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# Role of Islet Glucokinase, Glucose Metabolism, and Insulin Pathway in the Enhancing Effect of Islet Neogenesis-Associated Protein on Glucose-Induced Insulin Secretion

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**Objective:** To demonstrate the role of islet glucokinase, glucose metabolism, and intracellular insulin mediators in the enhancing effect of islet neogenesis-associated protein pentadecapeptide (INGAP-PP) on glucose-induced insulin secretion.

**Methods:** Islets from normal rats were cultured for 4 days in the absence or presence of 10 μg/mL INGAP-PP, with/without Wortmannin or LY294002. Islets were incubated with different glucose concentrations to measure insulin secretion and content, hexokinase and glucokinase activity, glucose oxidation and utilization, glucokinase, insulin receptor, insulin receptor substrate (IRS)-1/2, and PI3K concentration and phosphorylation.

**Results:** The INGAP-PP significantly increased insulin release at high but not at low glucose concentration, glucokinase activity, glucose metabolism, glucokinase, insulin receptor, IRS-2 and PI3K protein concentration, insulin receptor and IRS-1/2 tyrosine phosphorylation, and the association of p85 with IRS-1. Wortmannin and LY294002 blocked INGAP-PP effect on insulin secretion and glucokinase protein levels in a dose-dependent manner.

**Conclusions:** The enhancing effect of INGAP-PP on glucose-induced insulin release could be partly ascribed to its effect on glucokinase activity and glucose metabolism and is mainly mediated by the PI3K/AKT pathway. These results, together with the low hypoglycemia risk associated with the use of INGAP-PP, offer a new alternative for diabetes prevention and treatment.

**Key Words:** INGAP-PP, PI3K pathway, insulin secretion, glucose metabolism

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Type 2 diabetes represents a serious health care problem due to its increasing prevalence worldwide, high care cost, and negative impact on the quality of life.[1] The disease is characterized by an early and progressive loss of pancreatic β-cell mass and function.[2,3] Because the available treatments can only provide partial benefits, the development of strategies targeted at optimizing their outcomes may offer new approaches to prevent diabetes development and progression.

Islet neogenesis-associated protein (INGAP), a compound produced in the islets, duct, and exocrine cells of the pancreas,[4] is a member of the Reg proteins family with high homology to RegIIIδ, but with different regulatory sites.[5] To date, the only known Reg receptor (EXTL3) that specifically binds Reg1 is a transmembrane glycosyl transferase homologous to the multiple exostoses-like gene family.[6] However, it is not known whether this receptor binds INGAP or other Reg protein or whether a different receptor is involved in the mechanism of INGAP action. A pentadecapeptide with the 104 to 118 amino acid sequence of INGAP (INGAP-PP) reproduces the stimulatory effect of the intact molecule on thymidine incorporation into both pancreatic duct cells and a duct cell line.[7] Its administration to normal hamsters induced an increase of insulin secretion in response to glucose[8] and of β-cell mass in normal hamsters,[8,9] mice[10] and streptozotocin-induced diabetic mice.[9] The latter effect resulted from the combination of enhanced β-cell replication and islet neogenesis rate, with a concomitant decrease in the rate of β-cell apoptosis.[8] These changes can be partially ascribed to the INGAP-PP–induced increase in the mRNA expression of Pdx-1 and neurogenin-3 [8,10] and the decreased expression of p38MAPK and JNK.[11] In this context, INGAP might be an attractive preventive and therapeutic alternative.

