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Islet Neogenesis Associated Protein (INGAP) modulates gene expression in cultured neonatal rat islets

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Abstract

The Islet Neogenesis Associated Protein (INGAP) increases pancreatic β-cell mass and potentiates glucose-induced insulin secretion. We currently studied the effects of a pentadecapeptide having the 104–118 amino acid sequence of INGAP (INGAP-PP) on insulin secretion and on transcript profile expression in 4-day-cultured normal pancreatic neonatal rat islets. Islets cultured with INGAP-PP released significantly more insulin in response to 2.8 and 16.7 mM glucose than those cultured without the peptide. The macroarray analysis showed that 210 out of 2352 genes spotted in the nylon membranes were up-regulated while only 4 were down-regulated by INGAP-PP-treatment. The main categories of genes modified by INGAP-PP included several related with islet metabolism, insulin secretion mechanism, β-cell mass and islet neogenesis. RT-PCR confirmed the macroarray results for ten selected genes involved in growing, maturation, maintenance of pancreatic islet-cells, and exocytosis, i.e., Hepatocyte nuclear factor 3beta (HNF3β), Upstream stimulatory factor 1 (USF1), K^+-channel proteins (SUR1 and Kir6.2), PHAS-I protein, Insulin 1 gene, Glucagon gene, Mitogen-activated protein kinase 1 (MAP3K1), Amylin (IAPP), and SNAP-25. INGAP-PP also stimulated PDX-1 expression. The expression of three transcripts (HNF3β, SUR1, and SNAP-25) was confirmed by Western blotting for the corresponding proteins. In conclusion, our results show that INGAP-PP enhances specifically the secretion of insulin and the transcription of several islet genes, many of them directly or indirectly involved in the control of islet metabolism, β-cell mass and islet neogenesis. These results, together with other previously reported, strongly indicate an important role of INGAP-PP, and possibly of INGAP, in the regulation of islet function and development.

Keywords: cDNA array; Gene expression; Insulin secretion; INGAP-PP; Pancreatic islets culture

1. Introduction

At the embryonic period, pancreas development and islet-cell differentiation are controlled by the expression of several transcription factors, such as pancreatic duodenal homeobox-1 (PDX-1), neurogenin3 (ngn3), Nkx-1, as well as many others [1]. During the fetal and neonatal periods, adaptive changes of β-cell mass in response to different stimuli are mediated by a variety of hormonal, chemical and neural signals [2]. In both cases the majority of new β-cells are formed by neogenesis [3].

Neogenesis is also observed in adult animals after pancreatic injury provoked by streptozotocin injection [4], partial pancreatectomy [5], duct pancreatic ligation [6], cellophane wrapping of the pancreas head [7] and in insulin resistance state induced by sucrose feeding to normal hamsters [8,9].

INGAP was identified as the active part of a pancreatic protein complex isolated from normal hamsters, whose pancreatic heads were previously wrapped in cellophane [10]. The INGAP gene was expressed both in normal hamster islets and exocrine cells [11]. 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 [12]. We

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have reported that offspring from normal hamsters fed a sucrose diet during pregnancy have an increase in the mass of β-cells, 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 these cells would be early precursors of islet-cells [11]. On the other hand, it was reported an increase in β-cell mass and signs of neogenesis after intraperitoneal injection of this peptide to either normal or streptozotocin-induced diabetic mice [13]. More recently, we demonstrated that neonatal and adult normal rat islets cultured with INGAP-PP presented an increase in the size of their β-cells and released significantly more insulin in response to glucose [14]. Although these results indicate that INGAP may be involved in the regulation of islet function and neogenesis, its mechanism of action is not clear as yet.

In an attempt to answer this question, we have analyzed the changes that occurred in insulin secretion and gene expression of neonatal islets cultured with INGAP-PP for 4 days using two nylon arrays containing 2352 spotted genes. Our results showed that INGAP-PP-treatment increases simultaneously insulin secretion and the expression of several genes involved in the control of growth and development of islets as well as in insulin secretion.

