

Islet neogenesis-associated protein signaling in neonatal pancreatic rat islets: involvement of the cholinergic pathway

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Abstract

Islet neogenesis associated protein (INGAP) increases islet mass and insulin secretion in neonatal and adult rat islets. In the present study, we measured the short- and long-term effects of INGAP-PP (a pentadecapeptide having the 104–118 amino acid sequence of INGAP) upon islet protein expression and phosphorylation of components of the PI3K, MAPK and cholinergic pathways, and on insulin secretion. Short-term exposure of neonatal islets to INGAP-PP (90 s, 5, 15, and 30 min) significantly increased Akt1^{Ser473} and MAPK3/1^{Thr202/Tyr204} phosphorylation and INGAP-PP also acutely increased insulin secretion from islets perfused with 2 and 20 mM glucose. Islets cultured for 4 days in the presence of INGAP-PP showed an increased expression of *Akt1*, *Frap1*, and *Mapk1* mRNAs as well as of the muscarinic M3 receptor subtype, and phospholipase C (PLC)-β2 proteins. These islets also showed increased Akt1 and

MAPK3/1 protein phosphorylation. Brief exposure of INGAP-PP-treated islets to carbachol (Cch) significantly increased P70S6K^{Thr389} and MAPK3/1 phosphorylation and these islets released more insulin when challenged with Cch that was prevented by the M3 receptor antagonist 4-DAMP, in a concentration-dependent manner. In conclusion, these data indicate that short- and long-term exposure to INGAP-PP significantly affects the expression and the phosphorylation of proteins involved in islet PI3K and MAPK signaling pathways. The observations of INGAP-PP-stimulated up-regulation of cholinergic M3 receptors and PLC-β2 proteins, enhanced P70S6K and MAPK3/1 phosphorylation and Cch-induced insulin secretion suggest a participation of the cholinergic pathway in INGAP-PP-mediated effects.

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Introduction

Islet cell differentiation is controlled by several transcription factors including pancreatic duodenal homeobox-1 (PDX-1), neurogenin3, Nkx-1 and many others (Jensen *et al.* 2000). Hormonal, chemical, and neural signals are also essential for β-cell mass adaptation during its intrauterine development (Nielsen *et al.* 2001). During the fetal and neonatal periods more new β-cells are formed by neogenesis (Bouwens *et al.* 1994). Neogenesis is also observed in adult rodents after pancreatic injury such as streptozotocin injection (Fernandes *et al.* 1997), partial pancreatectomy (Lee *et al.* 1989), pancreatic duct ligation (Wang *et al.* 1995, Xu *et al.* 2008), cellophane wrapping of the pancreas head (Rosenberg & Vinik 1993), and in sucrose-induced insulin resistance (Del Zotto *et al.* 1999, 2000, Gagliardino *et al.* 2003).

Islet neogenesis associated protein (INGAP) was identified as the active part of a pancreatic protein complex isolated from

normal hamsters, whose pancreas heads had been wrapped in cellophane (Pittenger *et al.* 1992). The *Ingap* gene is expressed both in normal hamster islets and exocrine cells (Gagliardino *et al.* 2003) and it has been shown that a pentadecapeptide having the 104–118 amino acid sequence of INGAP (INGAP-PP) reproduces the effect of the intact molecule upon thymidine incorporation into ductal cells and a ductal cell line (Rafaeloff *et al.* 1997). We have reported that offspring from normal hamsters fed a sucrose rich diet during pregnancy have an increase in β-cell mass, an increased number of PDX-1 and INGAP-positive cells together with the appearance of a small population of cells that co-express PDX-1/INGAP. Since these cells have a high replication rate and do not stain with insulin-, glucagon-, somatostatin-, or PP-antibodies, we postulated that they would be early precursors of islet cells (Gagliardino *et al.* 2003). Otherwise, it was reported that the administration of the pentadecapeptide to either normal adult mice and dogs or streptozotocin-induced diabetic mice

induced an increase in β -cell mass and signs of neogenesis (Rosenberg *et al.* 2004, Pittenger *et al.* 2007). In addition, neonatal and adult normal rat islets cultured with INGAP-PP showed both increased β -cell size and insulin secretion in response to glucose (Borelli *et al.* 2005).

Since islet transplantation for therapy of type 1 diabetes is hampered by the shortage of islet donors, the search for alternative sources of β -cells has been intensified. In this sense, endogenous β -cell mass expansion and, consequently, the reversal of hyperglycemic states in animal models are being actively investigated (Rosenberg *et al.* 2004, Lipsett *et al.* 2006). Among the protocols used for this purpose INGAP-PP seems to be a suitable tool, but its mechanism of action is not yet completely elucidated.