We have previously demonstrated that INGAP-PP directly added to the culture media of either neonatal or adult normal rat islets increased significantly islet β-cell size and insulin release in response to glucose in a dose-dependent manner, amino acids (leucine and arginine),[12] as well as $K^+$, and tolbutamide.[13] The glucose effect was accompanied by marked changes in the expression of several genes involved in islet metabolism, insulin synthesis, insulin secretion machinery activation and β-cell mass regulation.[14] Addition of INGAP-PP to the culture medium also enhanced the glucose-induced increase of intracellular calcium concentration ($[Ca^{2+}]i$) both in adult islets[13] and MIN6 cells.[15]

In agreement with the data obtained in experimental animals, a clinical trial performed in patients with type 2 or type 1 diabetes showed that treatment with INGAP-PP induced a significant reduction of $HbA_{1c}$ at 90 days in the former and a significant increase in C-peptide secretion in the latter.[16] Although this peptide is in the phase II of clinical trials, its limited stability and a relatively short plasma half-life (<1 hour) leads to its administration in high doses, which produces local injection site reactions.[16] Thus, further studies are necessary to improve its pharmacological profile and the characterization of its mechanism of action before implementing new clinical trials.

Despite the clear experimental evidence on the multiple favorable effects of INGAP and INGAP-PP on β-cell function and mass, there are still many gaps in our knowledge about the intracellular pathway mediating their effect on insulin secretion. The

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J.J.G. conceived and designed the study. J.J.G., B.M., H.B.-S., and A.C.B. evaluated the data obtained, carried out the statistical analyses and drafted the manuscript. C.L.R. and B.M. handled the experimental animals and performed all the experimental work. All authors read and approved the final article.

present study was therefore conducted to simultaneously study the effect of INGAP-PP on insulin secretion and on several enzymes and mediators involved in glucose metabolism and the PI3-K (phosphoinositide 3-kinase)/Akt pathway in cultured islets isolated from normal adult rats.

## MATERIALS AND METHODS

### Chemicals and Drugs

Collagenase was obtained from Roche (Mannheim, Germany). Bovine serum albumin (BSA, fraction V), protein A Sepharose, Wortmannin, mouse monoclonal anti–β-actin antibody, and other reagents were from Sigma Chemical Co. (St. Louis, MO). LY294002 was purchased from Calbiochem (Darmstadt, Germany). Anti-insulin receptor (IR) β subunit (rabbit polyclonal, sc-711) and antiphosphotyrosine PY99 (mouse monoclonal sc-7020) antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti–insulin-receptor substrates (IRS)-1/2 and anti-PI3K (αp85 subunit) antibodies were from Upstate Biotechnology (Lake Placid, NY); anti-glucokinase (GK) (sheep anti–GST-GK fusion protein) antibody was kindly provided by Dr Mark Magnusson (Vanderbilt University, Vanderbilt, TN).

The INGAP-PP (NH-Ile-Gly-Leu-His-Asp-Pro-Ser-His-Gly-Thr-Leu-Pro-Asn-Gly-Ser-COOH) was kindly provided by Dr. G. Alexander Fleming (Kinexum LLC, Harper's Ferry, West Virginia). The scrambled peptide (NH-Ser-Ser-Thr-Gly-Gly-Gly-Asp-Ile-Pro-Pro-His-Leu-Leu-His-Asn-COOH) was provided by Gen Script (Scotch Plains, NJ). Quality control of the peptides (amino acid analysis and mass spectrometry) indicated greater than 95% purity and a molecular weight of 1501.63.

### Animals and Islet Isolation

AQ1 Adult male Wistar rats (230–260 g bw) were maintained under controlled conditions of 23°C and a fixed 12-hour light, 12-hour dark cycle (6:00 A.M.–8:00 P.M.), with free access to a standard commercial diet and water. Experiments were performed according to the "Ethical principles and guidelines for experimental animals" (3rd ed., 2005) from the Swiss Academy of Medical Sciences (mail@samw.ch). At the time of euthanasia, the whole pancreas from each animal was removed to isolate islets by collagenase digestion.[17]

