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Requirement of NF-kappaB signalling pathway for modulation of the cholinergic muscarinic M_3 receptor expression by INGAP-PP in insulin-producing cells

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ABSTRACT

The pentadecapeptide comprising the 104–118 amino acid sequence of the ilotropin-derived Reg3-related islet neogenesis-associated protein (INGAP-PP) has been implicated in beta cell neogenesis and enhancement of insulin secretion in pancreatic islets. The aim of this study was to investigate intracellular pathways by which INGAP-PP signals in insulin-producing cells. Treatment with INGAP-PP increased insulin secretion and intracellular calcium levels in MIN6 cells. INGAP-PP exposure activated c-Myc, serum and particularly nuclear factor-kappaB (NF-κB) response elements in insulin-producing cells (1.7 ± 0.1, 1.8 ± 0.1, 2.4 ± 0.3 for RINm5F, and 1.3 ± 0.1, 1.3 ± 0.1 and 1.6 ± 0.1 fold for MIN6 cells compared to controls, respectively). There was an increase in the proliferation rate of viable cells ($162 \pm 17\%$ for RINm5F and $155 \pm 13\%$ for MIN6) that was accompanied by an increase in proliferating cell nuclear antigen (PCNA) protein expression ($187 \pm 19\%$ and $170 \pm 8\%$ for RINm5F and MIN6 cells respectively) following INGAP-PP treatment. INGAP-PP increased the expression of the muscarinic M_3 receptor subtype ($169 \pm 4\%$ for RINm5F and $222 \pm 20\%$ for MIN6 cells). Activation of multiple serum response elements by foetal calf serum also increased muscarinic M_3 receptor expression ($173 \pm 9\%$ for RINm5F and $140 \pm 7\%$ for MIN6 cells). The blockade of NF-κB signalling pathway strongly decreased muscarinic M_3 receptor expression in response to both stimuli. In summary, a network of intracellular signals that includes activation of c-Myc signalling pathway and increased PCNA expression might be related to the increased proliferation rate of insulin-producing cells following incubation with INGAP-PP. NF-κB signalling plays an essential role in controlling the expression of the muscarinic M_3 receptor.

1. Introduction

Loss of pancreatic beta cell mass induces diabetes, and restoration of insulin-secreting cells is a feasible strategy for the treatment of both Type 1 and Type 2 diabetes mellitus (Rosenberg et al., 2004). INGAP (islet neogenesis-associated protein) was first identified in hamster pancreas following hypoxia/mechanical stress, conditions that induce proliferation of insular cells (Pittenger et al., 1992). INGAP is involved in the neogenesis of beta cells (Rafaeloff et al., 1997), and it has been demonstrated that a pentadecapeptide containing the amino acid sequence in the interval 104 to 118 (INGAP pentadecapeptide or INGAP-PP) induces the same effects as the whole molecule in many target cells (Rafaeloff et al., 1997). Therefore, INGAP-PP might be an interesting pharmacological agent in the treatment of diabetes.

There is little information available on the mechanisms by which INGAP-PP exerts its effects on pancreatic beta cells. Acute exposure of neonatal rat islets to INGAP-PP stimulates insulin secretion (Barbosa et al., 2008) and it is possible that INGAP-PP-induced increases in beta cell mass are secondary to the autocrine insulin signalling cascade that has been previously found to be important in maintaining pancreatic beta cell mass (Muller et al., 2006). Loss of beta cells in either Type 1 or Type 2 diabetes cannot be counteracted by the normal proliferation rate. In the particular case of Type 2 diabetes, a chronic process of impairment of beta cell function leads ultimately to cell death and consequently decreases beta cell mass (Butler et al., 2003). Consequently, strategies aiming at increasing beta cell mass may be of great value in the attempts to find a cure or novel therapies for diabetes.

Glucose oxidation, and the subsequent production of ATP, is the main stimulator of insulin secretion in beta cells (Boschero et al., 1990). The fine tuning of secretion is achieved by several pathways, including the autonomic nervous system that plays a major role in controlling insulin release (Ahren, 2000). While sympathetic activation

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of adrenergic alpha-2 receptors diminishes, parasympathetic activation of cholinergic muscarinic M_3 receptors enhances insulin release (Ahren, 2000; Boschero et al., 1995; Gautam et al., 2006). The muscarinic acetylcholine receptors are members of the G-protein coupled superfamily receptors (Gautam et al., 2006), and the INGAP-PP increases the muscarinic M_3 receptor expression in neonatal pancreatic islets (Barbosa et al., 2008).

In this study, we investigated cell signalling pathways activated by the INGAP-PP using two models for the in vitro study of pancreatic beta cells, namely the RINm5F and the MIN6 insulin-producing cell lines. For this purpose, screening of transcription factor-responsive element activation was carried out by means of reporter gene assays. Our results provide evidence that INGAP-PP signals in beta cell through activation of c-Myc, serum and, in particular, nuclear factor kappa B (NF-κB) response elements, increases cellular proliferation and increases muscarinic M_3 receptor expression. Stimulation of the NF-κB transcription factor is primarily responsible for the increase in the muscarinic M_3 receptor expression following INGAP-PP treatment.

2. Materials and methods

2.1. Materials

INGAP-PP, a pentadecapeptide with the 104–118 amino acid sequence of INGAP (NH-Ile-Gly-Leu-His-Asp-Pro-Ser-His-Gly-Thr-Leu-Pro-Asn-Gly-Ser-COOH), was provided by GenScript Corp. (Scotch Plains, NJ, USA). Quality control of the peptide (amino acid analysis and mass spectrometry) indicated >95% purity and a molecular weight of 1501.63. All SDS-PAGE and immunoblotting equipment were from Bio-Rad systems (Richmond, CA, USA). Anti-NF-κB p65 (C-20), a kind gift from Prof. L. A. Velloso (Campinas, Brazil), anti-p70 S6 kinase α (H-9) anti-Akt1/2/3 (H-136), anti-PI 3-kinase p85α (Z-8), and anti-PCNA (PC10) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-beta-actin antibody was from Abcam plc. (Cambridge, UK). Recombinant rat interleukin-1beta (IL-1beta) and tumour necrosis factor-alpha (TNF-alpha), Lipofectamine 2000, Top10 competent bacteria, and the Phospha-Light Secreted Alkaline Phosphatase Reporter Gene Assay System were from Invitrogen Corp. (Carlsbad, CA, USA). Reporter gene vectors were from Clontech (Mercury Pathway Profiling Systems, Clontech Laboratories, Inc., Mountain View, CA, USA). NF-κB p65 siRNA was from IDT (Coralville, IA, USA). Anti-muscarinic acetylcholine receptor M_3 antibody, carbachol, RPMI 1640 and DMEM medium, ammonium pyrrolidine dithiocarbamate (PDTC), and all other reagents were from Sigma Chemicals Co. (St. Louis, MO, USA).

2.2. Cell culture

RINm5F (passages 75–85) and MIN6 (passages 25–35) insulin-producing cells were cultured in RPMI 1640 or DMEM medium, respectively, supplemented with penicillin, and streptomycin in a humidified atmosphere at 37 °C and 5% CO_2. Unless otherwise indicated, foetal calf serum-free medium was used. Cells were plated at a density of 5×10^5 per 50-mm plastic dish and allowed to grow until reaching 90% confluence. When using 96-well plates, 5×10^4 for MTS or 1.5×10^5 starting cells for reporter gene analysis were used. Subsequently, cells were exposed to 10 μg/ml of INGAP-PP for the desired period. This concentration was selected on the basis of previous *in vitro* studies with pancreatic islets (Barbosa et al., 2008).

