg/kg), and after pancreas dissection, animals were killed by cervical dislocation.

Pancreatic β-cells were obtained following the technique previously described (5). Briefly, pancreatic islets were isolated and separated from the acinar tissue by collagenase digestion and a Ficoll gradient centrifugation; clean islets were then handpicked. Dissociation of the cells was achieved by incubating them in a shaker bath for 10 min at 37°C in calcium-free Spinner solution, with 15.6 mmol/l glucose, 0.5% bovine serum albumin, and 0.01% trypsin, followed by mechanical disruption. Single cells were cultured for 1 day (in the case of RHPA) and for 1–3 days (for electrophysiological experiments) in RPMI-1640 supplemented with 5% fetal calf serum, 200 units/ml penicillin G, 200 μg/ml streptomycin, and 0.5 μg/ml amphotericin B.

RHPA. To identify insulin-secreting cells, RHPA (16) was performed as described previously (17). Briefly, after 1 day in culture, islet cells were detached from culture dishes, and equal volumes of islet cells were mixed with protein A–coated sheep erythrocytes, introduced to Cunningham chambers previously treated with poly-L-lysine to promote cell attachment, and incubated for 45 min. Then, the chambers were rinsed and filled with Hank's balanced salt solution, which contained 5.6 or 15.6 mmol/l glucose with or without: *1*) NGF 2.5S (50 ng/ml), *2*) anti-NGF monoclonal antibody (0.5 μg/ml), *3*) K252a (200 nmol/l), or *4*) nifedipine (5 μmol/l). Under each condition, cells were incubated for 1 h in the presence of insulin antiserum (1:30 in Hank's balanced salt solution). 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 erythrocytes bearing insulin–anti-insulin complexes bound to protein A.

To demonstrate the specificity of the anti-NGF monoclonal antibody, we also made control experiments using an anticytochrome oxidase monoclonal antibody and found that including the last antibody does not affect insulin secretion (data not shown).

We measured the size of plaques by projecting the image of the cell on a monitor attached to a video camera and a Nikon Axiophot inverted microscope (Tokyo), with the aid of the JAVA video analysis software (Version 1.40; Jandel Scientific, Corte Madera, CA). The plaque size was expressed as area, and the cells that formed plaques were counted. These results were expressed as the percentage of insulin-secreting cells; at least 100 cells were counted per experimental condition. All experiments were performed 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 (17,18).

A frequency distribution of immunoplaque areas was constructed with data pooled from six different experiments by duplicate to identify functional subpopulations of β-cells and to determine whether these subpopulations were differentially affected by the experimental treatments (17–19). According to the size of immunoplaques formed by the cells, we classified them as follows: small plaque-forming cells (SP) (diameter of immunoplaques ≤2000 μm^2) and large plaque-forming cells (LP) (diameter >2000 μm^2) (20).

Electrophysiological recordings and analysis. The whole-cell patch-clamp configuration (21) was used to record calcium currents, using Ba as the charge carrier, at a temperature of 20–22°C with an Axopatch 200 amplifier (Axon Instruments, Forster City, CA). Patch electrodes were pulled from capillary tubes KIMAX-51 (Kimble Products) and had a resistance of 1.5–3 MΩ. Electrode tips were coated with Sylgard (Dow Corning, Midland, MI).

Total cell capacitance was determined by digital integration of capacitive transients with pulses of 10 mV, from a holding potential of −80 mV. In each cell, the capacity transient of the pipette was cancelled before accessing the cell. Capacity transients were cancelled and series resistance was compensated using the internal voltage-clamp circuitry. Remaining linear capacity transients as well as leakage currents were subtracted by a P/2 procedure (22).

The pulse protocol used for the analysis of the peak barium current (IBa) consisted of applying depolarizing test pulses of 15-ms duration, from −60 to +50 in 10-mV increments, from a holding potential of −80 mV.

NGF 2.5S (50 ng/ml) was added to cells in the external solution 5 minutes before electrophysiological recording. Then, this solution was washed, and cells were recorded in the presence of fresh external solution without NGF for no more than 10 min per plate. Control cells were never exposed to exogenously added NGF. In some experiments, nifedipine 5 μmol/l was added alone or with the NGF and remained in the bath solution throughout the recording time.

IBa current activation curves were obtained by converting the peak current values to conductances. We used the following equation:

$$gBa = IBa/(Vm - EBa)$$

where IBa is the peak current value, Vm is the command pulse potential, and EBa is the reversal potential obtained by extrapolation of the I-V relationships, which in all cases was nearly +56 mV.

Conductance values were normalized and fit to a Boltzmann relation:

$$g/gmax = \{1 + \exp[-(V - V_{1/2})/k\}^{-1}$$

where g is the Ba peak conductance, $gmax$ is the maximal Ba conductance, $V_{1/2}$ is the midpoint of the activation curve, and k is the activation steepness factor.

Recording solutions. The ionic composition of the solutions used in the experiments was as follows. External solution (mmol/l): 130 NaCl, 5 KCl, 2 $MgCl_2$, 10 $BaCl_2$, 10 HEPES, and 10 glucose. Internal solution (mmol/l): 120 CsAsp, 10 CsCl, 5 CsF, 2.5 Cs-BAPTA, 10 HEPES, and 10 tetramethylammonium-Cl. Na current was blocked with 100 nmol/l TTX in the external solution.