### Islet Culture

Freshly isolated islets were cultured in RPMI-1640 media (Gibco BRL, Carlsbad, CA) pH 7.4, containing 2 g/L $NaHCO_3$, 5% (v/v) fetal bovine serum, 1% penicillin/streptomycin, and 10 mM glucose at 37°C in a humid atmosphere (5% $CO_2$/95% $O_2$). Islets were cultured for 4 days in the absence (C, control) or presence of 10 μg/mL INGAP-PP or the scrambled peptide. This concentration was selected on the basis of previous in vitro studies with pancreatic islets and insulin-producing cells lines.[11,13,15,18] In parallel, half of the plates from each group were cultured for the same period with the addition of 2 PI3-K/Akt pathway inhibitors, Wortmannin (150 and 300 nM) or LY294002 (10 and 25 μM). The vehicle DMSO (1:20,000 dilution) was added to the media of groups without inhibitors (C and INGAP-PP), and the medium was renewed every other day. Thereafter, the islets were preincubated in Krebs-Ringer bicarbonate (KRB) buffer, pH 7.4, previously gassed with a mixture of $CO_2$/$O_2$ (5/95%), containing 1% (w/v) BSA and 3.3 mM glucose at 37°C for 45 minutes. Finally, islets were incubated with different glucose concentrations to study the processes detailed below.

### Insulin Secretion

Groups of 5 islets from all the study groups were incubated for 60 minutes in 0.6 mL KRB with different glucose concentration (3.3, 8.3, or 16.7 mM). At the end of the incubation period, aliquots of the medium were collected for insulin determination by radioimmunoassay. For this procedure, we used an antibody against rat insulin (Sigma Chemical Co.) and rat insulin standard (Novo Nordisk Pharma Argentina) and highly purified porcine insulin labeled with $^{125}I$.

### Islet Insulin Content

Groups of 20 cultured islets from each experimental group were homogenized in 200 μL of distilled water and stored at −70°C for subsequent measurement of insulin content by radioimmunoassay.

### Islet DNA Content

Islets from each experimental group were homogenized in TNE buffer and stored at −70°C for subsequent measurement of AQ2 DNA content by a fluorometric assay.

### Measurement of Islet Hexokinase and GK Activity

Groups of 20 islets were homogenized in 50 mM Hepes-NaOH buffer, pH 7.5, with 6 mM $MgCl_2$, 60 mM KCl, 10 mM $KPO_4H_2$, 1 mM EDTA, 1 mM L-cysteine and 0.02% BSA. They were incubated for 60 minutes at 37°C with the reaction mixture (Hepes-NaOH, 10 mM ATP, 20 μCi/mL [300 mCi/mmol] of D-[U-$^{14}$C]-glucose and 1 or 100 mM of unlabelled D-glucose). The D-[U-$^{14}$C]-glucose-6-phosphate was separated by ion exchange chromatography in an AG 1-X8 column.[19] The eluate was then mixed with scintillation fluid (Ultima Gold XR; Packard, Waltham, MA) and its radioactivity measured with a liquid scintillation spectrophotometer. The same mixture but without islet tissue was used as a blank. Under these conditions, the glucose-6-phosphate production obtained in the presence of 1 mM and 100 mM of unlabelled glucose corresponded to hexokinase (HK) and GK activity, respectively.

### Islet Glucose Oxidation and Utilization

Groups of 20 islets were incubated in a small tube containing 40 μL of KRB buffer supplemented with 10 mM Hepes, pH 7.4, D-[U-$^{14}$C]-glucose and D-[5-$^{3}$H]-glucose (10 μCi/mL [300 mCi/mmol]) in the presence of 3.3, 8.3, and 16.7 mM glucose. This tube, together with an empty one, was placed inside an airtight-sealed 20 mL glass vial containing 500 mL distilled water in the bottom. After 2 hours incubation at 37°C, the reaction was stopped by injecting 20 μL of 400 mM citric acid, 10 μM rotenone, and 3 mg KCN, pH 4.9, into the incubation media; at the AQ3 same time, 250 μL hyamine were added to the empty tube. After a second incubation for 60 minutes at 37°C, the $^{14}CO_2$ fixed to hyamine was measured in vials containing 5 mL of scintillation liquid (Ultima Gold XR, Packard). The islets were further incubated overnight at room temperature and glucose utilization was measured as $^{3}H_2O$ production captured by water in 5 mL of liquid scintillation.[20]