It has been reported that the pancreas retains the ability to regenerate a functioning β -cell mass in the postnatal period (Rosenberg 1995). This process of progenitor cell transformation that leads to islet neogenesis could be mediated, at least in part, by local pancreatic growth factors. It has been shown that INGAP-PP treatment of duct-like structures, obtained from quiescent adult human islets, induced their differentiation to islet-like structures with the four endocrine cell types showing a normal glucose-induced insulin secretion (Jamal *et al.* 2005). In clinical trials (Ratner *et al.* 2005a,b) INGAP-PP therapy has been found to reduce daily average blood glucose levels at 90 days in patients with type 2 diabetes and induce a significant increase in C-peptide secretion in patients with type 1 diabetes. Recently, we have further observed that in addition to its effects to stimulate insulin secretion, INGAP-PP treatment augmented the expression of several genes involved in the control of islet growth and development (Barbosa *et al.* 2006).

Different pathways are involved in the mechanism by which glucose and peptides such as incretin hormones stimulate β -cell growth and differentiation (Hui *et al.* 2003, Kluz & Adamiec 2006). Incretin effects are apparently mediated through stimulation of cyclic AMP/PKA, p42 MAPK and PI3K pathways associated with the transcriptional activity of cyclin D1 (Friedrichsen *et al.* 2006), while glucose promotes human β -cell mitogenesis by stimulating p44/p42 MAPKs and FRAP1/P70S6K phosphorylation (Guillen *et al.* 2006). These and other reports suggest that MAPK and PI3K cascades may be the major pathways involved in the stimulatory effect of different factors upon islet mass regulation.

In the present study, we show that the enhancing effect of short- and long-term exposure of neonatal rat islets to INGAP-PP upon dynamic insulin secretion is accompanied by an increase of both PI3K and MAPK protein phosphorylation, and of the expression of components of the cholinergic pathway, such as the M3 receptor subtype and phospholipase C (PLC)- β 2. Furthermore, Cch significantly increased the short-term induced phosphorylation of proteins from the PI3K and MAPK cascades as well as insulin secretion, suggesting a possible participation of the cholinergic pathway in this process.

Material and Methods

Reagents

Reagents used for RT-PCR were from Invitrogen and Sigma. The equipment for SDS-PAGE and immunoblotting was from Bio-Rad. All the chemicals used in the experiments for immunoblotting and buffers were from Sigma. Anti-muscarinic receptor M3 (rabbit polyclonal sc-9108), anti-PLC- β 2 (rabbit polyclonal, sc-9018), anti-Akt1 (rabbit polyclonal, sc-8312), anti P70S6K (mouse monoclonal sc-8418) anti-phospho Akt1^{-Ser473} (rabbit polyclonal, sc-7985), anti-MAPK3 (rabbit polyclonal, sc-94), anti-MAPK 2 (rabbit polyclonal, sc-154), and anti-phospho MAPK3/1^{-Thr202/Tyr204} (mouse monoclonal sc-7383) antibodies were from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; and anti-phospho P70S6K^{-Thr389} (mouse polyclonal, #9206S) was from Cell Signaling Technology Inc., Boston, MA, USA.

Islets isolation and culture

Islets of neonatal Wistar rats were obtained by collagenase digestion of pancreas in Hanks' balanced salt solution and cultured in RPMI-1640 medium supplemented with 5% fetal bovine serum, 10 mM glucose, 100 IU penicillin/ml, 100 μ g streptomycin/ml at 37 °C in a 5% CO₂/air atmosphere. In short-term experiments groups of 300 islets were cultured for two days to minimize contamination by exocrine tissue. In other studies the islets were cultured for 4 days in the absence (controls) or the presence of 10 μ g/ml of INGAP-PP, as reported (Barbosa *et al.* 2006), renewing the medium every day. All animal experiments were approved by the Committee for Ethics in Animal Experimentation of the State University of Campinas (CEE/IB/UNICAMP).

Insulin secretion

To study dynamic secretion groups of 60 neonatal rat islets, previously cultured for two days in control conditions, were placed in individual chambers and perfused with Krebs ringer buffer (KRB), previously gassed with a mixture of CO₂/O₂ (5/95%), pH 7.4 and maintained at 37 °C in a temperature-controlled environment (Persaud *et al.* 1989). The islets were first pre-perfused for 1 h at 2 mM glucose, followed by a 70 min perfusion with the medium containing 2 or 20 mM glucose in the absence or presence of INGAP-PP (10 μ g/ml). Medium samples were collected at 2 min intervals. To study static insulin secretion groups of five neonatal rat islets, previously cultured for 4 days in the absence or presence of INGAP-PP, were then pre-incubated in 0.6 ml of KRB containing 1.5% (w/v) BSA and 5.6 mM glucose at 37 °C for 45 min. After this period, the islets were further incubated in 1 ml KRB supplemented with 8.3 mM glucose for 1 h in the absence or presence of 200 μ M Cch and 1–100 nM 4-DAMP (Boschero *et al.* 1995). Aliquots from the static incubation and perfusion experiments were stored frozen for insulin

measurement by RIA. Islets from all experiments were lysed in alcohol–acid solution for insulin extraction and subsequent insulin immunoassay (data not shown). All results were normalized against insulin content from each well or chamber.