2. Materials and methods

2.1. Animals and islets

Islets of neonatal Wistar rats were isolated by collagenase digestion and cultured in RPMI-1640 medium supplemented with 5% fetal bovine serum, 10 mM glucose, 100 IU of penicillin/ml, 100 μg of streptomycin/ml at 37 °C in a 5% CO_2/air atmosphere for 7 days. At day 3 of culture, we added 5 μg/ml of INGAP-PP to half of the plates, using the remaining ones as control (no INGAP-PP). The medium was renewed every other day. All animal experiments were approved by the Committee for Ethics in Animal Experimentation of the State University of Campinas, SP (CEEA/IB/UNICAMP).

2.2. Insulin secretion

Cultured neonatal isolated islets were rinsed in HEPES-bicarbonate buffer, pH 7.4, previously gassed with a mixture of CO_2/O_2 (5/95%), and preincubated in 1.0 ml of HEPES-bicarbonate containing 1.5% (w/v) BSA and 5.6 mM glucose at 37 °C for 45 min. After this period, groups of 5 islets were incubated in 1.0 ml HEPES-bicarbonate with the addition of 2.8 or 16.7 mM glucose for 1 h. At the end of the incubation period, aliquots of the medium were collected for insulin determination by radioimmunoassay.

2.3. Macroarray analysis

The arrays used were the Atlas Rat 1.2 and 1.2 II, Clontech Labs (Palo Alto, CA), containing 1176 spotted genes each one. Total RNA was obtained from approximately 1000 cultured islets using the Trizol method followed by DNase I (Invitrogen, USA) treatment. The RNA integrity was checked by electrophoresis in agarose denaturating gel and lately quantified by spectrophotometry (GeneQuant, Amersham Biosciences). Radiolabeled cDNA ([α-^{33}P] dATP) was obtained using 5–10 μg of total RNA and a reaction containing a gene-specific CDS primer mix (Clontech Labs, Palo Alto, CA) performed according to the manufacturer's recommendations. Membranes were submitted to an overnight hybridization in a solution containing the radiolabeled cDNA, followed by exposition to a phosphor-Imager screen (Molecular Dynamics, San José, CA), and finally scanned with a Storm 840 Scanner (Molecular Dynamics). The images obtained were analyzed (Quantity One software, Bio-Rad, USA), the spots were normalized by membrane housekeeping genes and the results expressed as the INGAP-PP-treated/control ratio. Only results higher than the cut-off of two fold or lower than a half, in two independent experiments, were considered as significant. The evaluation of fold change was

Table 1
RT-PCR primer sets with predicted product sizes

Gene	GeneBank	Forward primer	Reverse primer	TM (°C)	Product (bp)
HNF3β	L09647	CTG AGT GGA AAC ATT GGG G	GAT TTG TGG AACTCT GGC CA	60	570
USF1	AF026476	GAG GGC TCA ACA TAA CGA AGT	AAT CAC ACT TGC CCA ACT CC	63	462
SUR1	AB052294	TTC CAC ATC CTG GTC ACA CCG	AGA AGG AGC GAG GAC TTG CCA C	60	425
Kir6.2	AB043638	TTA GCG CCA CCA TTC ATA TG	TCC GGA GAG ATG CTA AAC TTG	54	401
MKK1	U48596	AGT GAG GAG ACG GCA TTC ACC C	CTC GTT CGC TTT GGT ATG CCC	58	384
Insulin	V01242	ATT GTT CCA ACA TGG CCC TGT	TTG CAG TAG TTC TCC AGT T	57	340
SNAP25	NM030991	GAA TTC AAT GGC CGA GGA CGC AGA	ACT TAA CCA CTT CCC AGC ATC TTT GT	60	621
IAPP	J04544	ATT GCT GCC ACT GCC CAC TG	CCT CTG CCA CAT TCC TCT TCC C	58	280
PHAS1	U05014	GGA AAT TCC TGA TGG AGT G	CTG GAG TAG CAG CTC AGT ATC	58	401
Glucagon	NM012707	CGC CAG ATC ATT CCC AGC TTC C	CGC CCA AGT TCC TCA GCT ATG G	58	345
RPS29	NM012876	AGG CAA GAT GGG TCA CCA GC	AGT CGA ATC ATC CAT TCA GGT CG	55	202
PDX-1	NM022852	AAC CGG AGG AGA ATA AGA GG	GTT GTC CCG CTA CTA CGT TT	56	225

SUR1 = Sulfonylurea receptor; Kir6.2 = Inwardly rectifying K^+ channel 6.2 family; RPS29 = Ribosomal protein S29; PDX-1 = Pancreatic duodenal homeobox-1. The remaining gene abbreviations are listed in Table 2.

done using a Microcal Origin program, version 4.10 (Northampton, USA).

