An endogenous peptide isolated from the gut, NK-lysin, stimulates insulin secretion without changes in cytosolic free Ca^{2+} concentration

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Abstract We have recently isolated and cloned a novel endogenous peptide from pig intestine, NK-lysin (NKL). In the present study we show that NKL (1–100 nM) potently and reversibly stimulates insulin secretion in rat pancreatic islets and in the β-cell line HIT T15. This effect of NKL was not accompanied by changes in cytoplasmic free calcium concentration. The stimulatory activity of NKL on insulin release was also observed in permeabilized islets under Ca^{2+}-clamped conditions. Preincubation of HIT T15 cells with NKL for 1 h or 24 h did not influence cell viability. Possible mechanisms of insulinotropic activity of NKL are discussed.

Key words: NK-lysin; Insulin release; Cytoplasmic free Ca^{2+} concentration; Pancreatic β-cell; Exocytosis

1. Introduction

Insulin plays a key role in maintaining blood glucose homeostasis. Increase in glucose concentration leads to secretion of insulin from the pancreatic β-cell. The molecular mechanisms underlying insulin secretion in the pancreatic β-cell are not fully understood. Cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) plays an important role in insulin secretion [1,2]. The increase in $[Ca^{2+}]_i$ results from metabolism of glucose and thereby an increase in the ATP/ADP ratio with subsequent closure of ATP-dependent K^+ channels (K_{ATP} channels), membrane depolarization and opening of voltage-dependent Ca^{2+} channels [1,2]. However, recent data point to the possibility of exocytosis of insulin being promoted without a concomitant increase in $[Ca^{2+}]_i$, by activation of more distal steps in the secretory machinery [3–9]. Insulin secretion independent on concomitant changes in $[Ca^{2+}]_i$ involves glucose metabolism and is dependent on the activation of protein kinases A and C [4–8].

Recently we have isolated and cloned a novel endogenous 78 residue basic peptide from pig intestine. This peptide was named NK-lysin (NKL) and we have shown that it possesses antimicrobial and antitumor activity in the μM concentration range [10]. The present study further explores the mode of action of NKL on mammalian cells. We now show that NKL potently stimulates insulin secretion in the nM range, without concomitant changes in $[Ca^{2+}]_i$. This means that NKL belongs to the group of several gut peptides [11,12] modulating pancreatic β-cell function.

2. Materials and methods

2.1. Materials

NKL was purified from pig intestine as previously described [10] and concentrations were based on dry weight of the peptide. Other chemicals used were from Sigma (USA) and of analytical grade.

2.2. Isolation and incubation of islets of Langerhans

Pancreatic islets were isolated from male Wistar rats (200–250 g) by collagenase digestion as previously described [13] and maintained overnight in RPMI 1640 culture medium (Flow Laboratories, Scotland, UK), containing 11 mM glucose and supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin.

For measurements of insulin release, islets were preincubated in Krebs-Ringer bicarbonate buffer (KRB) containing (in mM): 115 NaCl, 4.7 KCl, 2.56 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 20 $NaHCO_3$ and 16 HEPES, pH 7.4, supplemented with 1 mg/ml bovine serum albumin, with 3.3 mM glucose for 30 min at 37°C. For batch incubation experiments groups of 3 islets were selected and incubated in 300 μl KRB for 60 min at 37°C with different concentrations of NKL or without the peptide (control). The incubation was terminated by cooling the samples on ice. Samples were stored at −20°C until insulin content was analyzed by radioimmunoassay, using rat insulin (mixture of the two rat insulins) as a standard (Novo Nordisk, Copenhagen, Denmark). To measure insulin release in permeabilized islets, islets were washed three times in a permeabilization buffer containing (in mM): 140 potassium glutamate, 5 NaCl, 1 $MgCl_2$, 10 EGTA, 25 HEPES and 0.25 mg/ml bovine serum albumin. pH was adjusted to 7.00 with KOH and pCa^{2+} ($-\log[Ca^{2+}]$) was adjusted to 7.00 with $CaCl_2$. Islets were electropermeabilized in this buffer by 5 pulses of a 3 kV/cm electric field, washed and collected in groups of 3 in 300 μl of modified permeabilization buffer containing 2 mM ATP and an ATP-regenerating system consisting of 2 mM creatine phosphate, 10 U/ml creatine phosphokinase and pCa^{2+} varying from 8 to 5. The actual pCa^{2+} in the buffer was adjusted using a Ca^{2+}-selective electrode (Orion Research, Boston, MA, USA) and solutions with standard Ca^{2+} concentrations (World Precision Instruments, USA). Islets were incubated for 15 min at 37°C and insulin release was measured by radioimmunoassay.