RT-PCR

Semi-quantitative RT-PCR was performed using specific primers to analyze gene expression of Akt1 (NM 033230), FRAP1 (NM 019906), and MAPK1 (M 64300). Reverse transcription was carried out with 3 µg total RNA using Moloney murine leukemia virus reverse transcriptase (Super-script II) and random hexamers, according to the manufacturer’s instructions (Invitrogen). RT-PCR assays were done in quadruplicate using recombinant Taq DNA polymerase (Invitrogen) and 10 pM of each primer in a master mix of 50 µl. The primer sets used in the RT-PCR analyses are shown in Table 1. The number of cycles for each gene was defined after titration using 20 to 42 cycles and was within the logarithmic phase of amplification. PCR products were separated on 1.5% EtBr-agarose gels and the band intensities were determined by digital scanning (GelDoc 2000, Bio-Rad) followed by quantification using Scion Image analysis software (Scion Corp., Frederick, MD, USA). The results were expressed as a ratio of target to ribosomal protein (RP)S-29 signals. The RNAs used for RT-PCR analysis were obtained from three sets of experiments.

Tissue extracts and immunoblotting

Cultured islets were homogenized in 100 µl solubilizing buffer (10% Triton-X 100, 100 mM Tris, pH 7.4, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, and 2 mM phenylmethylsulphonyl fluoride) for 30 s using a Polytron PT 1200 C homogenizer (Brinkmann Instruments, Westbury, NY, USA) and boiled for 5 min. The extracts were then centrifuged at 12 600 *g* at 4 °C for 20 min to remove insoluble material. The protein concentration in the supernatants was assayed using the Bradford dye method and the BioRad reagent. The proteins obtained were treated with Laemmli sample buffer containing dithio-threitol and boiled for 5 min. Seventy micrograms of protein from each sample were then applied to a 10% polyacrylamide gel and separated by SDS-PAGE in a BioRad miniature slab gel apparatus. The protein fractions were thereafter electrotransferred from the gel to nitrocellulose at 120 V for 90 min in a BioRad miniature transfer apparatus. Before incubation with the primary antibody, the nitrocellulose filters were treated with

a blocking buffer (5% non-fat dried milk, 10 mM Trizma, 150 mM NaCl, and 0.02% Tween 20) for 2 h at 22 °C. The membranes were then incubated for 4 h at 22 °C with antibodies against muscarinic receptor M3 (1:500), PLC-β2 (1:1,000), p-MAPK3/1 (1:500), MAPK3/1 (1:1,000), P70S6K (1:500), p-P70S6K (1:1,000), Akt1 (1:500) or p-Akt1 (1:500) diluted in blocking buffer with 3% non-fat dried milk, and then washed for 30 min in blocking buffer without milk. The blots were subsequently incubated with peroxidase-conjugated second antibody for 1 h. Specific protein bands were revealed using commercial enhanced chemiluminescence reagents with exposure to photographic film. The images were obtained by digital scanning on GelDoc 2000, BioRad, followed by quantification using Scion Image analysis software (Scion Corp).

Statistical analysis

Results are shown as means ± S.E.M.; appropriate statistical comparisons between INGAP-PP-treated islets and the respective control groups were carried out using Student’s unpaired *t*-test. Differences were considered significant when *P* values were <0.05.

Results

Short-term effect of INGAP-PP on islet Akt1 and MAPK3/1 phosphorylation and on insulin secretion

Akt1 phosphorylation increased twofold when neonatal rat islets were exposed to 10 µg/ml INGAP-PP for either 90 s or 5 min (*P*<0.05), returning to control values after 15 min (Fig. 1A). INGAP-PP also significantly increased MAPK3/1-Thr202/Tyr204 phosphorylation that lasted for 30 min after the peptide exposure, being 4.1-, 4.0-, 2.2-, and 2.8-fold higher than control at 90 s, 5, 15, and 30 min respectively (*P*<0.05; Fig. 1B). To verify whether insulin secretion was also affected by exposure to INGAP-PP, islets were perfused with a buffer medium containing 2 mM or 20 mM glucose in the absence or presence of the peptide (10 µg/ml). At 2 mM glucose (Fig. 2A) addition of INGAP-PP provoked a rapid and sustained twofold increase in insulin secretion (min 11–30). This effect was rapidly reversed by the removal of INGAP-PP from the perfusion medium. To determine whether the islets maintained their viability after INGAP-PP exposure, the glucose concentration was increased to 20 mM (min 51–70) and this resulted in a significant increase in the insulin