2.3. Pseudoislet generation

MIN6 cells were grown in non-adherent Petri plates for 1 week in DMEM medium supplemented with 10% foetal calf serum. Thereafter, naturally-formed pseudoislets were hand-picked using a micropipette and immediately used for the experiments.

2.4. Insulin secretion

The effects of INGAP-PP on insulin secretion from MIN6 pseudoislets was measured by radioimmunoassay as previously described (Barbosa et al., 2008; Luther et al., 2006).

2.5. Calcium measurements

MIN6 cells attached to glass coverslips were incubated in a physiological salt solution containing 10 mmol/l Hepes, 2 mmol/l glucose, 2 mmol/l calcium chloride, and supplemented with 5 μmol/l fura-2 acetoxymethyl ester for 30 min at 37 °C. Measurements of changes in intracellular free Ca^{2+} were performed as previously described (Persaud et al., 2002).

2.6. MTS cell proliferation assay

The number of proliferating viable cells was determined by the colorimetric method of reduction of tetrazolium salt into soluble formazan, according to the manufacturer's instructions.

2.7. Western blotting analyses

Control and INGAP-PP-incubated cells were homogenised by sonication in ice-cold medium containing protease inhibitors and centrifuged at 12,000 *g* and 4 °C for 1 min. Either 30 μg of the total protein for PCNA, 35 μg for the muscarinic M_3 receptor and p65 NF-κB subunit, or 50 μg for all other protein expression analysis from the supernatants were resolved by electrophoresis in 10% SDS-polyacrylamide gel and electroblotted onto nitrocellulose membranes. After blocking in 5% non-fat milk solution overnight, immunodetection was performed after an incubation period of 6 h with either a rabbit polyclonal p85 alpha antibody (1:500 dilution), a rabbit polyclonal Akt antibody (1:500 dilution), a mouse monoclonal $p70^{S6k}$ antibody (1:500 dilution), a mouse monoclonal PCNA antibody (1:1000 dilution), a rabbit polyclonal muscarinic M_3 receptor antibody (1:2000 dilution), and a rabbit polyclonal p65 antibody (1:1000 dilution). A rabbit polyclonal beta-actin antibody (1:10,000 dilution) was used as a housekeeping antibody. Membranes were then exposed to 150 ng/ml specific secondary peroxidase-conjugated antibody (anti IgG (H + L)–HRP, Invitrogen) for 2 h at 22 °C, and visualized by chemiluminescence (SuperSignal, Pierce Biotechnology Inc., Rockford, IL, USA). The bands were quantified using the Scion Image software (Scion Corp., Frederick, MD, USA).

2.8. Plasmid transfection and secreted alkaline phosphatase (SEAP) activity quantification for the reporter gene assay

Well characterized plasmids containing responsive elements to nuclear factor kappa B (NF-κB), c-Myc, activator protein 1 (AP1), serum, cAMP (CRE), and nuclear factor of activated T-cells (NFAT) were propagated in bacteria and purified using minipreps (PureLink Quick Plasmid Miniprep Kit, Invitrogen). Insulin-producing cells were plated in appropriate medium containing 10% foetal calf serum and allowed to attach for 24 h. The medium was then replaced by foetal calf serum-free antibiotic-free medium and cells were separately transfected with each vector construct (Lipofectamine 2000, Invitrogen). Four hours after transfection, foetal calf serum (final concentration of 1%) and antibiotics were added to the medium and cells were allowed to grow for an additional 18 h. Thereafter, medium was replaced again by foetal calf serum-free phenol red-free medium, which was used until the end of the protocols. After 24 h, either INGAP-PP or specific compounds for positive controls were added to the medium and samples were collected after 6 and 24 h incubation. The reporter signalling of the SEAP enzyme was measured using a commercial luminescent kit, according to the manufacturer's instructions

(Phospha-Light Secreted Alkaline Phosphatase Reporter Gene Assay System, Invitrogen).

2.9. Isolation of cytosolic and nuclear fractions

Nuclei were isolated according to the instructions contained in the "Nuclei Isolation Kit: Nuclei EZ Prep" (Sigma Chemicals Co.). The composition of the hypotonic lysis buffer, from which cytosolic fraction is obtained from the supernatant, was: Hepes (10 mmol/l), $MgCl_2$ (2 mmol/l) and KCl (25 mmol/l). Nuclei-containing pellets were washed twice in the nuclei resuspension buffer, composed of hypotonic lysis buffer added of 250 mmol/l of sucrose. Finally, nuclear proteins were obtained by incubating and vortexing the nuclei pellets with the nuclei extraction buffer, composed of the hypotonic lysis buffer with the addition of 1% Triton X100. All buffers contained a protease inhibitor cocktail, composed of phenylmethylsulphonyl fluoride (2 mmol/l), sodium orthovanadate (10 mmol/l), sodium fluoride (100 mmol/l), aprotinin (0.1 mg/ml) and EDTA (200 mmol/l).

2.10. siRNA transfection

RINm5F and MIN6 cells were seeded on 6-well culture plates until reaching 80% confluence. Thereafter, cells were transfected with 200 nmol/l (final concentration) siRNA raised against a consensus rat–mouse sequence of the p65 subunit of NF-κB (CGCAAACTG-CGAGUUAUAGCUUCAGGAGUACCCUGAAGCUACAGTTTGCG) using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Mock transfection was used as control. Cells were harvested for Western blotting measurements after 48, 72, 96, and 120 h of transfection.

2.11. Statistics

All data are expressed as means ± S.E.M. Statistical analyses were performed using Student's *t*-test or one-way ANOVA followed by Bonferroni's or Dunnett's test, as required. $P<0.05$ was considered statistically significant.

3. Results

3.1. Validation of insulin-producing cells as a model to study INGAP-PP signalling

We first tested whether MIN6 cell-derived islet-like structures (pseudoislets), which are well-known to respond to glucose and other nutrient and non-nutrient stimuli (Luther et al., 2006), show stimulated insulin secretion in response to acute exposure to INGAP-PP as do neonatal rat islets (Barbosa et al., 2008). Exposure of MIN6 pseudoislets to INGAP-PP caused an acute, reversible increase in insulin secretion that was nearly half of the amplitude of the increase observed in response to 20 mmol/l glucose, which is the main physiological signal for insulin secretion (Fig. 1A). This observation confirmed that INGAP-PP acutely stimulates insulin secretion from MIN6 β-cells, as it does in primary islets (Barbosa et al., 2008). To reinforce further the validation of insulin-producing cells as a model and to provide molecular insights into the effects of INGAP-PP that may have functional relevance for insulin secretion, we measured the intracellular calcium handling by MIN6 cells upon INGAP-PP stimulation. Fig. 1B shows that 10 μg/ml INGAP-PP stimulated a reversible increase in intracellular calcium at 2 mmol/l glucose in fura 2-loaded MIN6 cells, and that the cells showed a similar magnitude increase in response to 20 mmol/l glucose. INGAP-PP also further increased MIN6 cell intracellular calcium levels when it was administered in the presence of 20 mmol/l glucose (Fig. 1B, starting at 17 min incubation). The cells remained viable after exposure to INGAP-PP as they were able to mount an appropriate elevation in calcium in response to the purinergic agonist, ATP.