Statistical analysis. All data are reported as the mean ± SE; n denotes the number of cells studied or experiments performed. The statistical significance was obtained with the one-way analysis of variance, followed by Fisher's multiple range test using the Number Cruncher Statistical System (NCSS, 4.2; Dr. J.L. Hintze, Kaysville, UT). All results are expressed as mean ± SE.

RESULTS

Effect of NGF on insulin secretion of single β-cells. After 1 day in culture, control and NGF-treated cells are capable of discriminating between different glucose concentrations, as shown by their insulin secretion index (Fig. 1). The secretion index is 2.5-fold higher in 15.6 than in 5.6 mmol/l glucose in control cells and nearly twofold in NGF-treated cells.

A 1-h incubation of β-cells with NGF (50 ng/ml) increases individual insulin secretion, given by the mean immunoplaque area, by 1.7-fold and 1.4-fold in 5.6 and 15.6 mmol/l glucose, respectively (Table 1). In addition, NGF increases the percentage of β-cells that respond to glucose-forming insulin-immunoplaques by nearly 1.3-fold in both glucose concentrations. Consequently, NGF increases total insulin secretion, given by the insulin secretion index, by 2.3-fold in 5.6 mmol/l glucose and by 1.7-fold in 15.6 mmol/l glucose with respect to their own controls (Fig. 1 and Table 1). The cellular mechanism that explains this increase involves the amplification of hormone secretion by individual cells and an increase in the percentage of insulin-secreting cells.

To explore an autocrine effect of endogenous NGF secreted by single β-cells on insulin secretion, an NGF monoclonal antibody was included in the incubation medium in the presence of different glucose concentrations for 1 h to neutralize the NGF secreted by β-cells. In 5.6 mmol/l glucose, the NGF-neutralizing antibody has no effect on the insulin secretion index (Fig. 1*A*). In contrast, in 15.6 mmol/l glucose, the NGF antibody decreased the insulin secretion index by 41%. This effect results from a decrement of insulin secreted by single cells, whereas the percentage of secreting cells is not affected by the antibody (Table 2).

To analyze whether NGF stimulation of insulin secretion is mediated by the activation of the high-affinity NGF receptor TrkA, we incubated the cells with the specific inhibitor of TrkA phosphorylation K252a in the presence and in the absence of exogenous NGF. Figure 1*B* shows that K252a blocks the effects of exogenous NGF in both glucose concentrations without affecting basal insulin

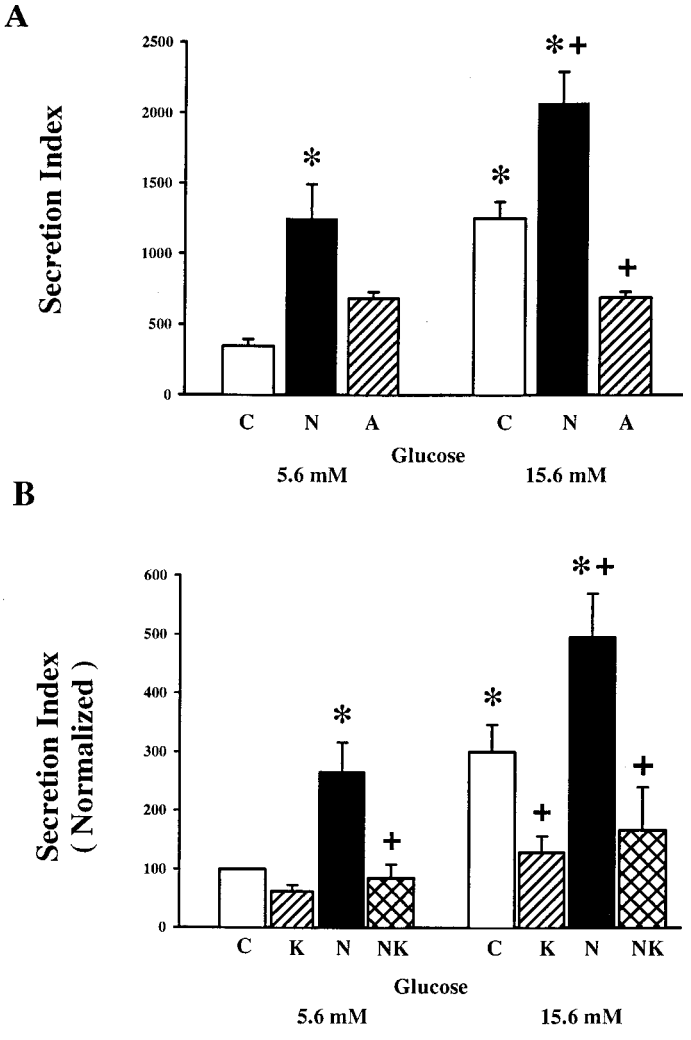

FIG. 1. Effect of NGF, a monoclonal NGF-antibody, and K252a on insulin secretion. *A*: Insulin secretion by single β-cells was measured with RHPA. The overall secretory activity of β-cells under a given experimental condition is expressed as a secretion index. C, control cells; N, cells exposed for 1 h to NGF (50 ng/ml); A, cells exposed for 1 h to a monoclonal NGF antibody. Data are the mean ± SE of four different experiments by duplicate. Symbols denote statistically significant differences with respect to control cells in each glucose concentration (+) and with respect their own control (∗), $P < 0.01$. *B*: Bars represent the normalized insulin secretion index, measured with the RHPA in control cells (C), in cells exposed to K252a (200 nmol/l) (K), in cells exposed to NGF (N), and in cells incubated with NGF + K252a (NK). Data were normalized to values of control cells in 5.6 mmol/l glucose. Data are the mean ± SE of four different experiments by duplicate. Symbols denote statistically significant differences with respect to 5.6 mmol/l glucose (∗) and with respect their own control (+), $P < 0.01$.

secretion. In 15.6 mmol/l glucose, the effect of K252a is similar to that observed with the neutralizing NGF antibody.