Table 1 RT-PCR primer sets with predicted product sizes

	Genebank	Forward primer	Reverse primer	TM (°C)	Cycle number	Product (bp)
Gene						
Akt1	NM 033230	CCTCAAGTACTCATTCCAGAC	CTCATACACATCTTGCCACAC	58	26	619
Frap1	NM 019906	AAGGAGATGCAGAAGCCTCAGT	TGTCCCAAAGCCCATTAGGTCT	58	29	249
Mapk1	M 64300	GACCCAAGTGATGAGCCATTG	AAGCCACTACGACCAGACTGCC	56	24	253

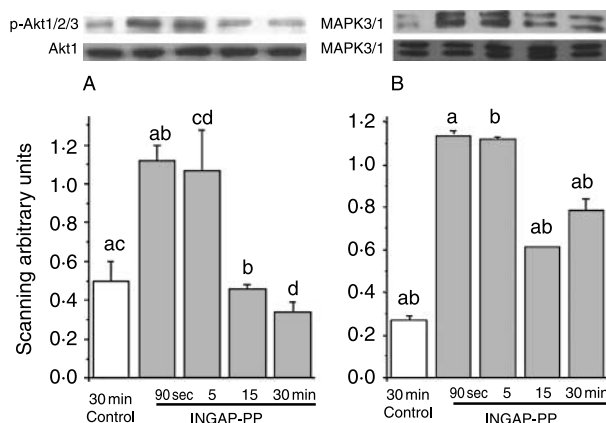


Figure 1 Acute effects of INGAP-PP on Akt1 and MAPK3/1 phosphorylation in neonatal rat islets. Two days cultured control islets were acutely exposed to 10 μ g/ml of INGAP-PP for 90 s, 5, 15, and 30 min and islet extracts were immunoblotted with anti-phospho-Akt1 (A) and anti-phospho-MAPK3/1 (B) antibodies. The bars represent the relative phosphorylation of the proteins (normalized against the total protein) measured by optical densitometry, and are the means $\pm$ S.E.M. of four experiments. The same letters indicate the pair comparisons that are significantly different ($P < 0.05$).

secretion. Figure 2B shows that increasing the glucose concentration from 2 to 20 mM (min 11–30) stimulated insulin secretion from perfused islets and that this secretory response was further potentiated by INGAP-PP (min 31–50) to levels approximately twofold higher than those obtained with 20 mM glucose alone. However, the potentiation of insulin secretion induced by INGAP-PP was transient and insulin levels returned to values comparable with those elicited by 20 mM glucose before INGAP-PP withdrawal.

Long-term effect of INGAP-PP on islet Akt1, Frap1 and Mapk1 gene expression, and Akt1 and Mapk3/1 phosphorylation

RNA obtained from islets that had been maintained in the presence of INGAP-PP for 4 days was submitted to semi-quantitative RT-PCR analysis and normalized against the RPS-29 housekeeping gene. INGAP-PP induced in cultured islets a significant increase in Akt1, Frap1 and Mapk1 gene expression attaining 1.9-, 1.6-, and 1.5-fold higher values than control islets ($P < 0.05$; Fig. 3A). Islets that had been exposed to INGAP-PP for 4 days also showed increased basal phosphorylation of Akt1 and MAPK3/1, with levels 1.8- and 1.5-fold respectively higher than control islets ($P < 0.05$; Fig. 3B).

Long-term effect of INGAP-PP on the expression of muscarinic M3 subtype receptor, PLC- β 2 proteins, and on Cch-induced acute phosphorylation of P70S6K and MAPK3/1

Due to the role of cholinergic pathway in the mechanism of insulin secretion, induced either by acetylcholine or glucose,