### Quantitative Real-Time PCR

Total RNA was isolated from cultured islets in 5 independent experiments using Trizol reagent (Invitrogen), following the manufacturer's instructions. The RNA integrity was checked by agarose-formaldehyde gel electrophoresis. Possible contamination with protein or phenol was controlled by measuring the 260:280 nm absorbance ratio, whereas DNA contamination was

avoided by treating the sample with DNase I (Invitrogen); 1 μg of total RNA was used for reverse transcription with SuperScript III Reverse Transcriptase (Invitrogen) and oligo-dT. Specific pairs of primers based on rat cDNA sequences were designed as follows: Glucokinase (NM_012565.1) forward primer 5′GTGTACAAGCTGCACCCGA3′; reverse primer 5′CAGCATGCAAGCCTTCTTG-3′. Real-time PCRs were run in triplicate using FastStart SYBR Green Master (Roche) in the iCycler 5 (BioRad). The cycling profile used was: 1 cycle of 2 minutes at 50°C (uracil-DNA glycosylase (UDG) activation), 1 cycle of 2 minutes at 95°C (DNA denaturation and UDG inactivation), 40 cycles of 15 seconds at 95°C and 30 seconds at 60°C, 1 cycle of 1 minute at 95°C followed by a melting curve from 55°C to 90°C. Quantified values were normalized against the housekeeping gene β-actin (NM_031144.3; forward primer, 5′AGAGGGAAATCGTGCGTGAC3′; reverse primer, 5′CGATAGTGATGACCTGACCGT3′), using the individual efficiency calculated with a standard curve for each gene.

## Islet Solubilization and Immunoprecipitation

For immunoprecipitation, cultured islets were preincubated for 45 minutes with 3.3 mM glucose for their stabilization and thereafter incubated for 60 minutes with 16.7 mM glucose. Islets were then homogenized in 100 mM Tris, pH 7.4, 10 mM EDTA, 1% Triton-X100, 2 mM phenyl-methylsulphonil-fluoride, 0.1 mg/mL aprotinin, 100 mM sodium pyrophosphate, 10 mM sodium vanadate, and 100 mM sodium fluoride. Insoluble material was removed by centrifugation for 20 minutes at 12,600 *g*, and the supernatant was then centrifuged for 90 minutes at 4°C at 100,000 *g* to separate the particulate (pellet) and cytosolic (supernatant) fractions. The pellet was then suspended in the specific assay buffer. Protein concentration from the different fractions (homogenate, particulate, and cytosolic) was quantified using the Bio-Rad protein assay. Twenty, 50, or 100 μg of protein were treated with Laemmli sample buffer, boiled for 5 minutes and stored at −20°C until electrophoresis.

For immunoprecipitation, 500 μg of homogenate protein were incubated at 4°C overnight with antiphosphotyrosine or anti–IRS-1 antibodies at a 5 μg/mL final concentration. After incubation, 20 μL protein A-Sepharose (50% v/v; Sigma) were added to the mixture. Additional samples were incubated in the absence of immunoprecipitating antibody to corroborate that the precipitated proteins were specifically recognized by the immunoprecipitating antibody and not by protein A-Sepharose. The preparation was further incubated with constant rocking for 2 hours at 4°C and then centrifuged at 1000 *g* for 1 minute. The supernatant was discarded, and the precipitate was washed with 50 mM Tris, 2 mM sodium vanadate, and 1% w/v Triton X-100, pH 7.4. The final pellet was resuspended in Laemmli buffer, boiled for 5 minutes, and stored at −20°C until electrophoresis.