2.4. RT-PCR analysis

Semi-quantitative RT-PCR was done using specific primers to confirm the differential expression of ten up-regulated genes detected in the macroarray analysis. Reverse transcription was done with 3 μg of total RNA using Moloney murine leukemia virus-reverse transcriptase (Superscript II) and random hexamers, according to manufacturer's instructions (Invitrogen, USA). RT-PCR assays were done in quadruplicate using recombinant *Taq* DNA polymerase (Invitrogen, USA) and 10 pmol of each primer in a master mix of 50 μl. The primer sets used in RT-PCR analysis 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). The results were expressed as a ratio of target to RPS-29 signals. The RNAs used for RT-PCR analysis were obtained from three to four sets of experiments.

2.5. Western blotting

Cultured islets were homogenized in 100 μl of solubilization 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 PMSF) for 30 s using a Polytron PT 1200 C homogenizer (Brinkmann Instruments, NY) 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 were treated with Laemmli sample buffer containing dithiothreitol and boiled for 5 min. Seventy μg of protein from each sample was applied to a 10% polyacrylamide gel and separated by SDS-PAGE in a Bio-Rad miniature slab gel apparatus. The electrotransfer of proteins from the gel to nitrocellulose was done at 120 V for 50 min or 1 h 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 incubated for 4 h at 22°C with antibodies against HNF3β (1:500), SUR1 (1:1000) or SNAP-25 (1:1000) (Santa Cruz, CA, USA) 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 band intensities were quantified by optical densitometry (Scion Image, Frederick, MD).

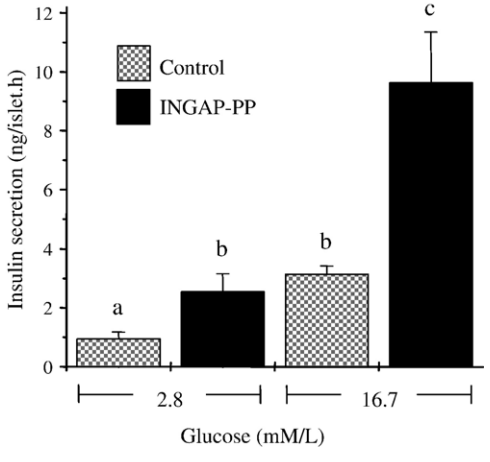

Fig. 1. Effect of INGAP-PP-treatment on insulin secretion in isolated neonatal rat islets. After isolation, the islets were cultured for 4 days in RPMI-1640 medium with or without INGAP-PP (10 μg/ml). After culture, groups of 5 islets were incubated in HEPES-bicarbonate buffer containing 2.8 or 16.7 mM glucose/l. The columns represent the cumulative 1 h insulin secretion and are the means ± SEM of 3 independent experiments ($n = 18$). Means without a common letter differ ($P < 0.05$).

Table 2
Pancreatic islet-related mRNA specificities modulated by INGAP-PP-treatment