These results suggest that the effects of NGF on insulin secretion are mediated by the activation of TrkA receptors and that endogenous NGF is an important regulator of stimulated insulin secretion.

NGF increases macroscopic Ba currents. Due to the effects of NGF on insulin secretion and the importance of calcium channels in this process, we investigated the effects on calcium currents of a brief exposure (5 min) of β-cells to this factor.

Figure 2*A* and *B* illustrates families of whole-cell barium currents of control and NGF-treated cells; Fig. 2*C* shows the mean current-voltage relationships of IBa. In both cases, maintained inward currents activate around −30 mV and reach a maximum around +20 mV during the 15-ms depolarizing pulse. NGF potentiation of barium current is not dependent on the holding potential of the cells, as shown in Fig. 2*D* and *E*.

Maximum Ba current in cells exposed to NGF is, on average, 66% larger than in control cells. NGF-treated cells have a small but significant increase in average cell capacitance compared with control cells; considering cell capacitance, NGF increases β-cell Ba current density by 32% (Table 3).

To determine whether NGF induced changes on the activation kinetics of the current, we calculated the voltage dependence of peak calcium conductances and used them to construct a Boltzmann relationship (as described in RESEARCH DESIGN AND METHODS) for control and NGF-treated β-cells (Fig. 3). NGF exposure does not modify the midpoint of activation or the steepness factor of the activation curve of IBa (Table 4).

Effect of nifedipine on NGF-stimulated insulin secretion and barium current. Because L-type Ca channels are important for insulin secretion, we studied the effect of

TABLE 1
Insulin secretion by single β-cells in response to a 1-h incubation to NGF in different extracellular glucose concentrations

Treatment	Glucose (mmol/l)	Percentage of plaque-forming cells	Plaque area (μmol/l)
Control	5.6	41 ± 2	1,280 ± 113
	15.6	51 ± 3*	2,299 ± 119*
NGF	5.6	56 ± 3†	2,152 ± 160†
	15.6	66 ± 2*†	3,189 ± 198*†
Nifedipine	5.6	29 ± 4‡	1,078 ± 270
	15.6	32 ± 2†	1,160 ± 191†
NGF + nifedipine	5.6	32 ± 3§	1,186 ± 174§
	15.6	29 ± 4§	1,828 ± 506§

Data are mean ± SE unless otherwise indicated. *Denotes significance level with respect to its own control in 5.6 mmol/l glucose, in both groups ($P < 0.01$). Significance level of experimental cells with respect to control cells in both glucose concentrations: $P < 0.01$ (†), $P < 0.05$ (‡). Significance level of NGF + nifedipine with respect to NGF-treated cells in both glucose concentrations: $P < 0.01$ (§); n = 13 experiments by duplicate for control and NGF, and n = 5 for nifedipine alone or with NGF.

TABLE 2
Effect of a monoclonal anti-NGF antibody on insulin secretion by single β-cells

Treatment	Glucose (mmol/l)	Percentage of plaque-forming cells	Plaque area (μmol/l)
Control	5.6	41 ± 2	1,279 ± 113
	15.6	51 ± 3*	2,299 ± 119*
Monoclonal anti-NGF	5.6	49 ± 3	1,371 ± 69
	15.6	47 ± 3	1,415 ± 67*

Data are mean ± SE unless otherwise indicated. *Significance level with respect to its own control in 5.6 mmol/l glucose, n = 6 experiments by duplicate for monoclonal anti-NGF.