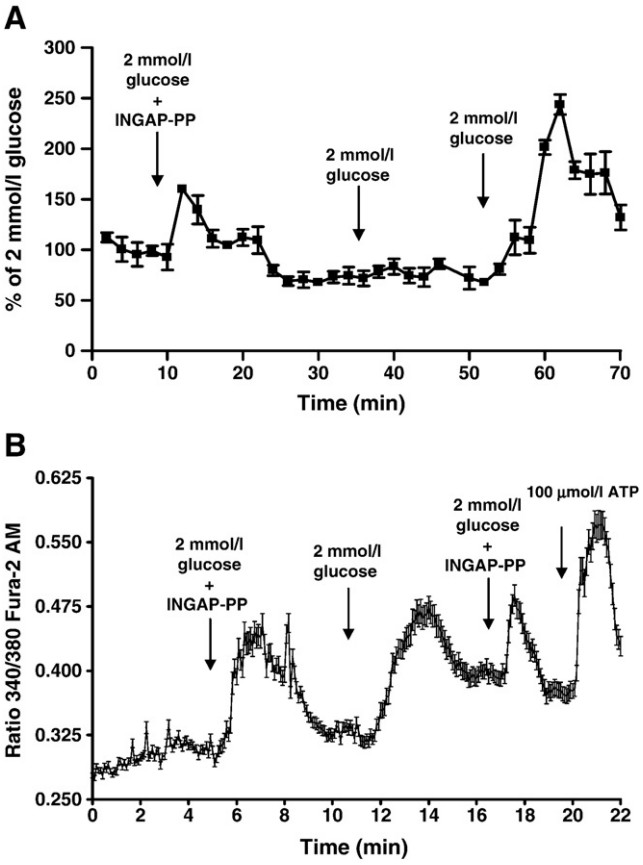

Fig. 1. Kinetics of INGAP-PP-evoked stimulation of insulin secretion and intracellular calcium handling. A) MIN6 pseudoislets were continuously perifused with a physiological salt solution at 37 °C. After a pre-perifusion period of 1 h (2 mmol/l glucose) the effluent was collected every 2 min for 70 min. INGAP-PP (10 μg/ml) was applied, as indicated, in the presence of 2 mmol/l glucose. Results are expressed as% of mean values calculated for min 0 to 10 (2 mmol/l glucose), and normalised against total insulin content ($N=3$ independent experiments). Peak of INGAP-PP-induced insulin release was significantly increased ($P<0.05$; *t*-test) compared to basal insulin secretion (2 mmol/l glucose). B) Fura 2-loaded MIN6 beta cells responded to INGAP-PP (10 μg/ml) at 2 mmol/l and 20 mmol/l glucose with a reversible increase in intracellular calcium. Peak of intracellular calcium increase was 86 ± 2% of 100 μmol/l ATP response. $N=36$ separate cells.

3.2. Effect of INGAP-PP on proliferation of insulin-producing cells

Since insulin-producing cells comprise very pure populations (Bargsten, 2004; Miyazaki et al., 1990), making them very useful for proliferation studies, we next explored a possible pro-proliferative effect of INGAP-PP using a well-validated MTS cell proliferation kinetic assay (Zhang et al., 2004). Exposure of both RINm5F and MIN6 insulin-producing cells to 10 μg/ml of the INGAP-PP for 72 h increased the number of proliferating viable cells as shown by the MTS test (Fig. 2). This effect was observed in both the presence (Fig. 2A) and absence (Fig. 2A and B) of 10% foetal calf serum. In the presence of INGAP-PP alone, MTS reduction to formazan increased to 162 ± 17% and 155 ± 13% of control values for RINm5F and MIN6 insulin-producing cells, respectively, compared to cells grown in the absence of the peptide (Fig. 2B).

3.3. Effect of INGAP-PP on the expression of the p85 alpha catalytic subunit of phosphatidylinositol 3-kinase (p85), RAC-alpha/beta/gamma serine/threonine-protein kinase (Akt1/2/3), ribosomal protein S6 kinase beta ($p70^{S6k}$), proliferating cell nuclear antigen (PCNA) and muscarinic M_3 receptor in insulin-producing cells

Incubation of insulin-producing cells with INGAP-PP for 72 h did not change the expression of the downstream insulin signalling

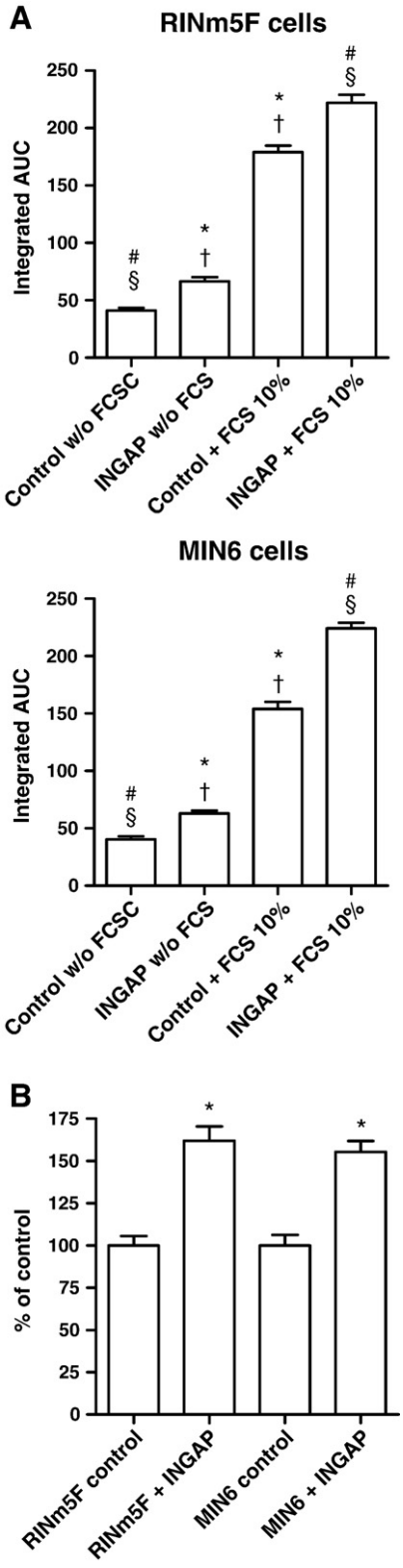

Fig. 2. Cell proliferation after exposure of insulin-producing cells to the INGAP-PP in the presence or absence of 10% foetal calf serum (FCS). A) RINm5F and MIN6 cells were cultured in the absence (w/o FCS) or presence (+10% FCS) of foetal calf serum and exposed to 10 μg/ml INGAP-PP for 72 h (INGAP groups). Control cells were grown in the absence of INGAP-PP. MTS conversion to a soluble formazan product by viable cells was determined by kinetic measurement of absorbance at 490 nm. N = 6. Symbols above bars represent $P<0.05$ vs. Control w/o FCS (*), INGAP w/o FCS (#), Control + 10% FCS (§), INGAP + 10% FCS (†); ANOVA followed by Bonferroni. B) Percentage values related to exposition of insulin-producing cells to INGAP-PP alone. N = 6. * $P<0.05$; *t*-test against respective controls.

pathway proteins p85, Akt1/2/3 and $p70^{S6K}$. However, there were increases of 187 ± 19% and 170 ± 8% in the expression of PCNA, as a direct indicator of proliferation, and of 169 ± 4% and 222 ± 20% in the expression of the muscarinic M_3 receptor subtype, for RINm5F and MIN6 cells, respectively (Fig. 3).

3.4. Effect of INGAP-PP on intracellular signalling pathways in insulin-producing cells

A gene reporter screening analysis of RINm5F and MIN6 cells in response to INGAP-PP exposure is shown in Table 1. INGAP-PP (10 μg/ml) exposure increased c-Myc, as well as serum response element activation, in both cell types. A strong activation of NF-κB signalling in both cells starting already at 6 h incubation was also observed. At 24 h incubation NFAT was activated by INGAP-PP only in RINm5F cells while AP1 was activated only in MIN6 cells.