## Western Blotting

Samples were fractioned under reducing conditions by SDS/PAGE (7.5% gel) and electroblotted to polyvinylidene difluoride transfer membranes (Amersham Hybond-P, GE Healthcare, UK). The amount of protein loaded onto the gel was quantified using the Bio-Rad protein assay. Nonspecific binding sites were blocked with a nonfat milk solution at 4°C overnight for GK, IR, PI3K, and β-actin quantification or with T-TBS buffer containing 3% w/v BSA at 4°C for 2 hours for IRS-1 and IRS-2 quantification. The membranes were then incubated with specific antibodies against GK (1:2,000 dilution), IR β (1:2,000 dilution), PI3K p85 (1:6,000 dilution), and β-actin (1:10,000 dilution) at 4°C for 90 minutes or overnight with antibodies against IRS-1 (1:1,000 dilution) or IRS-2 (1:500 dilution). After rinsing with T-TBS, the blots were incubated with streptavidin-peroxidase conjugate anti-sheep immunoglobulin (Ig)G or peroxidase-conjugated second antibody (goat anti-rabbit IgG-HRP sc-2004; Santa Cruz Biotechnology, CA) for 1 hour at room temperature. For β-actin, the horseradish-peroxidase–conjugated antimouse IgG antibody was used as secondary antibody.

To determine the tyrosine phosphorylation of IR, IRS-1 and IRS-2, the antiphosphotyrosine immunoprecipitates, were analyzed by Western blotting using the respective specific antibodies. To determine the amount of the p85 subunit of PI3K associated with IRS-1, membranes corresponding to anti–IRS-1 immunoprecipitates were incubated with anti-p85 antibody.

For the specific IR, PI3K, GK, and β-actin protein bands, diaminobenzidine (DAB; Sigma) was used for color development. The other proteins were revealed by using and enhanced chemiluminescence detection system (ECL Prime, Amersham, GE Healthcare, UK). Finally, the bands were quantified using the Gel-Pro Analyser 4.1 software.

## Statistical Data Analysis

The experimental data were statistically analyzed using analysis of variance and the Student *t* test for independent samples followed by Bonferroni test. Results are expressed as means ± SEM. Differences between groups were considered significant when $P$ values are less than 0.05. Results of GK protein levels from the cytosol and particulate fractions correspond to the percentage of each one from the total band intensity measured in the homogenate.

# RESULTS

## Insulin Secretion

Islets cultured during 4 days and thereafter incubated for 60 minutes with different glucose concentrations released insulin in a dose-dependent manner (Fig. 1A). Addition of INGAP-PP F1 (10 μg/mL) to the culture medium significantly enhanced this release at high (8.3 and 16.7 mM) but not at low (3.3 mM) glucose concentration. The effect was larger at 16.7 (94%) than at 8.3 mM glucose (49%); thus, such enhancing effect of INGAP-PP upon glucose-induced insulin release was clearly glucose concentration-dependent. No significant changes in glucose-induced insulin secretion were observed when the islets were cultured for 4 days with 10 μg/mL of scrambled peptide (Fig. 1A).

## DNA Content

Islet DNA content of cultured islets was not significantly modified by the presence in the medium of neither INGAP-PP (INGAP-PP vs C, 114.2 ± 1.1 and 120.1 ± 7.6 ng DNA/islet, respectively), nor scrambled peptide (Scrambled vs C, 106.6 ± 7.1 and 120.1 ± 7.6 ng DNA/islet, respectively). In all cases, values represent means ± SEM of 6 different experiments.