mRNA	GenBank accession	Fold modulation
1. Liver specific transcription factor (LF-B1; HNF1)	J03170	2.42
2. Upstream stimulatory factor 1 (USF1)	AF026476	2.61
3. Hepatocyte nuclear factor 3beta (HNF3β; FOXA2)	L09647	2.57
4. Calcium-activated potassium channel rSK3	U69884	2.30
5. Calcium channel, voltage-dependent, L type, alpha 1E subunit	L15453	2.50
6. Potassium inwardly-rectifying channel, subfamily J, member 2	AF021137	2.48
7. Potassium channel, voltage gated, KV3.4; RAW3; KCNC4	X62841	2.58
8. Calcium channel beta 1 subunit	X61394	2.52
9. Calcium-transporting ATPase	M93017	2.02
10. Synaptotagmin 4	L38247	2.05
11. Synapsin 1	M27812	2.10
12. Secretogranin 2	M93669	2.32
13. Syntaxin binding protein Munc18-2	U20283	2.01
14. Synaptosomal-associated protein, SNAP-25	AB003991	2.17
15. L-type pyruvate kinase	M11709	2.20
16. Islet Amyloid Polypeptide (IAPP)	J04544	2.13
17. Thiol-specific antioxidant protein (1-Cys peroxiredoxin)	Y17295	2.29
18. Eukaryotic initiation factor 4E-binding protein (4EBP1); PHAS-1	U05014	3.28
19. Insulin 1 gene	V01242	3.51
20. Insulin-like growth factor II, somatomedin A	M13969	2.16
21. Insulin-like growth factor I	M15480	2.07
22. Glucagon gene	K02813	3.55
23. Mitogen-activated protein kinase kinase 1(MAP3K1; MKK1)	U48596	2.20
24. Pyruvate dehydrogenase kinase 1	L22294	2.00
25. Cholinergic receptor, muscarinic 3	M18088	2.10

Atlas Rat 1.2 and 1.2 II Array: fold modulation as compared to expression in cultured neonatal normal islets.