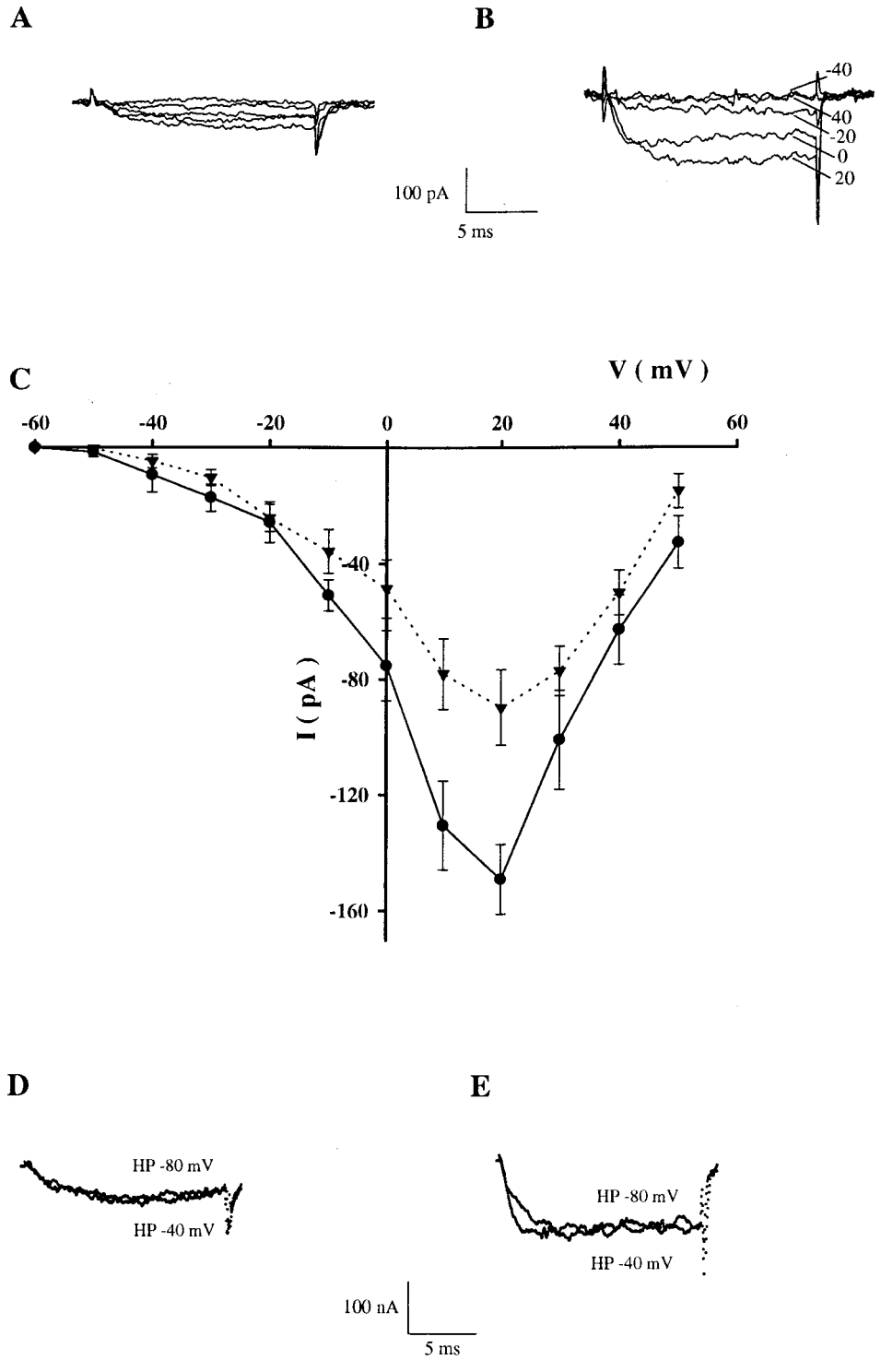

FIG. 2. Effect of NGF on Ba currents and current-voltage relationship. The illustrated currents were recorded during 15-ms increments to indicated voltages, from a holding potential of −80 mV in control cells (*A*) and in cells pre-exposed for 5 min to NGF (50 ng/ml) (*B*). *C*: The mean-peak current was plotted as a function of voltage under control conditions (▼) and in cells exposed for 5 min to NGF (●). Data points are means ± SE, $n = 11$ for control and $n = 9$ for NGF-exposed cells. The illustrated currents were recorded on the same cell from different holding potentials (−80 and −40 mV), control cell (*D*) and cell preincubated (*E*) with NGF for 5 min.

nifedipine (5 μmol/l) on this process. Figure 4 and Table 1 show the effects of nifedipine on insulin secretion and RHPA parameters.

Basal insulin secretion in 5.6 mmol/l glucose is not affected by nifedipine. However, the L-type channel blocker completely inhibits the NGF-induced insulin secretion. In the presence of a stimulating glucose concentration (15.6 mmol/l), nifedipine decreases the insulin secretion index by 63% in control cells and by 68% in NGF-treated cells, compared with their respective controls (Fig. 4).

It is interesting to note that in both control and NGF-treated cells exposed to 15.6 mmol/l glucose and in NGF-treated cells in 5.6 mmol/l glucose, nifedipine decreased the percentage of insulin-secreting cells as well as the amount of insulin secreted by individual cells (Table 1).

To determine the role of NGF on L-type calcium currents, we studied the effect of nifedipine (5 μmol/l) on IBa in controls and cells treated for 5 min with NGF. Figure 5*A* and *B* illustrates the effect of nifedipine on maximum IBa during steps to +20 mV, from a holding potential of −80 mV, in control and NGF-treated cells, respectively.

Figure 5*C* and *D* shows the effect of nifedipine on

TABLE 3
Effects of NGF on β-cell Ba^{2+} current

Treatment	Capacitance (pF)	IBa density (pA/pF)	Peak current (pA)
Control	8.4 ± 0.4	11.2 ± 1.2	−89.7 ± 13
NGF	10.5 ± 0.9*	14.8 ± 1.1*	−149 ± 12*

Data are mean ± SE. *A significance level of $P < 0.05$ with respect to control cells ($n = 11$ for control and $n = 9$ for NGF-treated cells).

current-voltage relations of IBa in control and NGF-treated β-cells, respectively. These figures show that nifedipine blocks nearly 60% of the average peak IBa in both conditions, with no shift on the voltage dependence of the current. However, a heterogeneous response to the blocker was observed among β-cells. In nearly 30% of control cells, there was no evident block of IBa, and in 15% of cells treated with NGF, nifedipine completely blocked the current (these cells were not included in the analysis shown in Fig. 5).