3.5. Effect of INGAP-PP on the nuclear factor kappa B (NF-κB) translocation to the nucleus in insulin-producing cells

Translocation of NF-κB was measured using a specific antibody against the p65 subunit in both cytosolic and nuclear fractions. A time course for incubation of insulin-producing cells with INGAP-PP is shown in Fig. 4. There was an increase in the nucleus/cytosol optical density ratio for p65 expression at 6 h, which was enhanced at 24 h in both RINm5F and MIN6 cells. No significant differences were found at the 72 h exposure time though activation was still greater than controls. These results confirm that activation of the NF-κB signalling pathway in insulin-producing cells occurs as early as 6 h after exposure to INGAP-PP.

3.6. Contribution of NF-κB signalling to INGAP-PP-induced upregulation of muscarinic M_3 receptor expression in insulin-producing cells

There was an increase in the muscarinic M_3 receptor expression in insulin-producing cells exposed to INGAP-PP for 24 h (Fig. 5), similar in magnitude to that obtained after 72 h incubation of RINm5F and MIN6 cells with INGAP-PP (as shown in Fig. 3). Blocking NF-κB translocation by incubation of cells with the specific inhibitor PDTC (pyrrolidine dithiocarbamate) caused a significant ($P<0.05$) decrease of muscarinic M_3 expression in both control and INGAP-PP-treated cells. Thus, RINm5F cells showed a decrease of 49% and 62% and MIN6 cells showed a reduction of 52% and 57% after NF-κB inhibition for control and INGAP-PP-treated cells, respectively.

3.7. Differential contribution of serum response elements and the NF-κB response element for INGAP-PP-stimulated expression of the muscarinic M_3 receptor in insulin-producing cells

There was an increase in the muscarinic M_3 receptor expression in both RINm5F and MIN6 insulin-producing cells incubated in the presence of 10% foetal calf serum for 24 h compared to cells grown in foetal calf serum-free medium (Fig. 6). This effect was further enhanced in cells incubated with both foetal calf serum and INGAP-PP. Blocking NF-κB translocation with PDTC caused a strong decrease of muscarinic M_3 expression in both tested situations, especially in the MIN6 cells incubated with foetal calf serum alone, which were very sensitive to the NF-κB inhibition. While RINm5F cells presented a decrease of 60% and 61%, MIN6 cells presented a decrease of 85% and 61% after NF-κB blockade for cells treated with foetal calf serum alone or foetal calf serum plus INGAP-PP, respectively.

3.8. Confirmation of the NF-κB-dependent modulation of the muscarinic M_3 receptor expression by small interfering RNA (siRNA)

Confirmation of the involvement of NF-κB in controlling muscarinic M_3 receptor expression was obtained using a siRNA raised

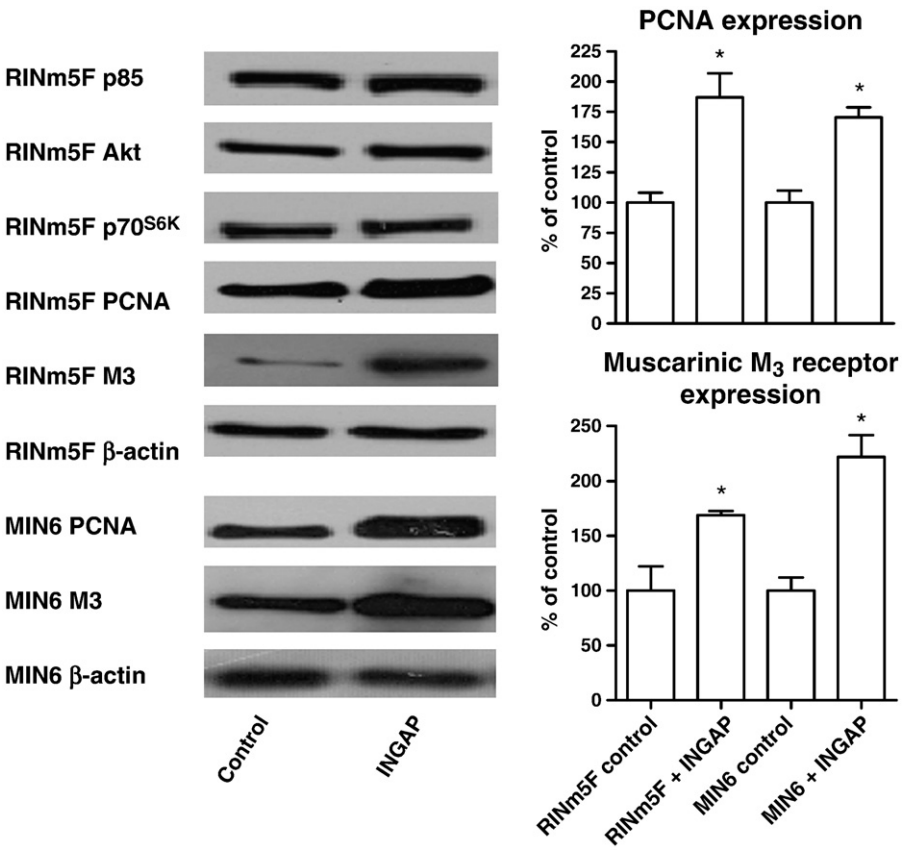

Fig. 3. Effect of INGAP-PP on the expression of proteins implicated in cell proliferation and of the cholinergic muscarinic M_3 receptor subtype in insulin-producing cells. RINm5F and MIN6 cells were seeded 24 h before incubation with 10 μg/ml INGAP-PP (INGAP group). After 72 h of incubation, the cells were lysed and used for Western blot analyses. Blots on the left are representative of 5 independent experiments. Graphics on the right are mean values for proliferating cell nuclear antigen (upper graphic) or muscarinic M_3 receptor (lower graphic) expression, both normalised to the housekeeping protein beta-actin. P85, p85 alpha catalytic subunit of phosphatidylinositol 3-kinase; Akt, RAC-alpha/beta/gamma serine/threonine-protein kinase; $p70^{S6k}$, ribosomal protein S6 kinase beta; PCNA, proliferating cell nuclear antigen; M3, muscarinic M_3 receptor. * $P<0.05$; *t*-test against respective control.

Table 1
Intracellular signalling pathways after exposure of insulin-producing RINm5F and MIN6 cells to INGAP peptide.

	6 h			24 h		
	Control	INGAP-PP	Positive control	Control	INGAP-PP	Positive control
RINm5F insulin-producing cells						
AP-1	1.0 ± 0.1	1.2 ± 0.1	3.0 ± 0.4	1.0 ± 0.1	1.2 ± 0.4	3.3 ± 0.4
CRE	1.0 ± 0.1	1.0 ± 0.1	1.3 ± 0.1	1.0 ± 0.3	1.0 ± 0.1	1.5 ± 0.4
c-Myc	1.0 ± 0.1	1.4 ± 0.2	2.6 ± 0.2	1.0 ± 0.1	1.7 ± 0.1[a]	4.6 ± 0.4
NFAT	1.0 ± 0.2	0.9 ± 0.1	1.5 ± 0.3	1.0 ± 0.1	1.5 ± 0.2[a]	2.4 ± 0.2
NFκB	1.0 ± 0.0	1.8 ± 0.4[a]	2.8 ± 0.5	1.0 ± 0.2	2.4 ± 0.3[a]	5.8 ± 0.3
Serum	1.0 ± 0.1	1.2 ± 0.1	1.5 ± 0.1	1.0 ± 0.1	1.8 ± 0.1[a]	2.4 ± 0.2
MIN6 insulin-producing cells						
AP-1	1.0 ± 0.1	1.3 ± 0.1	2.8 ± 0.3	1.0 ± 0.1	1.4 ± 0.1[a]	2.5 ± 0.2
CRE	1.0 ± 0.0	1.0 ± 0.1	1.2 ± 0.1	1.0 ± 0.0	1.0 ± 0.1	1.5 ± 0.1
c-Myc	1.0 ± 0.1	1.5 ± 0.2[a]	2.5 ± 0.3	1.0 ± 0.0	1.3 ± 0.1[a]	3.2 ± 0.1
NFAT	1.0 ± 0.1	1.0 ± 0.2[a]	1.5 ± 0.2	1.0 ± 0.1	1.2 ± 0.0	1.7 ± 0.2
NFκB	1.0 ± 0.1	2.2 ± 0.1[a]	3.1 ± 0.3	1.0 ± 0.1	1.6 ± 0.1[a]	3.2 ± 0.1
Serum	1.0 ± 0.1	1.2 ± 0.0	1.2 ± 0.1	1.0 ± 0.0	1.3 ± 0.1[a]	3.0 ± 0.1