2.3. Cell culture and study of insulin release from HIT T15 cells

Hamster insulinoma tumor HIT T15 cells were cultured at 37°C in RPMI 1640 medium containing 11 mM glucose and supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 10^{-7} M selenious acid

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Abbreviations: NKL, NK-lysin; $[Ca^{2+}]$, cytoplasmic free Ca^{2+} concentration; K_{ATP} channels, ATP-dependent K^+ channels; BSA, bovine serum albumin; KRB, Krebs-Ringer bicarbonate buffer; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

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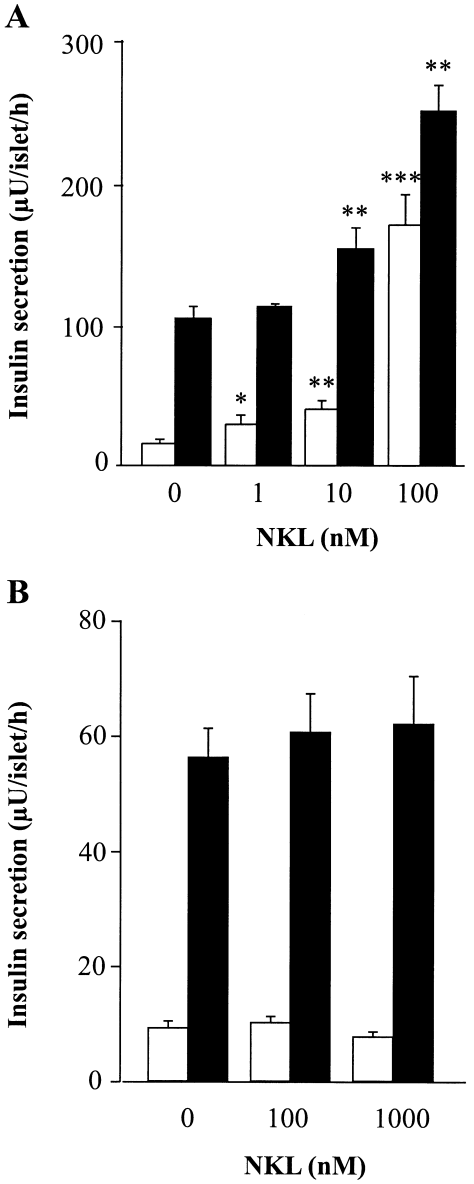

Fig. 1. A: Concentration-dependent effect of NKL on insulin release in rat islets incubated for 30 min at 3.3 mM (open bars) and 16.7 mM (black bars) glucose. Each value represents the mean ± S.E.M. for four experiments. B: Effect of 30 min preincubation of islets with 100 nM or 1 μM of NKL on their subsequent insulin secretion; open and black bars correspond to 3.3 mM and 16.7 mM glucose, respectively. $*P<0.05$, $**P<0.01$, $***P<0.001$, relative to insulin release in the absence of NKL at a corresponding glucose concentration.