Mean cell capacitance of nifedipine-treated cells was not different between control and NGF-exposed cells (6.65 ± 0.05 and 6.70 ± 0.7 pF, respectively), and it was significantly different than in their respective controls, as shown in Table 3 ($P < 0.05$ for control cells and $P < 0.01$ for NGF-treated cells). The observations that cell capacitance is variable, according to the treatment, and that this could not be attributed to apparent differences between cell sizes suggest that this parameter reflects the secretory activity of the cells.

The fact that the nifedipine-induced decrease of IBa is quantitatively similar to the current increase induced by the acute exposure of β-cells to NGF suggests that the effect of NGF on the whole IBa currents could be due to an increase on the L-type Ca currents.

NGF modulates the behavior of functional β-cell subpopulations. Distribution analysis of insulin immunoplaque areas shows a multimodal distribution that corresponds to the behavior of functional subpopulations of pancreatic β-cells, as previously reported (17,19,20,23). In Fig. 6, cells are grouped according to the size of immunoplaque area and expressed as the percentage of total cells. It is clear that in both glucose concentrations, NGF increases the percentage of LP cells by threefold and nearly

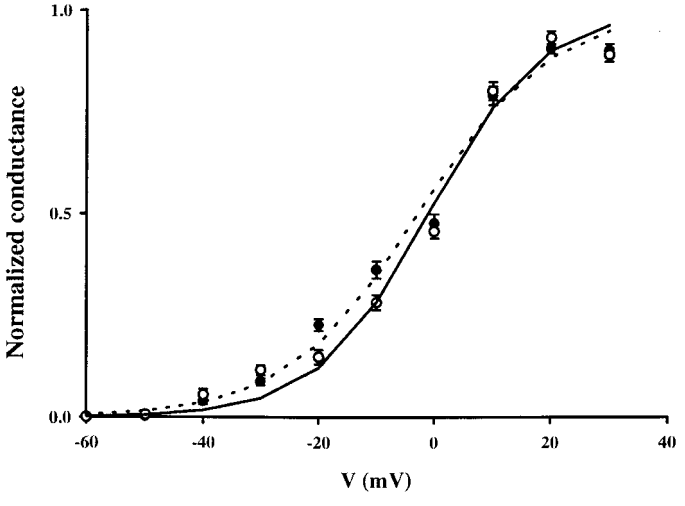

FIG. 3. Voltage-dependence of Ba current activation in control and in NGF-exposed cells. At each voltage, the Ba conductance was calculated (as described in RESEARCH DESIGN AND METHODS) and plotted as a function of voltage. Experimental data points are means ± SE, $n = 10$ for control and $n = 9$ for NGF-exposed cells, and are plotted as symbols: control β cells (○) and NGF-treated β-cells (●). The smooth curves represent the Boltzmann fit to the data (as described in RESEARCH DESIGN AND METHODS). Boltzmann parameters are shown in Table 4.

TABLE 4
Boltzmann parameters of Ba conductance

Parameter	Control	NGF
$Va_{1/2}$ (mV)	−2.97 ± 1.03	1.16 ± 0.51
Ka	10.39 ± 0.36	12.63 ± 0.96

Data are mean ± SE. These parameters correspond to the smooth curves of Fig. 3 and represent the Boltzmann fit to the data (as described in RESEARCH DESIGN AND METHODS); $n = 10$ control cells and $n = 9$ NGF-treated cells.