## Insulin Content

A comparable insulin content (expressed either per islet or per islet DNA) was measured in islets cultured with or without INGAP-PP (INGAP-PP vs C, 4.52 ± 0.39 vs 4.65 ± 0.35 ng insulin/islet and 0.040 ± 0.003 vs 0.039 ± 0.003 ng insulin/ng DNA). On the other hand, addition of scrambled peptide to the culture medium did not modify the insulin content expressed either per islet or per islet DNA (4.58 ± 0.41 ng insulin/islet and 0.042 ± 0.004 ng insulin/ng DNA). In all cases, values represent means ± SEM of 5 different experiments.

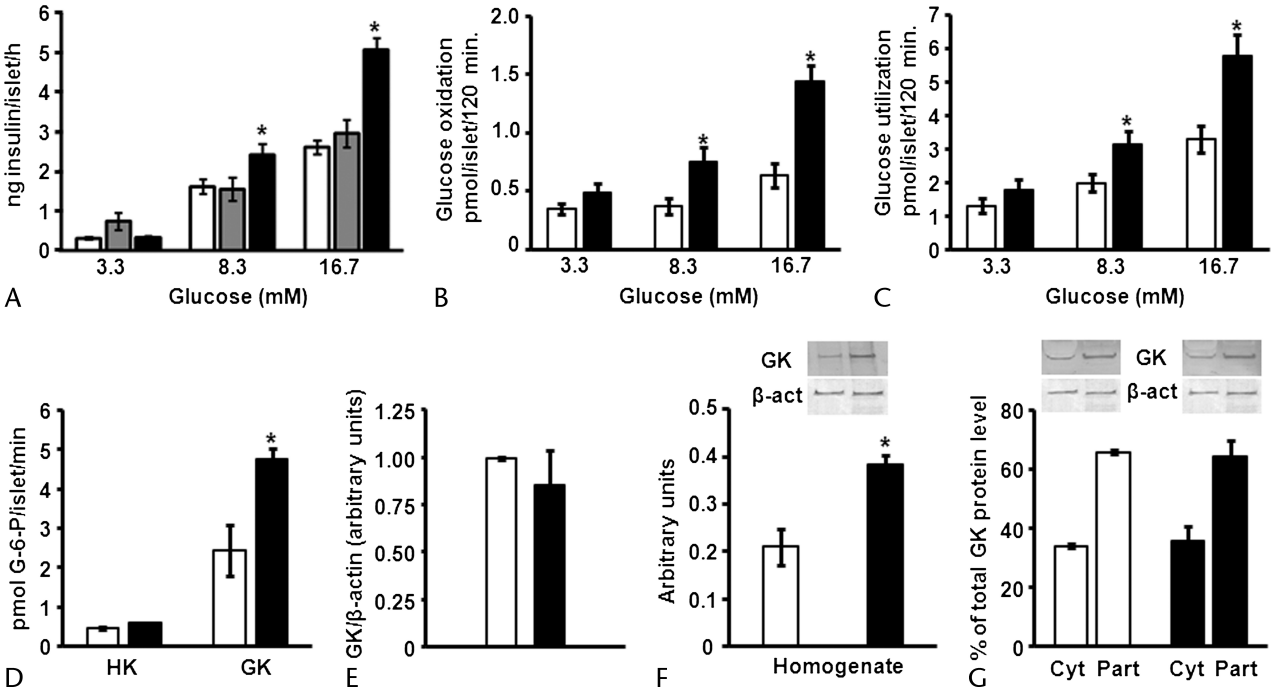


**FIGURE 1.** Effect of INGAP-PP on β-cell function. (A) Glucose-induced insulin secretion. (B and C) Glucose oxidation and utilization measured as $^{14}CO_2$ produced from D-[U-$^{14}$C]-glucose and $^{3}H_2O$ from D-[5-$^{3}$H]-glucose, respectively. (D) Glucose phosphorylation measured in islet homogenates at 100 (GK activity) and 1 mM glucose (HK activity). (E) GK mRNA levels normalized with the housekeeping gene (β-actin). (F and G) GK protein levels (representative blot of five independent experiments) measured in islet homogenate as arbitrary units and in cytosolic and particulate fractions as the percentage that each one represents of the total band intensity measured in the homogenate. Values were normalized using β-actin as housekeeping protein. In all cases, bars represent means ± SEM of 5 different experiments performed with islets cultured for 4 days in the absence (C, white bars) or presence of 10 μg/mL INGAP-PP (black bars) or 10 μg/mL scrambled peptide (gray bars). *$P < 0.05$ compared to C bars.