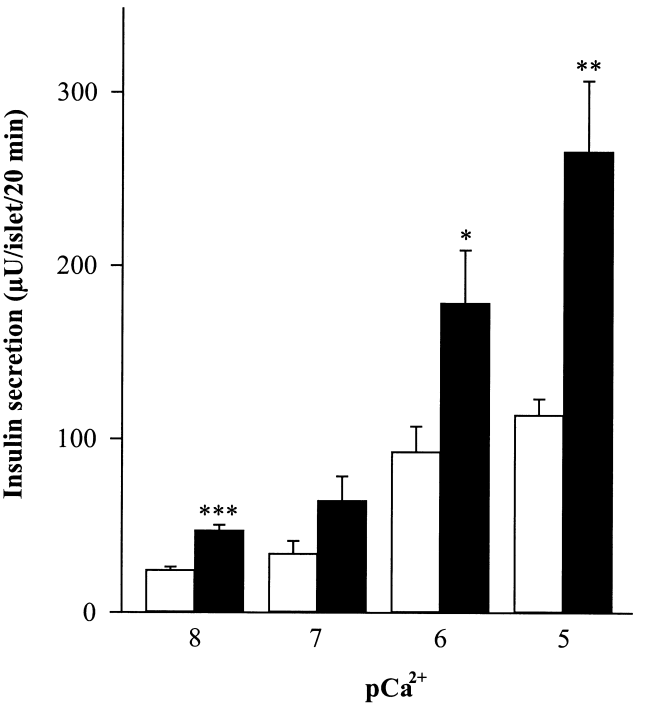

Fig. 2. Stimulation of insulin release by NKL (100 nM) in electropermeabilized rat islets at different ambient free concentrations of Ca^{2+}. Open and black columns show effects in the absence and in the presence of NKL, respectively. Each value represents the mean ± S.E.M. for three experiments. $*P<0.05$, $**P<0.01$, $***P<0.001$, relative to insulin release in the absence of NKL and at appropriate Ca^{2+} concentration.

and 10 μg/ml glutathione as previously described [14]. Measurements of insulin release in HIT T15 cells were performed in medium consisting of (in mM): NaCl 125, KCl 5.9, $CaCl_2$ 1.3, $MgCl_2$ 1.2 and HEPES 25, pH 7.4, supplemented with 1 mg/ml bovine serum albumin according to the procedure previously described [15].

2.4. Measurements of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ measurements were performed according to the procedure previously described [4,16] in a medium consisting of (in mM): glucose 3.3, NaCl 125, KCl 5.9, $CaCl_2$ 1.3, $MgCl_2$ 1.2 and HEPES 25, pH 7.4, supplemented with 2 mg/ml bovine serum albumin and other additions as shown in the figure. Single islets were loaded with 2 μM fura-2/AM for 1 h in a medium containing 3.3 mM glucose. After loading, the single islet was placed under a microscopic grid in a custom built open perfusion chamber for microscopic work and maintained at 37 ± 0.2°C. Single islets were perfused at a flow rate of 0.15 ml/min and measurements of the 340/380 nm fluorescence ratio, reflecting $[Ca^{2+}]_i$, were performed.

2.5. Assessment of cell viability

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess viability of HIT T15 cells, according to the procedure previously used for neuronal cells [17]. HIT T15 cells were incubated with 100 nM or 1 μM NKL in 24-well multiplates for 1 or 24 h. The assay was initiated by removing the old medium and adding MTT tetrazolium salt dissolved in serum-free culture medium (0.3 mg/ml, filter-sterilized) for 1 h at 37 C. The medium was then aspirated and 0.5 ml of isopropanol added. The culture plates were shaken by hand to ensure that all the MTT formazan crystals were dissolved and aliquots (100 μl) of solution were pipetted into 96-well microplates. The absorbance was recorded at 570 nm with reference at 630 nm in an Anthos htIII microplate reader (Anthos Labtec Instruments, Salzburg, Austria) and the results were expressed as percentage of intact cells as compared to control untreated cells.

2.6. Data analysis

Data analysis was performed using the programs Sigma Plot for Windows (version 1.02, Jandel Corp.) and Graph PAD (version 2.0, Graph PAD software). All results are expressed as means ± S.E.M. for the indicated number of experiments. Statistical significance of differences between means was assessed by Student's *t*-test for unpaired data. Differences were considered significant at $P<0.05$.

3. Results

3.1. Effect of NKL on insulin secretion

Rat islets showed a dose-dependent release of insulin when incubated with NKL (Fig. 1A). When islets were incubated at

3.3 mM glucose, a significant release of insulin was observed at 1 nM NKL whereas the highest NKL concentration tested, 100 nM, showed a 10-fold increase of insulin release compared to basal levels. In the presence of high glucose (16.7 mM) a significant stimulation of insulin release could be seen at 10 nM of NKL, while at 100 nM the stimulatory effect reached 150%. The effect of NKL on insulin release at high or low glucose was reversible, since exposure of islets to 100 nM or even to 1 μM NKL followed by washing off the peptide from the medium did not affect subsequent insulin release either at 3.3 mM or 16.7 mM glucose (Fig. 1B).