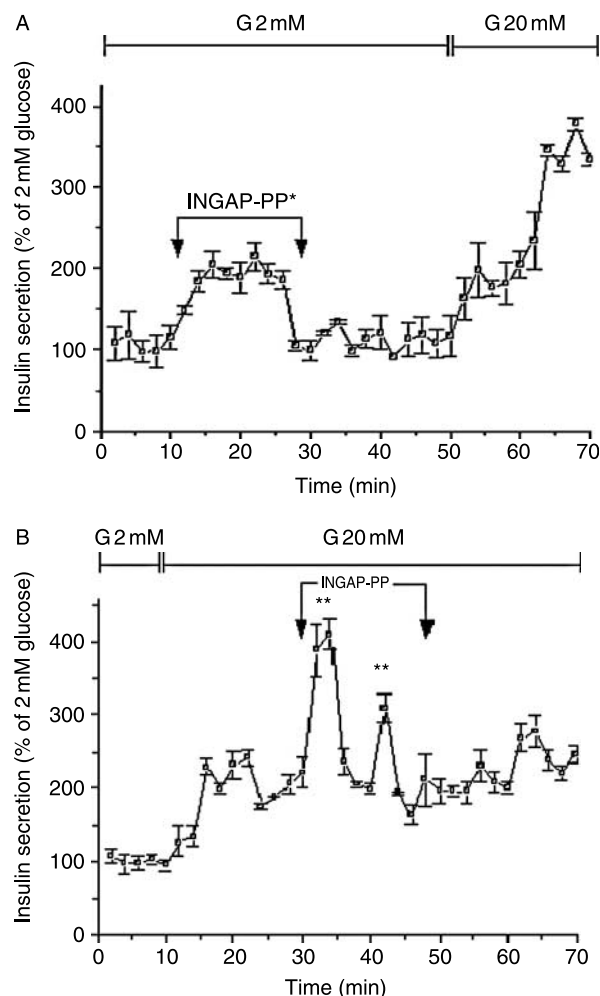


Figure 2 Kinetics of INGAP-PP-evoked stimulation of insulin secretion. Two-day cultured control islets were continuously perfused with KRB buffer at 37 °C. After a pre-incubation period of 1 h (2 mM glucose) the effluent was collected each 2 min for 70 min. INGAP-PP (10 μ g/ml) was applied as indicated either in the presence of 2 mM (A) or 20 mM glucose (B). Results are expressed as % of mean values calculated for min 0–10 (2 mM glucose), and normalized against total insulin content. Insulin secretion rates are means $\pm$ S.E.M. from three different experiments, * $P < 0.05$ compared with basal insulin secretion (2 mM glucose) and ** $P < 0.05$ compared with 20 mM glucose.

and considering that gene expression of the M3 receptor subtype was up-regulated by INGAP-PP after 4 days culture, in the next series of experiments we analyzed M3 receptor and PLC- β 2 expression as well as the effect of Cch on protein phosphorylation of the PI3K and MAPK pathways. Protein levels of the muscarinic M3 receptor subtype and PLC- β 2 were significantly higher in islets cultured for 4 days in the presence of INGAP-PP than in controls (Fig. 4A and B). Basal P70S6K phosphorylation was similar in INGAP-PP-treated and control islets and exposure of these islets to 200 μ M Cch for short periods of time (90 s, 5 and 15 min)

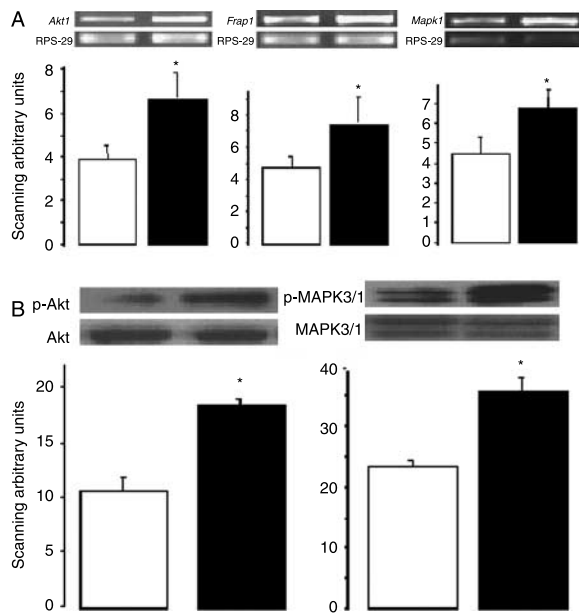


Figure 3 Effects of culture with 10 µg/ml of INGAP-PP for 4 days on mRNA levels of Akt1, Frap1, and Mapk1 (A), and on Akt1 and Mapk3/1 (B) phosphorylation. A: RT-PCRs annealing temperatures and cycle numbers used were as follows: 58 °C and 26 cycles for Akt1; 58 °C and 29 cycles for Frap1; 56 °C and 24 cycles for MAPK1. Results were normalized to the RPS-29 housekeeping gene. B: islet extracts were immunoblotted with anti-phospho-Akt1 and anti-phospho-MAPK3/1 antibodies (normalized against the total protein). Plotted columns are means $\pm$ S.E.M. of 12 experiments for mRNA and four experiments for protein phosphorylation. * $P < 0.05$ versus control.

significantly increased phosphorylation in both groups. However, Cch-stimulated P70 S6K phosphorylation was significantly higher in INGAP-PP-treated islets than in control islets (4.4-, 6.9- and 4.3-fold for INGAP-PP-treated versus 2.5-, 1.5- and 2.0-fold for control islets at 90 s, 5, and 15 min respectively; $P < 0.05$; Fig. 5A). MAPK3/1 phosphorylation was also similar in INGAP-PP-treated and control islets and exposure to Cch for 90 s induced a similar and significant increase in MAPK3/1 phosphorylation in treated and control islets (3.2-fold) compared with appropriate controls (no Cch). While the Cch-induced MAPK3/1 phosphorylation increment returned to basal values after 5 min in control islets, it remained significantly higher in INGAP-PP-cultured islets at 5 and 15 min (3.7 and 2.3 versus control respectively; $P < 0.05$; Fig. 5B).