Based on the lack of effect of the scrambled peptide on the glucose-induced insulin secretion and DNA and insulin content, the rest of the studies were performed omitting the scrambled peptide-treated group.

## Glucose Oxidation and Utilization

The production of both $^{14}CO_2$ from D-[U-$^{14}$C]-glucose and $^{3}H_2O$ from D-[5-$^{3}$H]-glucose in cultured islets increased in a dose-dependent manner when the glucose concentration in the incubation medium rose from 3.3 to 16.7 mM (Fig. 1B and C). At high glucose concentrations (8.3 and 16.7 mM), INGAP-PP increased significantly the production of $^{14}CO_2$ (100% and 126%, respectively; $P < 0.05$; Fig. 1B) and $^{3}H_2O$ (59% and 76%, respectively; $P < 0.05$; Fig. 1C). No differences in $^{14}CO_2$ or $^{3}H_2O$ production were recorded at a lower glucose concentration.

## HK and GK Activity

In our assay system, the glucose phosphorylation rate measured in islet homogenates at 1 and 100 mM glucose represents HK and GK activity, respectively. A significantly higher GK activity (95%) was measured in homogenates of islet cultured in the presence of INGAP-PP ($P < 0.05$), whereas the peptide did not significantly affect HK activity (Fig. 1D). Consequently, the GK-to-HK activity ratio was significantly higher in islets exposed to INGAP-PP (INGAP-PP vs C, 8.45 ± 0.44 vs 5.19 ± 0.95; $P < 0.05$).

## GK mRNA

There were no significant differences in GK mRNA levels recorded in islets cultured with or without INGAP-PP (INGAP-PP vs C, 100% ± 0.7% vs 85% ± 17%; Fig. 1E).

## GK Protein Concentration

Western blot performed with a GK-specific antibody in homogenates from 4-day cultured islets with or without INGAP-PP showed a band with a molecular weight of about 50 kDa.

The GK protein levels were significantly higher (82%) in homogenates from islets cultured in the presence of INGAP-PP ($P < 0.05$, Fig. 1F). However, no differences were recorded in GK compartmentalization rate among the experimental groups: GK band intensities measured in the cytosolic fraction represented 35.65% ± 5.33% and 34.18% ± 0.64% of the total intensity measured in homogenates of INGAP-PP and C islets, respectively (Fig. 1G).

## Protein Concentration of Intracellular PI3K/AKT Pathway Mediators

Western blot performed with IR-, IRS-1/2–, and PI3K-specific antibodies in homogenates from cultured islets showed a band with a molecular weight of about 75, 185, and 85 kDa, respectively.

The INGAP-PP induced a significant increase above control values in the protein concentration of IR (63%), IRS-2 (70%), and PI3K (155%) (Fig. 2A, C and D; $P < 0.05$). However, no significant changes were depicted in IRS-1 protein content (Fig. 2B). F2