Insulin-producing RINm5F and MIN6 cells were exposed to the INGAP-PP (10 μg/ml) in serum-free medium and samples collected after 6 and 24 h thereafter. Results are presented as -fold ± S.E.M. and compared against control values obtained from cells incubated without INGAP-PP. Positive controls were 10% foetal calf serum (AP-1 and c-Myc responsive elements), 25 μmol/l Forskolin (CRE), 50 ng/ml TNF-alpha (NFAT and NFκB), 2 ng/ml IL-1beta (Serum). AP-1, activator protein 1 responsive element; CRE, cAMP responsive element; c-Myc, c-Myc responsive element; NFAT, nuclear factor of activated T-cells responsive element; NFκB, nuclear factor kappa B responsive element; Serum, serum responsive element. N = 9. [a] $P<0.05$; *t*-test against respective controls.

against the p65 subunit of NF-κB. Fig. 7 shows that treatment of RINm5F insulin-producing cells with the NF-κB siRNA decreased the expression of the NF-κB over the time, with maximal effect at 120 h, when a 56 ± 8% decreased was observed (Fig. 7A and B, $P<0.05$), whilst MIN6 insulin-producing cells showed an even greater decrease of 67 ± 11% at the same time-point (Fig. 7D and E, $P<0.05$). This was paralleled by a decrease of 59 ± 5% in the expression of muscarinic M_3 receptor in both cell lines RINm5F at the same time-point (Fig. 7A and C, and Fig. 7D and F, $P<0.05$), confirming that NF-κB modulates the muscarinic M_3 cholinergic receptor expression. The additional lower band in the muscarinic M_3 protein expression experiments, which is strongly presented in the MIN6 cells blots, is unknown.

3.9. Activation of the NF-κB signalling pathway by the cholinergic agonist carbachol (CCh) in the presence and in the absence of INGAP-PP

Translocation of NF-κB was measured using a specific antibody against the p65 subunit in both cytosolic and nuclear fractions. A time course for incubation of insulin-producing cells with CCh is shown in Fig. 8A. There was an increase in the nucleus/cytosol optical density ratio for p65 expression from RINm5F (upper graphic) and MIN6 (lower graphic) insulin-producing cell lines of 178% and 214% respectively for each cell line after 10 min incubation with CCh. This effect was slight enhanced at 15 min incubation (207% for RINm5F and 269% for MIN6 cells). This maximum activation was kept up to 1 h after CCh exposure (not shown). A concentration–response curve to CCh is shown in Fig. 8B. In RINm5F cells (upper graphic) translocation

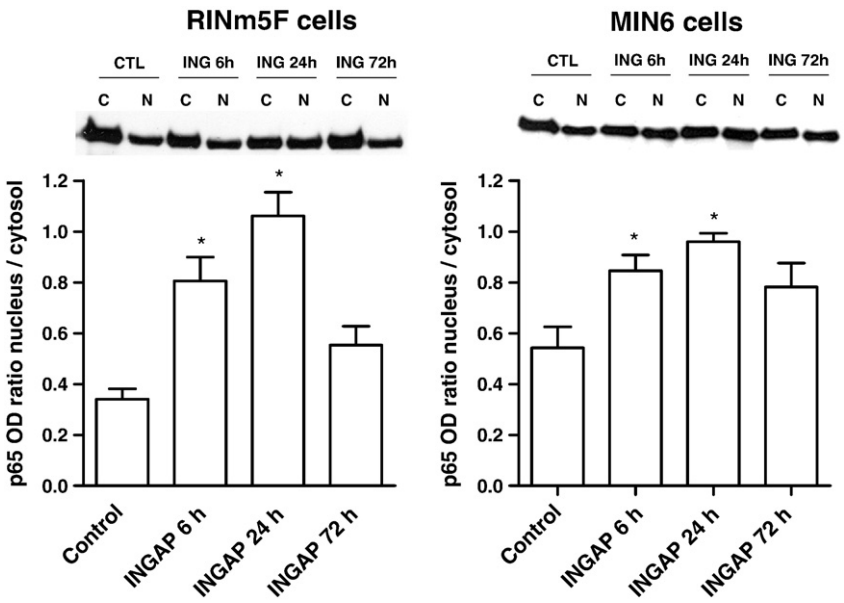

Fig. 4. Effect of INGAP-PP on NF-κB p65 subunit translocation into the nucleus in insulin-producing cells. RINm5F and MIN6 cells were exposed to 10 μg/ml INGAP-PP for the indicated incubation periods. Thereafter, cells were lysed and cytosolic and nuclear fractions were separated and used for Western blot analyses. Blots on the top are representative of 3 independent experiments. C, cytosolic fraction; N, nuclear fraction. CTL, control; ING or INGAP, INGAP-PP-treated cells. * $P<0.05$ against controls; ANOVA followed by Dunnett.

of p65 subunit of NF-κB was induced at concentrations of CCh as low as 10 μmol/l (237% of control), and maximum activation was achieved at 100 μmol/l (257% of control) after 15 min of incubation. In MIN6 cells (lower graphic) translocation of p65 subunit of NF-κB was induced at concentrations of CCh as low as 1 μmol/l (162% of control), and maximum activation was also achieved at 100 μmol/l (209% of control) after 15 min of incubation. In both cell lines, a decline of NF-κB translocation occurred at the concentration of 1000 μmol/l, especially in the RINm5F cells. Fig. 8C shows the effect of co-incubation of INGAP-PP and CCh in RINm5F (left graphic) and MIN6 (right graphic) insulin-producing cells lines. Both compounds were able to induce translocation of NF-κB to the nucleus. However, there was no increase in NF-κB activation by CCh after pre-incubation with INGAP-PP compared to incubation with CCh alone.