Effects of Cch on insulin release from INGAP-PP-cultured and control islets

Insulin secretion at 8.3 mM glucose was higher in INGAP-PP-cultured islets compared with control islets ($P < 0.05$; Fig. 6). When 200 µM Cch was added to the incubation medium insulin secretion increased 5.7 times in the INGAP-PP-cultured and 1.5 times in the control islets. This

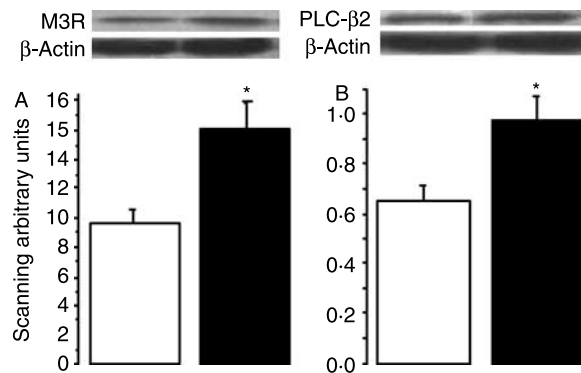
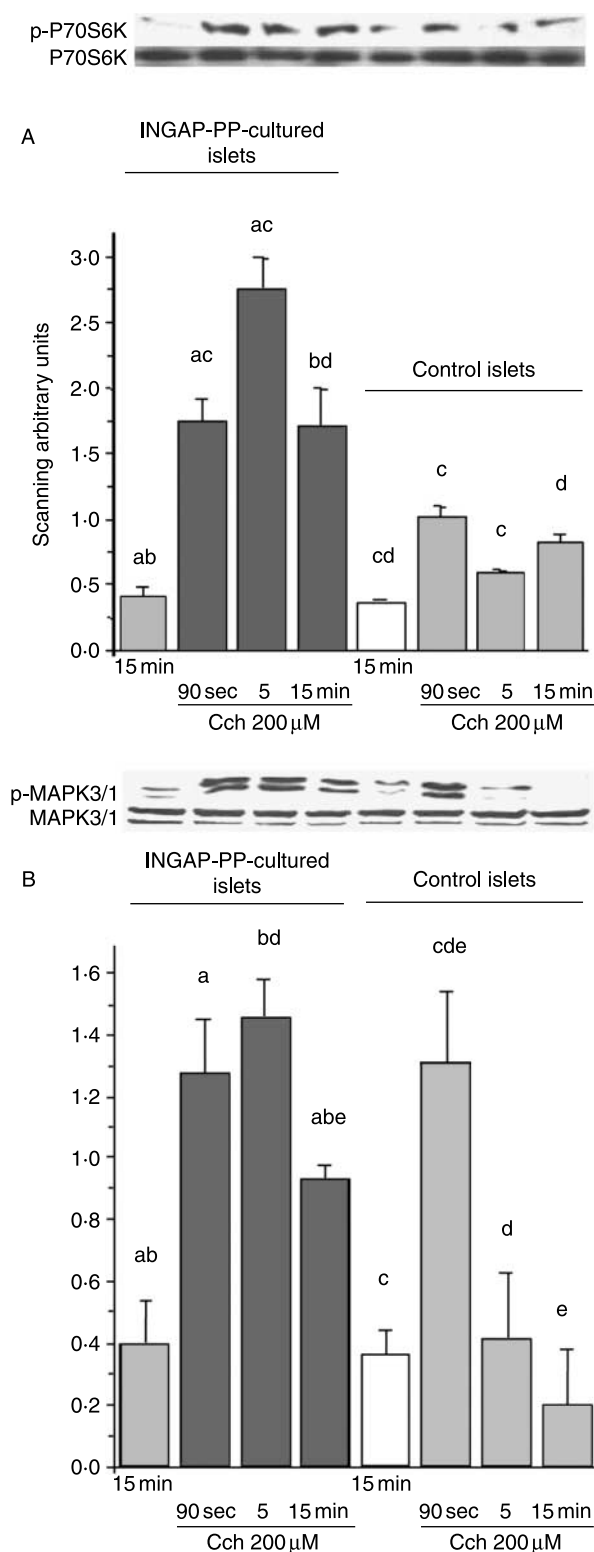