The stimulatory effect of NKL on insulin release was also observed in permeabilized islets under Ca^{2+} -clamped conditions (Fig. 2). At all Ca^{2+} concentrations (10^{-8}–10^{-5}) 100 nM NKL increased insulin release by about 100–150%.

In the β-cell line HIT T15, 100 nM NKL stimulated insulin secretion both in the absence and in the presence of high glucose (Fig. 3). Insulinotropic effect of the peptide was also reversible in HIT T15 cells. After preincubation of cells with 100 nM NKL at 0 glucose for 18 h followed by washing off NKL from the medium HIT T15 cells were fully responsive to a subsequent addition of either 10 mM glucose or 100 nM NKL, the responses being 98 ± 11 and 126 ± 34% of control (not preincubated) cells, respectively.

3.2. Effect of NKL on $[Ca^{2+}]_i$

Experiments performed on single rat pancreatic islets did not show any effect of NKL on $[Ca^{2+}]_i$ in the presence of either 3.3 or 16.7 mM glucose (Fig. 4).

3.3. NKL effects on cell viability

To assay the effect of NKL on cell viability, HIT T15 cells were incubated with 100 nM or 1 μM NKL for 1 h or 24 h and then analyzed for residual living cells using the MTT test.

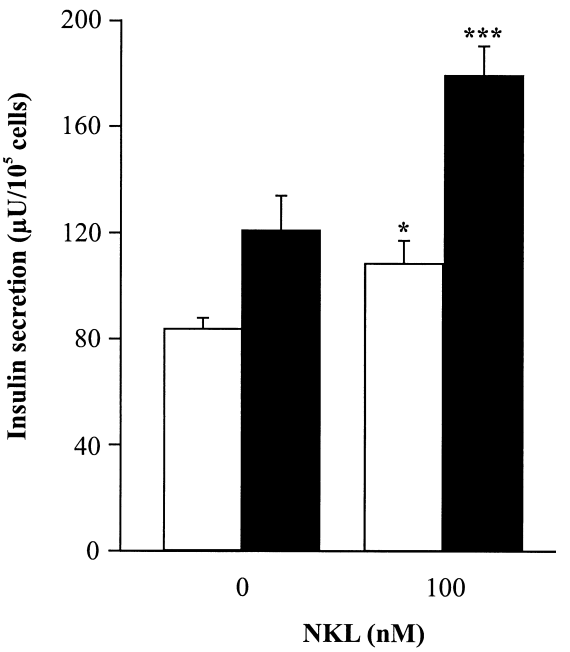

Fig. 3. Effect of NKL (100 nM) on insulin release in HIT T15 cells in the absence (open bars) and presence (black bars) of glucose (10 mM). Each value represents the mean ± S.E.M. for four experiments. $*P < 0.05$, $***P < 0.001$, relative to insulin release in the absence of NKL at a corresponding glucose concentration.