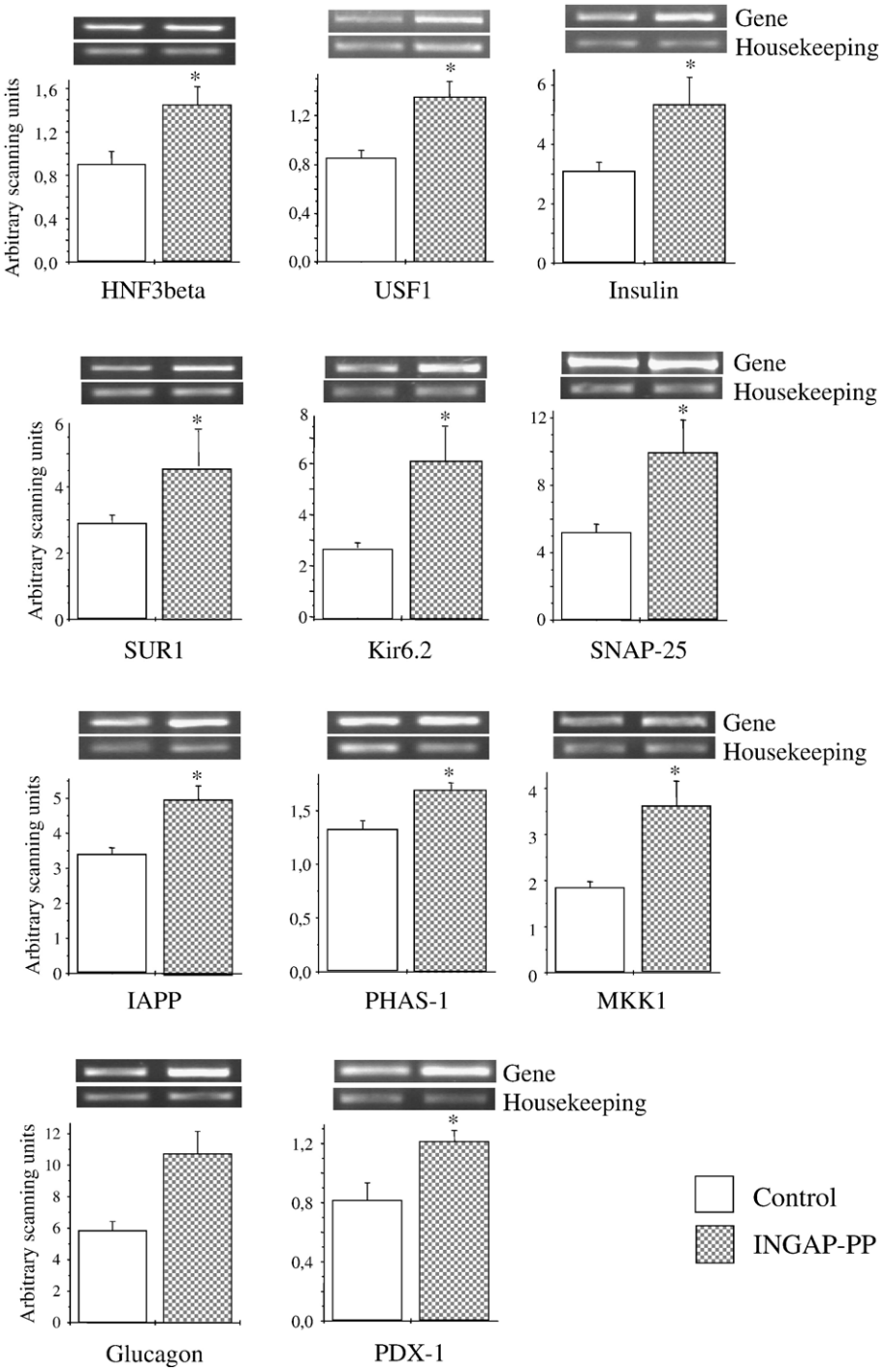

Fig. 2. Confirmation by RT-PCR of 11 selected genes that were significantly up-regulated in neonatal pancreatic islets after INGAP-PP-treatment (10 μg/ml). The bars represent the mean ± SEM of 3 experiments done with specific primer sets (Table 1) and normalized against the ribosomal protein S29 (RPS29). See Table 2 for definition of gene abbreviations. *$P<0.05$, with P values calculated from t test on the average difference between CTL and INGAP-PP.

2.6. Statistical analysis

Results are shown as means ± SEM, where appropriate statistical comparisons between INGAP-treated islets and the respective control groups were done using the Student's unpaired t test. P values <0.05 indicated a significant difference.

3. Results

3.1. Effect of INGAP-PP on insulin secretion

Normal neonatal islets cultured with INGAP-PP and then incubated with 2.8 and 16.7 mM glucose released significantly more insulin than those cultured in the control medium (Fig. 1).

3.2. Macroarray analysis

Of the 2352 genes spotted in the membranes, 210 were up-regulated and only 4 were down-regulated by INGAP-PP-treatment; complete data are presented in a Supplemental Table. According to the macroarray manufacturer, the main categories of these genes were: Basic Transcription Factors; Facilitated Diffusion Proteins; Voltage-Gated Ion Channels; Targeting and Exocytosis Proteins; Carbohydrate and Amino Acid Metabolism; Chaperones, Heat Shock Proteins and Proteins Modification Enzymes; Post-translational Modifications Proteins and Ribosomal Proteins; Translation Factors; RNA Processing, Turnover and Transport Proteins; DNA-binding and DNA Damage Repair Proteins; Hormones and Neurotransmitter Receptors; Growth Factors, Cytokines and Chemokines Receptors; Hormones; Intracellular Kinase Network Members; G-Proteins and Protein-Coupled Receptors; GTP/GDP Exchangers, GTPase Activity, Oncogenes and Tumor Suppressors Proteins, and others (Table 2).

A closer analysis of each one of the above categories indicated that, in neonatal islets, INGAP-PP modulated several genes related to general cellular processes and others specifically related to islet metabolism (pyruvate dehydrogenase kinase and phosphatase, MAP3K1), the mechanism of insulin secretion (K^+- and Ca^{2+}-channels, calcium/calmodulin-dependent protein kinase), exocytosis (synapsin, SNAP25), insulin biosynthesis (insulin) and islet neogenesis (HNF-3β, USF-1).