Figure 4 Effect of culture with 10 µg/ml of INGAP-PP for 4 days on muscarinic receptor M3 subtype (A) and PLC-β2 (B) expression. Islet extracts were immunoblotted with anti-muscarinic receptor and anti-PLC-β2 antibodies. The bars represent the relative protein expression as determined by optical densitometry, and are the means $\pm$ S.E.M. of three experiments, normalized to β-actin protein expression, * $P < 0.05$ versus controls. Open and closed bars represent islets from control and INGAP-PP-cultured rat islets, respectively.

stimulatory effect of Cch was dose-dependently inhibited by increasing concentrations of 4-diphenylacetoxy-N-methylpiperidine (DAMP), a selective antagonist of M3 receptors, with complete inhibition observed at 10 nM for INGAP-PP-cultured islets and at 1 nM for control islets.

Discussion

It is generally accepted INGAP-PP increases β-cell mass and insulin secretion in response to different stimuli (Borelli *et al.* 2005, Jamal *et al.* 2005, Barbosa *et al.* 2006, Lipsett *et al.* 2006). We have also recently demonstrated that INGAP-PP simultaneously enhanced insulin secretion and up-regulated several genes related to protein synthesis and islet maturation in neonatal rat islets (Barbosa *et al.* 2006). However, the mechanism by which INGAP-PP produced these effects is not yet fully elucidated. It has been reported that culturing isolated adult human islets for 10 days in a simple medium resulted in the disappearance of β-cells from the islet core and when these ghost islets were cultured with INGAP-PP for 4 days the islets were rebuilt and the newly formed β-cells released insulin in response to glucose at levels comparable with freshly isolated adult islets. Using a variety of blockers it was concluded that the effects of INGAP-PP were due to activation of the PI3K pathway (Jamal *et al.* 2005). It is still a matter of debate whether most of the new β-cells in adult animals are formed by the replication of resident β-cells (Dor *et al.* 2004) or if they are derived from ductal or other pancreatic precursor cells (Noguchi *et al.* 2006, Yatoh *et al.* 2007, Xu *et al.* 2008). Jamal *et al.* (2005) have suggested that the enhancing effect of INGAP-PP upon β-cell mass was obtained by a direct effect upon the remaining external islet cells that presumably conserves characteristics of ductal cells.



Neonatal islets exposed to INGAP-PP for short periods of time (90 s, 5, 15, and 30 min) showed a significant increase in Akt1/2/3- and MAPK3/1 phosphorylation (Fig. 1) supporting the early proposal that both PI3K and MAPK pathways may act as intracellular mediators of the beneficial effect of INGAP-PP upon β -cell mass and viability (Jamal *et al.* 2005). These short-term effects of INGAP-PP upon the phosphorylation of proteins that belong to the PI3K and MAPK cascades were also observed when neonatal islets were cultured with INGAP-PP for 4 days (Akt1 and MAPK3/1) together with an increase of *Akt1*, *Frap1*, and *Mapk1* gene expression.

Long-term exposure of neonatal islets to INGAP-PP also increased expression of members of the cholinergic pathway, namely the M3 receptor subtype and PLC- β 2. Furthermore, 4-DAMP (a selective M3 inhibitor) significantly inhibited Cch-stimulated insulin release. These results strongly suggest that the enhanced insulin secretion induced by cholinergic stimulus in INGAP-PP-cultured islets was due, at least in part, to an increase in the expression of the M3 muscarinic receptor subtype. It has been reported that in MIN6 β -cells, PLC activation by Ca^{2+} is an essential step for the stimulatory effect of Cch upon insulin secretion (Thore *et al.* 2005); such a mechanism includes an intracellular Ca^{2+} mobilization phase and a sustained activation phase dependent on Ca^{2+} entry through non voltage-dependent channels present in the plasma membrane. Since the expression of PLC β 2 was increased in INGAP-PP-cultured islets it is conceivable that higher levels of intracellular $[\text{Ca}^{2+}]$ in these islets (Silva *et al.* 2008) could account for the increased secretion of insulin via a higher PLC- β 2 activity. The increased expression of the two $\text{K}^{+}_{\text{ATP}}$ dependent channels component genes (Barbosa *et al.* 2006) and the increase in intracellular $[\text{Ca}^{2+}]$, would also contribute to the enhanced release of insulin observed in INGAP-PP cultured islets. Furthermore, Cch significantly augmented P70S6K and MAPK3/1 protein phosphorylation in these islets. Altogether, these results suggest participation of the cholinergic pathway in the mechanism by which INGAP-PP increases insulin secretion.