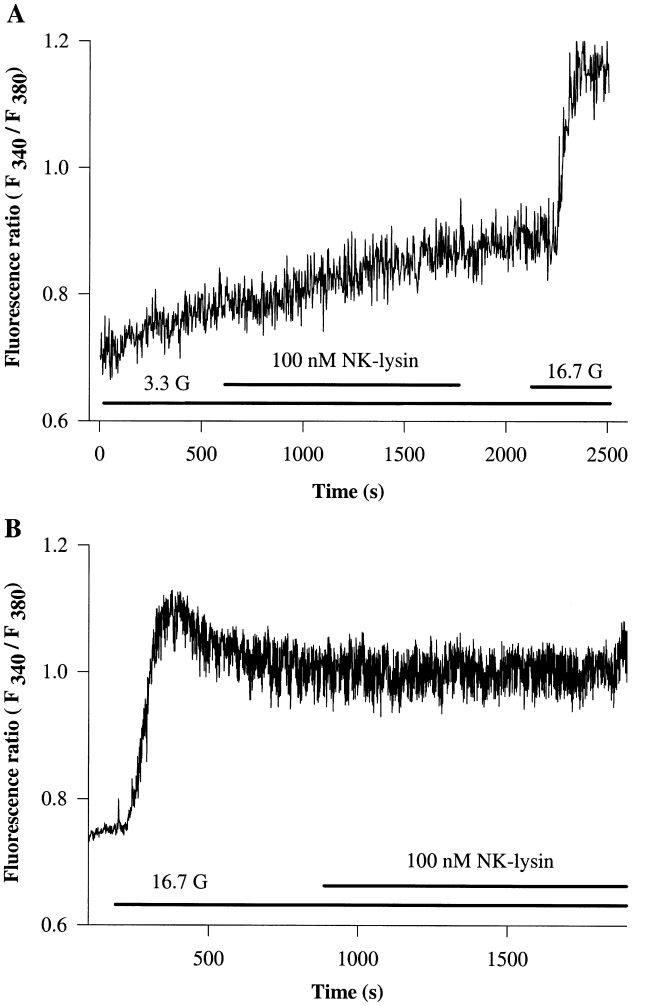

Fig. 4. Effects of NKL on fluorescence ratio F_{340}/F_{380} corresponding to $[Ca^{2+}]_i$ in a single rat pancreatic islet perfused with 3.3 mM glucose (A) or with 16.7 mM glucose (B). Addition of NKL is indicated in the figure. Representative traces out of five experiments.

The results obtained show that preincubation with NKL did not influence the viability of HIT T15 cells (Table 1).

4. Discussion

This study was performed with the aim to search for new endogenous substances which modulate insulin secretion. It appears that an endogenous peptide isolated from gut, NK-lysin, previously shown to possess antimicrobial and antitumor activity, at μM concentrations [10], is a potent stimulator of insulin secretion in the nM range. The stimulatory effect of NKL is due to a direct effect of the peptide on the β-cell, since the insulinotropic effect of the peptide could also be observed

Table 1
Effect of NKL on viability of HIT-T15 cells

	1 h incubation (%)	24 h incubation (%)
Control	100 ± 1.3	100 ± 1.6
100 nM NKL	99 ± 1.7	98 ± 1.7
1 μM NKL	100 ± 2.5	97 ± 2.4

Values are expressed as percentage of the untreated control, arbitrarily set to 100.

in the β-cell line HIT T15. NKL stimulated insulin secretion independent on concomitant changes in $[Ca^{2+}]_i$. This was evident from both experiments showing no increase in $[Ca^{2+}]_i$ after addition of NKL and by the observation that NKL significantly increased insulin release in permeabilized islets when Ca^{2+} concentrations were clamped to 10^{-8}–10^{-5} M.

NKL belongs to a class of physiologically active peptides and shares similarities with peptides of the saposin family [18]. It has recently been shown that some peptides of this family of proteins, e.g. the amoebapores, can damage or even destroy other cells by pore formation in the plasma membrane of the target cell [19,20]. However, our data argue against the possibility that NKL induced increase in insulin release by making pores in the plasma membrane of β-cells. First, preincubation with NKL did not affect a subsequent basal or glucose-stimulated insulin release. Second, if there was any kind of cell damage by NKL, which made the β-cell plasma membrane permeable and leaky it should have given rise to influx of extracellular Ca^{2+} and hence to an increase in $[Ca^{2+}]_i$. Our experiments did not reveal any increase in $[Ca^{2+}]_i$ at either basal or high glucose. Third, experiments performed on insulin secreting cells with the MTT test showed that NKL (up to 1 μM) did not affect the viability of these cells.

Although NKL appeared not to form pores under our experimental conditions, it is possible that a membrane activity of this peptide is responsible for its effect on insulin exocytosis. NKL is an amphiphilc peptide which binds to lipid bilayers, destabilizing them [18,21]. The membrane destabilizing activity of NKL may lead to facilitation of vesicle fusion with plasma membrane and hence to an increased exocytosis.

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