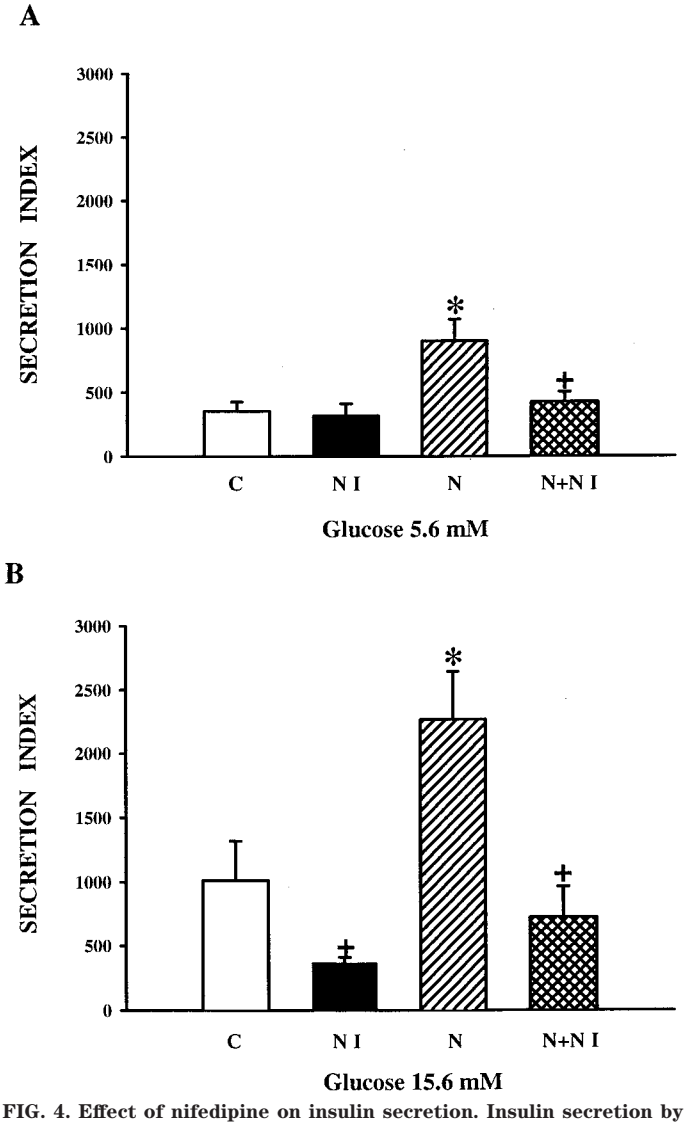

FIG. 4. Effect of nifedipine on insulin secretion. Insulin secretion by single β-cells was measured with RHPA. The overall secretory activity of β-cells under a given experimental condition is expressed as a secretion index. Cells were incubated for 1 h in 5.6 mmol/l glucose (*A*) and 15.6 mmol/l glucose (*B*), in the presence of an insulin antibody (see RESEARCH DESIGN AND METHODS) and the different experimental treatments. C, control cells; NI, cells exposed to nifedipine (5 μmol/l); N, cells exposed for 1 h to NGF (50 ng/ml). Data are the mean ± SE of six different experiments by duplicate. Symbols denote statistically significant differences with respect to the control cells (*) and with respect to its own control (+), $P < 0.01$.

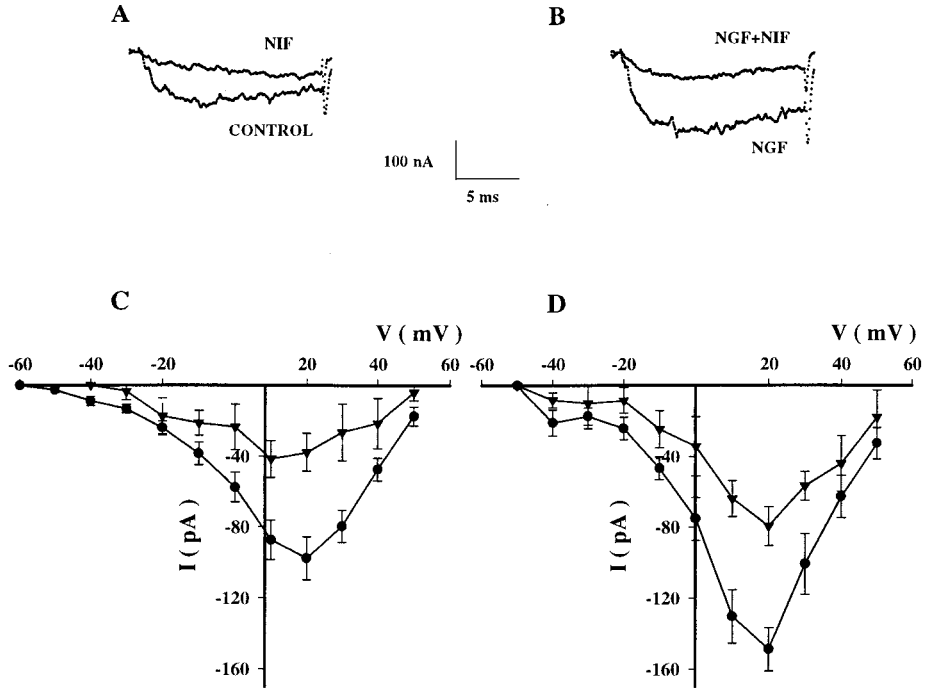

FIG. 5. Effect of nifedipine on whole-cell Ba currents. The illustrated currents were recorded during 15-ms increments to indicated voltages, from a holding potential of −80 mV in the absence or in the presence of nifedipine (NIF) in control cells (*A*) and in cells preexposed for 5 min to NGF (50 ng/ml) (*B*). *C*: Recordings were made in control cells, in the absence (●) and in the presence of nifedipine (5 μmol/l; ▼). Currents were elicited by steps from a holding potential of −80 mV, to voltages between −60 and +40 mV in 10-mV increments, $n = 11$ for control and $n = 7$ for nifedipine-treated cells. *D*: Recordings were made in NGF-pretreated cells in the absence (●) and in the presence of nifedipine (5 μmol/l; ▼). Currents were elicited as in (*A*), $n = 9$ for NGF-treated cells and $n = 8$ for NGF + nifedipine-treated cells.

twofold in 5.6 and 15.6 mmol/l glucose, respectively, without affecting the percentage of SP cells.

The effect of nifedipine in control and NGF-treated cells is glucose-dependent. In 5.6 mmol/l glucose, nifedipine has no effect in control cells but completely blocks the effect of NGF on LP cells, without significantly affecting the percentage of SP cells. In 15.6 mmol/l glucose, nifedipine blocks the expression of LP cells in both control and NGF-treated cells.

DISCUSSION

This study represents the first description of the acute effects of NGF on insulin secretion and on barium currents of pancreatic β-cells. We observed that a brief exposure of β-cells to NGF increases both insulin secretion and Ba current density.