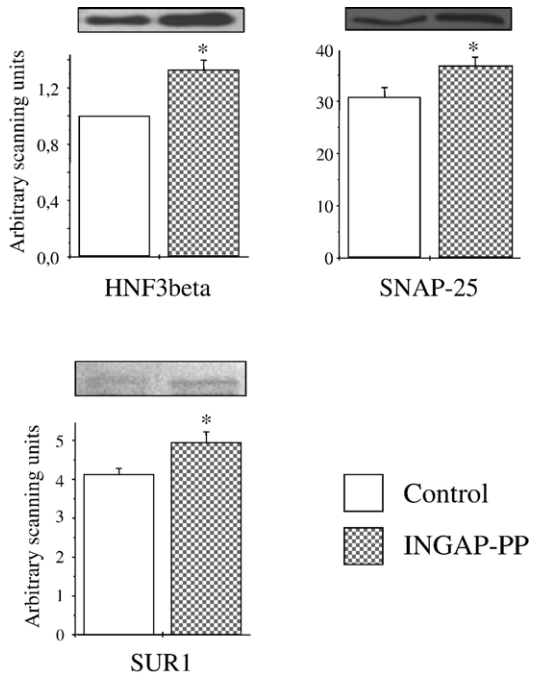

Fig. 3. Protein expression of three transcripts to validate the macroarray analysis. Neonatal pancreatic islets were cultured in the absence or presence of INGAP-PP (10 μg/ml). After 4 days, the proteins were extracted (see Methods) and equal amounts of protein from control and INGAP-PP-treated islets were resolved by SDS-PAGE on 10% gels and transferred to a nitrocellulose membrane. The proteins were identified with anti-HNF3b (a), anti-SNAP-25 (b), and anti-SUR1 antibodies (c). The values are the mean ± SEM of 3 experiments. *$P<0.05$ for CTL vs. INGAP-PP.

3.3. RT-PCR and Western blotting

Based on the reported effect of INGAP-PP upon insulin secretion [14] and islet neogenesis [13,15], we selected ten of the genes affected by this peptide directly and/or indirectly related to these two processes to perform RT-PCR (Fig. 2). Although not included in the macroarray membranes, we have also evaluated the transcription of PDX-1 due to its close relationship with islet neogenesis and its co-expression with INGAP-PP in islet-cells during dietary-induced neogenesis [11]. The data show that INGAP-PP increased the transcription of all the up-regulated genes.

When we tested the effect of INGAP-PP on the expression (Western blot) of three transcripts involved in the regulation of PDX-1 expression (HNF3β), β-cell membrane potential (SUR-1), and insulin exocytosis (SNAP-25), we observed a significant increase in the concentration of the corresponding proteins (Fig. 3).

4. Discussion

Our results showed that INGAP-PP increased significantly the expression of 210 out of 2352 genes, many of them involved in the regulation of function, growth and development of pancreatic β-cells.

The current data confirmed our own report that insulin secretion increased significantly in islets cultured with INGAP-PP [14]. It also provides some alternative mechanistic explanation for this effect. Accordingly, the ATP-sensitive K^+ channels (K_{ATP} channels) in pancreatic β-cells couple insulin secretion to the extracellular glucose concentration [16]: the increase in ATP/ADP ratio induced by glucose metabolism triggers a cascade of events (closes these channels → depolarizes the β-cell membrane → opens the voltage-dependent Ca^{2+} channels → promotes calcium influx → increases intracellular calcium concentration) that leads to an increase of insulin secretion [17]. The β-cell K_{ATP} channels comprise two components: a Kir6.2 subunit and a SUR1 subunit [18]. The up-regulation of SUR1 and Kir6.2 by INGAP-PP currently shown would play a role in its enhancing effect upon insulin secretion. Complementary, deletion of HNF3-β in pancreatic β-cells of mutant mice resulted in down-regulation of both K_{ATP} channel subunits [19,20], showing that HNF3β is required for maintaining the expression of SUR1 and Kir6.2. Thus, the INGAP-PP induced increase in HNF3β could also contribute to its effect upon K_{ATP} channels and thereby upon insulin secretion.