4. Discussion

The INGAP-PP has been formerly associated with neogenesis of pancreatic islets from ductal precursors (Lipsett et al., 2006), including those from animal models of diabetes (Rosenberg et al., 2004). Therefore, it is intriguing that in the current study INGAP-PP increased proliferation of insulin-producing cells since insulin is the major hormone expressed by RINm5F cells (Bargsten, 2004), and the only hormone secreted by MIN6 cells (Miyazaki et al., 1990),

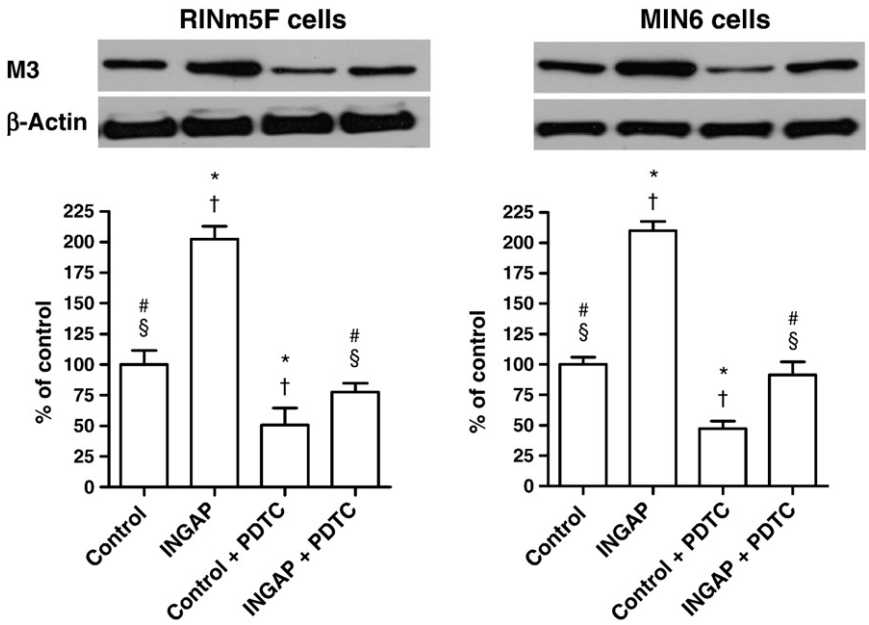

Fig. 5. Effect of blockade of NF-κB on the INGAP-PP-induced muscarinic M_3 receptor protein expression in insulin-producing cells. RINm5F and MIN6 cells were seeded 24 h before incubation with 10 μg/ml INGAP-PP. After 24 h of INGAP-PP (INGAP groups) incubation in the presence or absence of the NF-κB inhibitor PDTC, the cells were lysed and used for Western blot analyses. Blots on the top are representative of 5 independent experiments, normalised to the housekeeping protein beta-actin. Symbols above bars represent $P<0.05$ vs. Control (*), INGAP (#), Control + PDTC (§), INGAP + PDTC (†); ANOVA followed by Bonferroni.

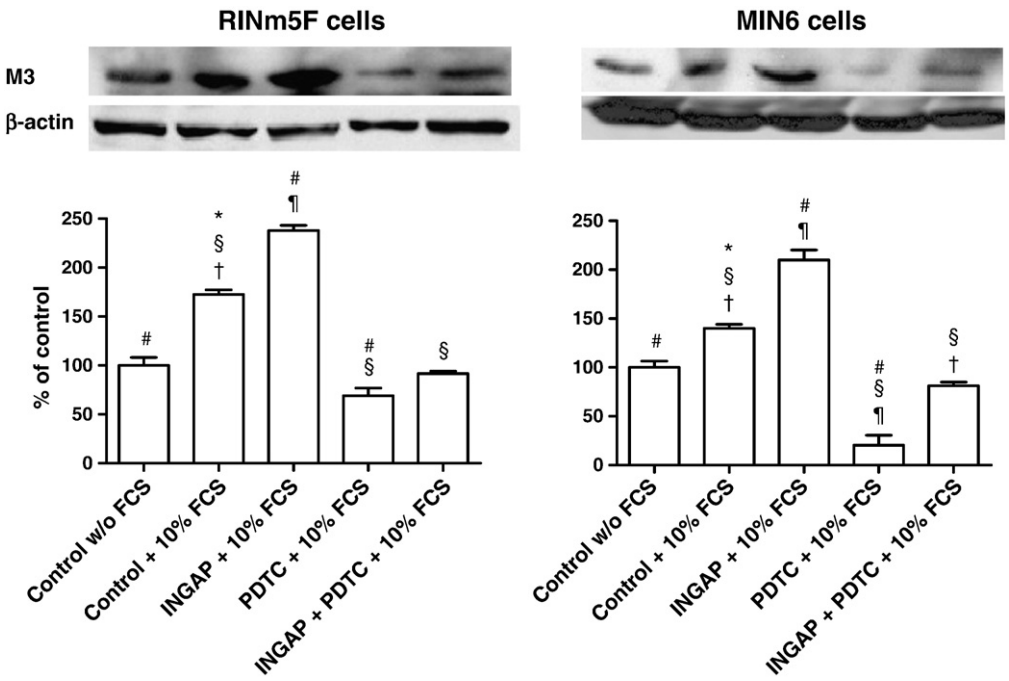

Fig. 6. Effect of foetal calf serum (FCS) on muscarinic M_3 receptor protein expression in insulin-producing cells with or without NF-κB blockade. RINm5F and MIN6 cells were seeded 24 h before incubation with 10% FCS or 10% FCS plus 10 μg/ml INGAP-PP (INGAP groups). After 24 h of FCS or FCS + INGAP-PP incubation in the presence or absence of the NF-κB inhibitor PDTC, the cells were lysed and used for Western blot analyses. Blots on the top are representative of 4 independent experiments, normalised to the housekeeping protein beta-actin. Symbols above bars represent $P<0.05$ vs. Control (*), Control + 10% FCS (#), INGAP-PP + 10% FCS (§), PDTC + 10% FCS (†), INGAP + PDTC + 10% FCS (¶); ANOVA followed by Bonferroni.

indicating their appropriate use as pancreatic beta cell models. Hence, due to the lack of ductal precursors in the insulin-producing cell lineages, our results indicate that INGAP-PP-dependent increase in cell mass might also occur through increased replication of pre-existing beta cells in addition to neogenesis. This effect is similar to the Reg1-associated replication of pre-existing beta cells in pancreatomized animals (Okamoto, 1999), and strengthens the concept of using INGAP-PP for restoration of beta cell mass, since pancreatic beta cells from the adult have a very limited capacity for proliferation (Bouwens and Rooman, 2005).

The activation of the transcription factor c-Myc may be involved in proliferation of beta cells following INGAP-PP treatment. Interestingly, activation of c-Myc may render pro-apoptotic or pro-proliferative effects in pancreatic beta cells depending on the period (Laybutt et al., 2002) and level of activation (Pascal et al., 2008). Relatively lower c-Myc activation increases beta cell proliferation, whereas higher activation causes death of beta cells (Pascal et al., 2008). Mild activation of c-Myc by INGAP-PP, as compared to the greater activation triggered by foetal calf serum (Table 1), is therefore an additional indication of pro-proliferative effects of the polypeptide. The interrelation of c-Myc and PCNA is another evidence for INGAP-PP-dependent induction of proliferation in beta cells, as PCNA is also a cell cycle-related gene playing an important role in replication, cell cycle progression and DNA repair (Tsurimoto, 1999). Supporting our interpretation, similar simultaneous induction of c-Myc activation and PCNA expression has been proposed to explain the stimulatory effect induced by other agents in proliferating epithelial cells (Srinivasan and Jewell, 2001). Taken together, these results indicate that c-Myc activation and PCNA expression may be ascribed as part of the mechanism by which INGAP-PP exerts its effect upon cell proliferation.