As previously described (17), insulin secretion by single β-cells in 15.6 mmol/l glucose is nearly 2.5-fold higher than in 5.6 mmol/l glucose (basal glucose concentration). Acute NGF treatment increases insulin secretion in both glucose concentrations. This effect is reflected by two different parameters: *1*) amplification of insulin secretion by individual cells because the immunoplaque area, which is proportional to the amount of hormone secreted by the cell, increases in response to NGF; and *2*) the recruitment of previously silent cells that do not secrete detectable insulin at a specific glucose concentration because the percentage of insulin-secreting cells is larger in the NGF-treated group (see Table 1).

Insulin secretion by single β-cells is heterogeneous, and it has been shown that there are functional subpopulations of adult rat β-cells (17,19,23–25). One subpopulation secretes more hormone (LP cells) than the other (SP cells), and it is important to note that LP cells have the highest secretion rate and are responsible for nearly 75% of the insulin secreted (17,20). The effects of NGF are easily observed in LP cells because their expression is increased by threefold and twofold in 5.6 and 15.6 mmol/l glucose, respectively. This may imply that a β-cell subpopulation is preferentially modulable by NGF and other secretagogues like carbachol (19). It is then possible that membrane receptors to these compounds are not homogeneously distributed among islet cells or that differences among cell types could be inherent to their ionic channel density (14,23,26) and/or biosynthetic and secretory machineries (24,25).

NGF initiates intracellular signaling processes by binding to transmembrane receptors that contain intrinsic tyrosine kinase activity. Adult and fetal pancreatic β-cells as well as insulin-producing cell lines, such as RINm5F and INS-1, express the TrkA high-affinity NGF tyrosine kinase receptors (8,9–11). NGF binding induces the receptor dimerization and autophosphorylation on tyrosine residues (27), and it has been demonstrated that within 5 min after addition of NGF to INS-1 cells, TrkA is phosphorylated on tyrosine residues (28). The fact that the specific inhibitor of TrkA phosphorylation K252a blocks the effects of NGF on insulin secretion demonstrates that the observed increase in insulin secretion is mediated by the activation of TrkA.

Glucose-stimulated insulin secretion depends on the closure of ATP-sensitive K^+ channels and membrane depolarization (29). This results in activation of voltage-sensitive Ca channels, calcium entry, and hormone exocytosis (30). The increase in insulin secretion could be explained, in part, by the fact that NGF induces changes in the whole-cell calcium currents of β-cells.

We found that ~60% of the total barium currents in control and NGF-treated cells are nifedipine-sensitive. A 5-min exposure of β-cells to NGF induces a 66% increase in the peak IBa (which corresponds to a 32% increase in barium current density). We propose that this increase could be due to L-type calcium channel phosphorylation, because it has been largely demonstrated that calcium channel activity can be regulated by several types of kinases, at different sites of the channel (31), and signal transduction through the NGF receptor activates some of

these kinases. Moreover, it has been shown that protein tyrosine kinase inhibitors decrease glucose-induced insulin release by adult rat islets, without affecting glucose metabolism, and the authors suggested that inhibitory effects of protein tyrosine kinase inhibitors may be due to dephosphorylation of voltage-dependent calcium channels (32). However, more experiments will have to be done to support this hypothesis.

It is interesting to note that the importance of L-type calcium-channel activity may be glucose-dependent. In Fig. 4, it is clear that in 5.6 mmol/l glucose, nifedipine does not block insulin secretion by single β-cells, but it blocks the effect of NGF. However, in 15.6 mmol/l glucose, nifedipine completely blocks glucose-stimulated insulin secretion.

Surprisingly, we found a small but significant increase in the cell capacitance of NGF-treated cells with respect to control cells (Table 3) and a significant reduction of capacitance in cells treated with nifedipine (in both control and NGF-treated cells). Because electrophysiological recordings were performed in 10 mmol/l glucose, we believe that the increase in cell capacitance could be reflecting the increase in membrane area produced by granule fusion with the plasma membrane during increased insulin exocytosis induced by NGF. Likewise, the decrease in cell capacitance could be reflecting the reduction in insulin secretion elicited by nifedipine, as can be observed in Fig. 4.

We have previously shown that NGF is secreted in response to elevated glucose concentrations, suggesting both an autocrine and an endocrine role for NGF in vivo (11). A similar type of positive feedback has been demonstrated recently in isolated mouse β-cells, in which insulin secreted by the cells interacts with insulin autoreceptors, leading to an increase in insulin secretion (33).

The possible autocrine effect of NGF on insulin secretion was studied by neutralizing endogenous NGF with an NGF monoclonal antibody, by blocking TrkA activation with K252a, and by challenging β-cells with different glucose concentrations. Both compounds only decreased the insulin secretion index in 15.6 mmol/l glucose (Fig. 1, Table 2). This result is in accordance with our previous observations, in which NGF secretion by β-cells is increased in 15.6 mmol/l glucose (11), and further confirms the hypothesis that NGF is a physiological regulator of β-cell function.

Interestingly, the experiments with both K252a and the monoclonal NGF antibody demonstrate that in 15.6 mmol/l glucose, the overall insulin secretion is enhanced by endogenous NGF through an increase in the amount of insulin secreted by individual β-cells, rather than affecting the percentage of secreting cells.