The increment of SNAP-25 (synaptosomal-associated protein-25), a t-SNARE protein, in INGAP-PP-treated islets may also contribute to the secretagogue effect of this peptide. In fact, the insulin granule extrusion needs the assembly of a complex between proteins, named soluble *N*-ethylmaleimide-sensitive factors (NSF)–attachment protein receptors (SNAREs). Among these proteins, those associated with the plasma membrane (t-SNAREs) and those anchored on the membrane of secretory vesicles (v-SNAREs) are involved in the membrane fusion step of the β-cell exocytosis process [21].

It is well established that for most eukaryotic genes, initiation of transcription is a critical and rate-limiting step in gene expression. In our study, INGAP-PP induced an up-regulation of

important transcription factors such as Maf2, CCAAT-binding transcription factor subunit B, Myocite-specific enhancer factor 2D (MEF2D), Hepatocyte nuclear factor 3beta (HNF3β, FOXA2), Upstream stimulatory factor 1 (USF-1) and others.

HNF3β is a member of the fork head/winged helix transcription factor family and is essential for endoderm cell lineages [22,23]. It increases PDX-1 transcription by binding to specific sequences in a nuclease hypersensitive site [24,25], being essential for the control of embryonic development of the endocrine pancreas [26,27]. HNF3β and Neuro-D act synergistically to induce PDX-1 expression in pancreatic islet-cells. Further, overexpression of HNF3β overcomes the blocking effect of glucocorticoids upon its own expression and activity in cultured islet-cells [28]. Thus, the stimulatory effect of INGAP-PP upon HNF3β transcription and expression (Figs. 2 and 3) may explain, at least partly, the reported neogenic effect of this peptide [7,10,13].

INGAP-PP also enhanced USF-1 expression, a factor that also regulates PDX-1 transcription by binding to its promoter [29]. Overexpression of a dominant-negative form of the USF2 protein (USF family proteins) reduces simultaneously PDX-1 transcription, its protein expression and insulin promoting activity and the insulin transcription level [29]. Conversely, the levels of USF-1 and PDX-1 mRNA increased significantly in islets of rats reared artificially on a high carbohydrate formula during the suckling period [30,31]. Similarly, newborns of normal hamsters fed a sucrose diet during pregnancy showed an increase in the mass of β-cells, PDX-1 and INGAP-positive cells, together with the appearance of PDX-1/INGAP-positive cells [11]. These results suggest that pancreas adaptation to a dietary-induced increased demand of insulin (insulin resistance state) includes up-regulation of this specific transcription factor gene in the islet-cells, thereby facilitating increased insulin gene transcription.

The plasticity of β-cell mass and function is important to maintain glucose homeostasis. In this regard, evidence in the literature has shown the importance of Insulin receptor substrate-2 (IRS-2) [32–34], as a mitogenic signal transduction pathway to pancreatic β-cell growth and survival. IRS-2 triggers at least two distinct signaling pathways in the β-cell, the Phosphatidylinositol 3-kinase (PI3K) pathway and Mitogen-activated protein kinase (MAPK) pathway [32,35]. The latter pathway involves the growth factor receptor-bound protein-2 (Grb2) and a cascade reaction that in its stream phosphorylates MAP/Erk kinase (MEK), leading to Erk-1/2 phosphorylation activation [36–38]. Thus, the increase observed in the MEKK1 transcription induced by INGAP-PP might indicate that this pathway is involved in the neogenic effect of this peptide upon islets, as previously described [13]. A recent report provides evidence of the participation of the PI3K pathway in islet plastic reactivity [15]. These authors showed that a short treatment of primitive duct-like structures, obtained from quiescent adult human islets, with INGAP-PP induced their reconversion to islet-like structures in a PI3-kinase-dependent manner. In their experimental conditions these neo-islets resembled freshly isolated human islets regarding the presence and topological distribution of the four endocrine cell types, islet gene expression, hormone production, insulin content and glucose-induced secretion of insulin.

In brief, our results show that INGAP-PP enhances specifically the secretion of insulin and, for the first time, of the transcription of several islet genes, many of them directly or indirectly involved in the control of islet metabolism, insulin synthesis/secretion, exocytosis and islet neogenesis. These results, together with other previously reported, strongly indicate the role of INGAP-PP, and possibly of INGAP, in the regulation of islet development and function.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.regpep.2006.04.015.

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