Our results also demonstrate, to our knowledge for the first time, a direct correlation between activation of NF-κB and expression of muscarinic M_3 receptors in insulin-producing cells. This is documented convincingly in the present work by both a pharmacological and a specific molecular approach by RNA interference. Regarding such a relationship, it has previously been shown that glucocorticoids both decrease NF-κB activity in the brain (Unlap and Jope, 1995) and decrease the expression of the cholinergic muscarinic M_3 receptor in smooth muscle (Emala et al., 1997). Notably, in the current study there was a relatively small activation of NF-κB by INGAP-PP, compared to the larger activation triggered by tumour necrosis factor (TNF)-alpha (Table 1). NF-κB may play a dual role in pancreatic beta cells, namely either pro-apoptotic (Souza et al., 2008) or pro-survival (Kim et al., 2007). Exposure of pancreatic beta cells to pro-inflammatory compounds *in vitro*, in particular interleukin-1beta, leads to a strong activation of the NF-κB response element, NF-κB expression and ultimately cell death by apoptosis (Souza et al., 2004, 2008). On the other hand, NOD mice expressing a constitutive form of IκB, which blocks NF-κB translocation to the nucleus, are more susceptible to develop diabetes than wild type controls (Kim et al., 2007). We speculate that these differences might be due to the degree of NF-κB activation and, hence, our results may provide a possible explanation for the opposing findings regarding the nature of the NF-κB effects on pancreatic beta cells, in the same fashion as that proposed for activation of c-Myc (Pascal et al., 2008). On the other hand, these effects might be context-dependent, since the suppression of NF-κB in the early stage of foetal pancreatic islets development (as it occurs in transgenic mice) impaired beta cell differentiation, but such an effect is not observed when blocking NF-κB signalling pathway in beta cells from the adult rat, which are able to keep normal pancreatic islet functions (Cnop et al., 2005). Additionally, beta cells and insulin-producing cells are somewhat protected against pro-inflammatory cytokines when NF-κB signalling is disrupted (Cnop et al., 2005) or attenuated (Souza et al., 2008). It reinforces the view of NF-κB activation as a pro-apoptosis rather than a pro-survival signal in pancreatic beta cells at the time of diabetes onset. Nevertheless, it does not rule out a physiological pro-survival role for the basal NF-κB activity.

As shown in the present study, modulation of the muscarinic M_3 receptor expression through foetal calf serum-dependent activation of multiple serum elements also depends on NF-κB activation.

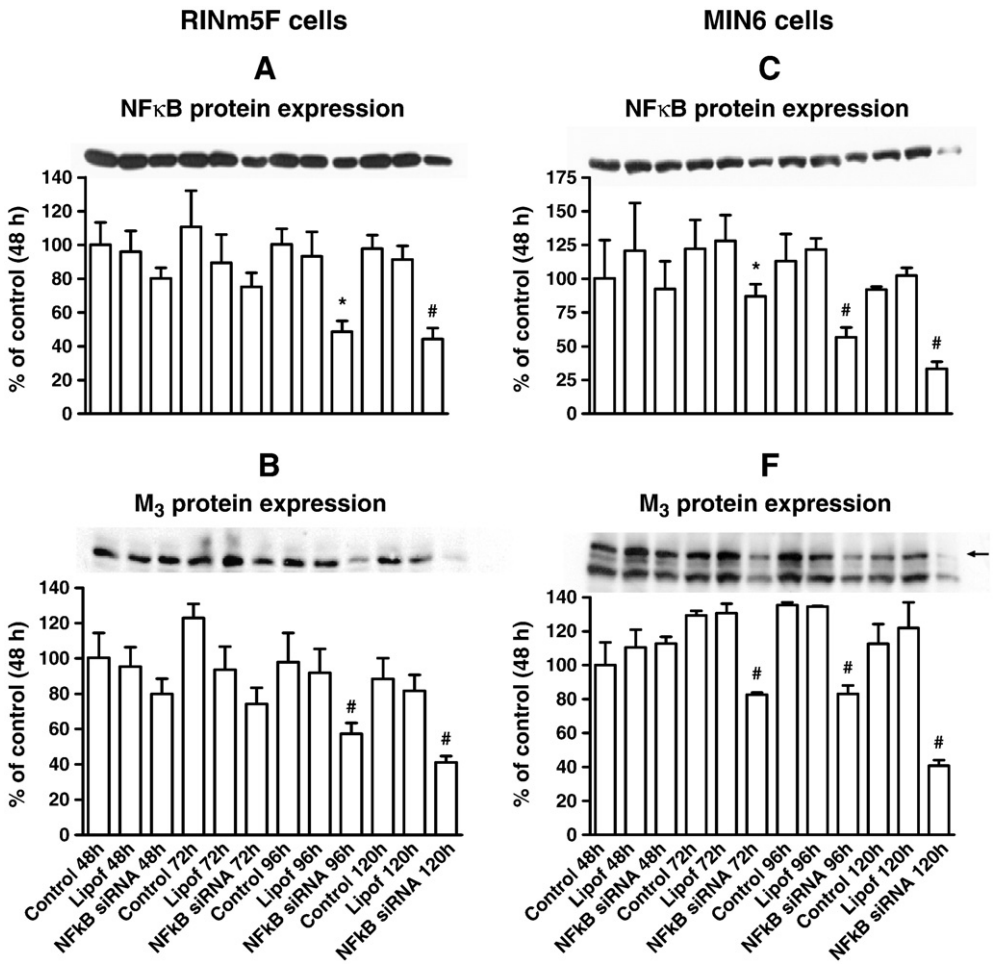

Fig. 7. Confirmation of the NF-κB-dependent modulation of the muscarinic M_3 receptor expression by siRNA. RINm5F and MIN6 cells were transfected with 200 nM NF-κB p65 subunit siRNA or mock transfected (Lipof group). After 48, 72, 96, and 120 h of transfection the cells were lysed and used for Western blot analyses of the NF-κB or the muscarinic M_3 cholinergic receptor protein expression. Blots on the top of the graphics are representative of 5 independent experiments. The arrow denotes the correct molecular weight. The expression of the housekeeping protein beta-actin did not change significantly among the groups (not shown). NF-κB, nuclear factor kappa B; M_3, muscarinic M_3 receptor subtype. * $P<0.05$ and # $P<0.01$ vs. respective time-point mock transfected control cells; ANOVA followed by Bonferroni.

Whether this modulation is direct or indirect is not known, since NF-κB can modulate other genes that might be able to (co-)modulate the muscarinic M_3 receptor expression. Nevertheless, our results show that NF-κB has an essential role in controlling muscarinic M_3 receptor expression in insulin-producing cells, despite the fact that the precise regulatory pathways must still be defined.

Although some controversy exist (Delhase et al., 2000), previous studies have shown that phosphatidylinositol 3-kinase/RAC serine/threonine-protein kinase (PI3K/AKT) signalling pathway might be involved in the activation of NF-κB (Beraud et al., 1999; Romashkova and Makarov, 1999). INGAP-PP activates the PI3K/AKT pathway in neonatal rat islets (Barbosa et al., 2008), and hence it is conceivable that PI3K/AKT activation might be involved in the upstream events leading to NF-κB activation in INGAP-PP-treated cells. However, INGAP-PP does not modulated the expressions of either p85 catalytic subunit of PI3K or AKT proteins, as shown in this study, and hence interaction between these pathways should be at the level of protein activity, as for instance modulation by phosphorylation of the proteins. Alternatively, mitogen-activated protein kinases (MAPK) might also be involved in these events since INGAP-PP activates MAPK in neonatal pancreatic islets (Barbosa et al., 2008) and a correlation between MAPK and NF-κB activation has already been proposed (Vanden Berghe et al., 1998). On the other hand, it is also possible that these pathways operate independently in the insulin-producing cell lines upon INGAP-PP stimulation. Further studies are necessary to determine the role played by PI3K/AKT and MAPK pathways on NF-κB activation in insulin-producing cells.