Activation of PI3K and MAPK pathway proteins occurs following both short- and long-term exposure to INGAP-PP. It has been shown that activation of those pathways is a common mechanism shared by several hormones, growth factors and substances that affect tissue growth and differentiation in many cell types. For example, MAPK3/1 phosphorylation is potentiated by glucose, incretins, IGF-1 and glyburide in INS-1 cells (Briaud *et al.* 2003). Prolactin also activates PI3K and

Figure 5 Acute effects of 200 μM Cch on P70S6K and MAPK3/1 phosphorylation in islets for 4 days in the absence or presence of 10 $\mu\text{g}/\text{ml}$ INGAP-PP. After culture, control and INGAP-PP islets were exposed to Cch for 90 s, 5, and 15 min. Islet extracts were immunoblotted with anti-phospho-P70S6K (A) and anti-phospho-MAPK3/1 (B) antibodies. The bars represent the relative phosphorylation of the proteins (normalized against the total protein) determined by optical densitometry, and are the means $\pm$ S.E.M. of four experiments. The same letters indicate the pairs comparisons that are significantly different ($P < 0.05$).

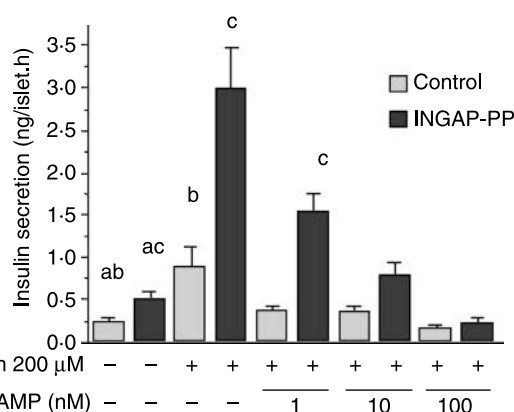


Figure 6 Effect of 4-DAMP on 200 μM Cch-induced insulin secretion in control and INGAP-PP-cultured (10 μg/ml) neonatal rat islets for 4 days. After culture, groups of 5 islets were incubated in KRB buffer containing 8.3 mM glucose in the absence or presence of 200 μM Cch and increasing concentrations of the M3 receptor antagonist 4-DAMP (1 nM, 10 nM and 100 nM). The bars represent cumulative 1 h insulin secretion and are means $\pm$ S.E.M. of 14–20 groups of islets from three different experiments. The same letters indicate the pairs comparisons that are significantly different ($P < 0.05$) between groups. Light and dark bars represent islets from control and INGAP-PP-cultured islets respectively.

MAPK cascades in cultured neonatal rat islets and in islets from pregnant rats, underlining the importance of these pathways in growth and development of islets in both periods of life (Amaral *et al.* 2003, 2004). In addition, MAPK6, a member of the MAPK cascade, is involved in the process of insulin granule exocytosis possibly through the complex MAPK6/MAP2, and via conventional PKCs activation (Anhe *et al.* 2006, Cunha *et al.* 2007). Moreover, it has been postulated that phosphorylation of synapsin I, a protein involved in glucose-induced insulin secretion, is dependent on MAPK3/1 activation (Longuet *et al.* 2005). Thus, our findings concerning INGAP-PP-induced phosphorylation of proteins of the MAPK and PI3K pathways are in good agreement with the literature mentioned above.

There is also evidence of participation of the cholinergic pathway in cell growth in other tissues. Thus, a) Cch increases $[Ca^{2+}]_i$, MAPK1 phosphorylation, protein synthesis, and cell proliferation in a human breast cancer cell line (Jimenez & Montiel 2005); b) M3 muscarinic cholinergic receptors activate p70S6K1 via PI3K signaling pathway in astrocytoma cells (Tang *et al.* 2003, Batty *et al.* 2004); c) a novel pathway of acetylcholine-stimulated activation of eNOS, involving the JAK2/IRS-1/PI3K/ Akt1 pathway, has recently been reported in aorta of obese rats that are simultaneously resistant to insulin and Cch (Zecchin *et al.* 2007); and d) islet M1 and M3 muscarinic receptor expression is increased during islet cell regeneration (Renuka *et al.* 2005). The increased expression of such receptors is associated with an increase in insulin secretion that, in turn, might modulate β -cell growth and differentiation via IRS2 activation (Velloso *et al.* 1995).

In conclusion, our data show that both short- and long-term exposure of neonatal normal rat islets to INGAP-PP activates islet PI3K and MAPK pathways and increases the expression of muscarinic M3 receptors and PLC- β 2 proteins. In INGAP-PP-cultured islets, a brief exposure to Cch enhanced the phosphorylation of several components of both signaling cascades leading to an increase in insulin secretion. This latter phenomenon suggests that the effect of INGAP-PP upon pancreatic islets is due, at least in part, to the activation of the cholinergic pathway. In light of the beneficial effects of INGAP-PP on β -cell function INGAP-PP supplementation of human islets, maintained in culture prior to transplantation therapy, could be a suitable strategy to optimize their viability and secretory function.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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