Cells exposed to a high glucose concentration secrete insulin and NGF that could lead to the activation of their respective autoreceptors, which could exert positive control over insulin secretion by activating several downstream proteins. Several recent studies indicate that β-cells express components of insulin signal systems (34). In our studies, we used the reverse hemolytic plaque assay, which includes an insulin antibody to measure insulin secretion by single β-cells. Insulin antiserum is probably neutralizing the positive feedback exerted by insulin on insulin secretion. We observed that when NGF receptors

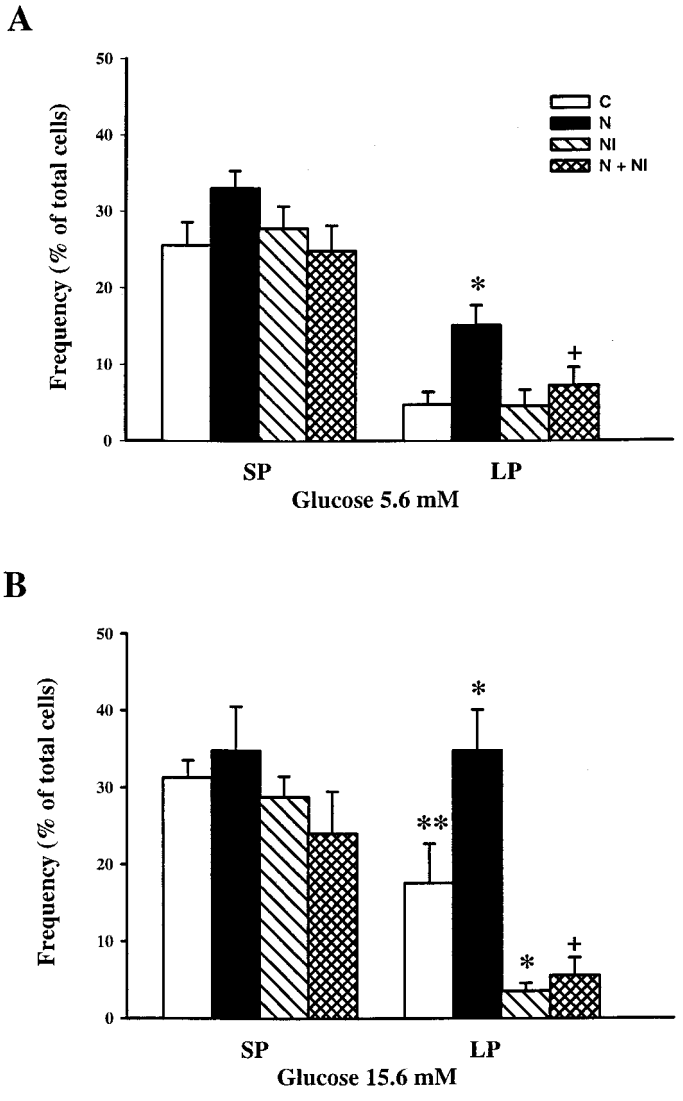

FIG. 6. NGF modulates the behavior of functional β-cell subpopulations. According to the size of immunoplaques formed by the cells, we classified them as follows: SP cells, diameter of immunoplaques ≤2000 μm², and LP cells, diameter >2000 μm². *A*: Cells incubated for 1 h in 5.6 mmol/l glucose, in the presence of an insulin antibody (see RESEARCH DESIGN AND METHODS), and the different experimental treatments. The total number of cells measured for each experimental condition was 354 for control (C), 238 for nifedipine (5 μmol/l) (NI), 392 for NGF (50 ng/ml) (N), 273 for N + NI. *B*: Cells incubated for 1 h in 15.6 mmol/l glucose, in the presence of an insulin antibody (see RESEARCH DESIGN AND METHODS), and the different experimental treatments. The total number of cells measured for each experimental condition was 210 for C, 237 for NI, 284 for N, and 194 for N + NI. Data are the mean ± SE of six different experiments by duplicate. Symbols denote statistically significant differences, $P < 0.01$ (*), $P < 0.05$ (**), with respect to the control cells; $P < 0.01$ (+) with respect to its own control, in each condition.

are blocked, the cells do not respond adequately to 15.6 mmol/l glucose; it is then possible that protein-phosphorylation cascades activated by both insulin and NGF play a permissive role by maintaining the phosphorylation of proteins necessary for exocytosis.

The data shown in this work suggest that two of the possible targets for the autocrine regulation of β-cell function by NGF are calcium channel activity and insulin secretion. Therefore, a deficient secretion of NGF by pancreatic β-cells may significantly contribute to decreased serum levels of NGF in patients with diabetic neuropathies (35) and may also lead to dysfunctional insulin secretion.

ACKNOWLEDGMENTS

This work was supported by grants from the Dirección General de Asuntos del Personal Académico (DGAPA), Universidad Nacional Autónoma de México (UNAM) (grant no. IN214298); the Consejo Nacional de Ciencia y Tecnología (CONACyT) (grant no. 3,174-N9607); and the Fundación Miguel Alemán, A.C., México.

We thank Carol Deutsch for discussion of a previous version of the manuscript; R. Vidaltamayo, A. Caso, and L. Islas for proofreading and discussion of the manuscript; and M. Elena Larrieta-Carrasco for actively participating in some of the RHPA experiments.

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