Recently, the crucial importance of the M_3 muscarinic acetylcholine receptor for regulated insulin secretion and glucose homeostasis has been elegantly demonstrated (Gautam et al., 2006). Additionally, muscarinic M_3 receptor stimulation activates MAP kinases (Jimenez et al., 2002; Ragheb et al., 2001), induces early growth genes (Albrecht et al., 2000), and may have potential survival and anti-apoptotic effects (Yang et al., 2005) in other cell types. In the present study we show that INGAP-PP not only triggers insulin release and increase intracellular calcium levels but also induces proliferation. These results allow us to hypothesize that insulin-producing cells might transduce, at least partly, the INGAP-PP signals into intracellular pro-proliferative events through the muscarinic M_3 receptors. A muscarinic M_3 receptor-dependent increase in PCNA expression has also been observed for other cell types exposed to muscarinic agonists (Arredondo et al., 2003). PCNA is also increased in INGAP-PP-treated insulin-producing cells, and this provides additional evidence for an interdependent role for the combined action of c-Myc, PCNA, and muscarinic M_3 receptor on the progression of the cell cycle.

It is also of note that stimulation of astroglial cells with the muscarinic agonist carbachol leads to proliferation, an effect mediated by the muscarinic M_3 receptor subtype and dependent on NF-κB activation (Guizzetti et al., 2003). These results not only provide a link between cholinergic pathway and NF-κB but also underline the

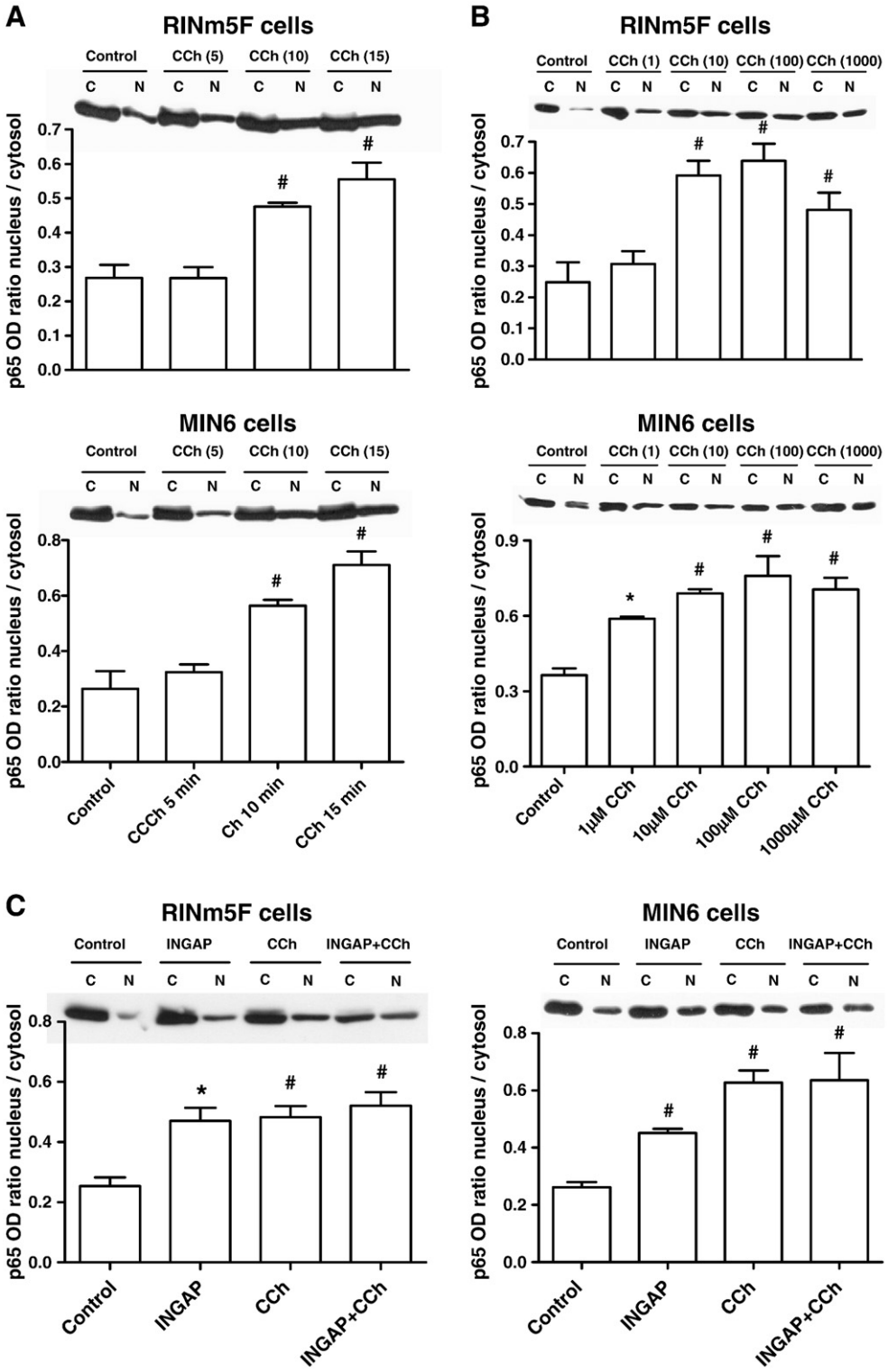

Fig. 8. Activation of the NF-κB signalling pathway by the cholinergic agonist carbachol in the presence and in the absence of INGAP-PP. RINm5F and MIN6 cells were seeded 24 h before incubation with carbachol (CCh). After 24 h of incubation in the presence (groups INGAP and INGAP + CCh in the graphics in C) or absence (all other groups in the graphics in A, B, and C) of 10 μg/ml INGAP-PP, cells were exposed to CCh, lysed and used for Western blot analyses of the translocation of the p65 NF-κB subunit from the cytosol to the nucleus. Blots on the top of the graphics are representative of 3-4 independent experiments. A) Time course of the effects of CCh on NF-κB activation in insulin-producing cells exposed to 100 μmol/l of CCh for (5), (10) and (15) min. B) Concentration–response curve of carbachol-stimulated insulin-producing cells exposed to (1), (10), (100), and (1000) μmol/l of CCh for 15 min. C) RINm5F and MIN6 cells were cultured in the absence (Control and CCh groups) or presence (INGAP and INGAP + CCh groups) of INGAP-PP for 24 h. Thereafter, cells were exposed to 100 μmol/l of CCh for 15 min (CCh and INGAP + CCh groups) or left in medium without CCh (Control and INGAP groups). C, cytosolic fraction; N, nuclear fraction. * $P<0.05$ and # $P<0.01$ vs. respective controls; ANOVA followed by Bonferroni.

importance of muscarinic M_3 receptor and NF-κB as main players in cellular proliferation events. However, there is no enhancement of carbachol-stimulated NF-κB activation after pre-incubation of insulin-producing cells with INGAP-PP. This is unexpected result, since INGAP-PP increases the expression of the muscarinic M_3 receptor and at the same time carbachol induces NF-κB activation in insulin-

producing cells. This provides additional evidence that INGAP-PP, like carbachol, might signal in insulin-producing cell lines through the muscarinic M_3 receptors and/or that INGAP-PP and carbachol share a common intracellular pathway that leads to NF-κB activation.

In summary, INGAP-PP is recognized by the insulin-producing MIN6 cells in the same way as occurs in rat islets, affecting simultaneously and significantly not only the secretion capacity, as previously observed, but also the calcium levels within cells. Significant changes in a network of intracellular signals that include c-Myc signalling pathway activation, increased PCNA expression and NF-κB-dependent cholinergic muscarinic M_3 receptor expression accompanies functional changes. INGAP-PP-dependent activation of the c-Myc signalling pathway and increased PCNA expression might be involved in proliferation of insulin-producing cells. Activation of NF-κB signalling pathway is critical for the modulation of cholinergic muscarinic M_3 receptor expression. Inversely, stimulation of the cholinergic pathway by the muscarinic agonist carbachol leads to NF-κB activation. A cooperative action of direct effects of INGAP-PP on beta cell replication and increased muscarinic M_3-dependent proliferation might occur in *in vivo* situations.

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