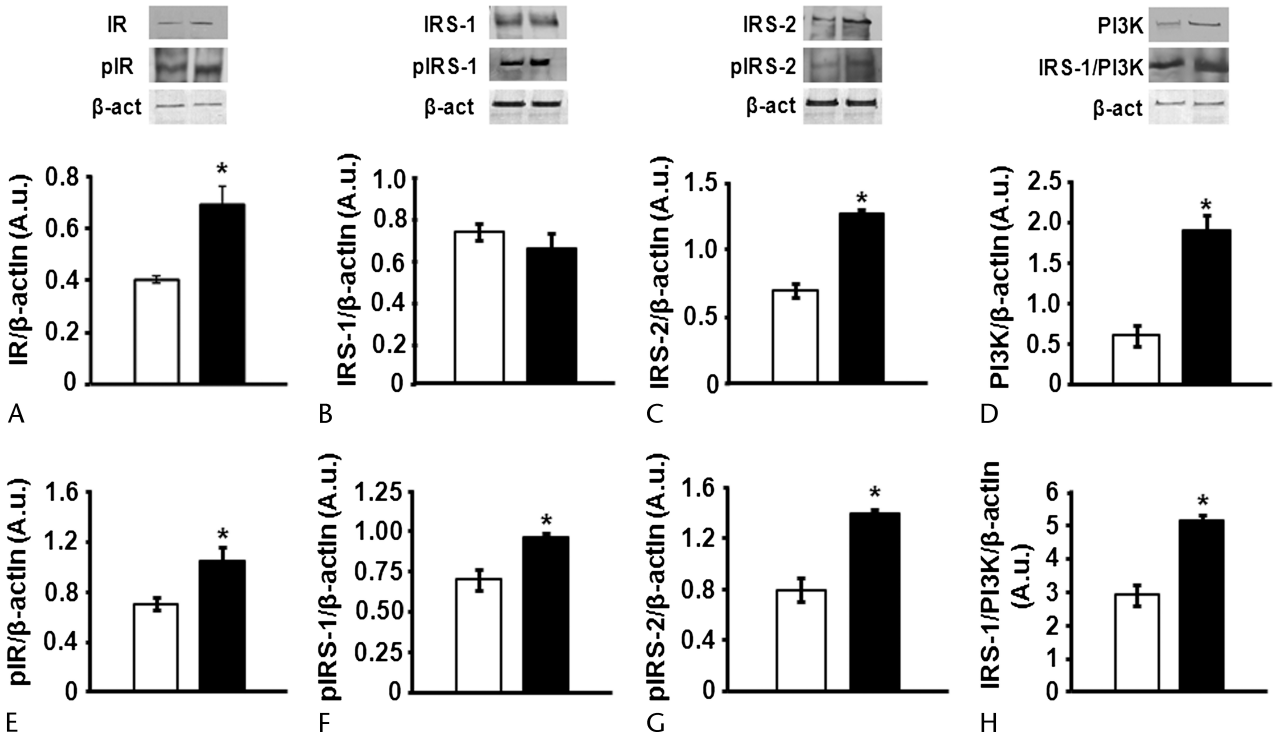


**FIGURE 2.** Effect of INGAP-PP on protein and tyrosine-phosphorylation levels of the PI3K/AKT pathway intracellular mediators. (A, B, C and D) Protein level of IR, IRS-1, IRS-2, and PI3K. (E, F, and G) Tyrosine-phosphorylation of IR, IRS-1 and IRS-2. (H) Association of the regulatory subunit of PI3K (p85) with tyrosine-phosphorylated-IRS-1 measured in islets cultured for 4 days in the absence (C, white bars) or presence of 10 μg/mL INGAP-PP (black bars). Band intensities (arbitrary units, A.u.) are represented below the bands from a representative blot of six independent experiments. Values were normalized using β-actin as housekeeping protein. *$P < 0.05$ compared to C bars.

Concomitantly, tyrosine phosphorylation of IR, IRS-1, and IRS-2 was significantly higher in INGAP-PP-exposed islets compared to C ones (71%, 38%, and 75% of control, respectively; Fig. 2E, F, and G; $P < 0.05$). The INGAP-PP also increased the tyrosine phosphorylation-to-total protein ratio of IR (INGAP-PP vs C, 3.2 ± 0.25 vs 2.38 ± 0.17), IRS-1 (INGAP-PP vs C, 1.66 ± 0.18 vs 0.98 ± 0.07) and IRS-2 (INGAP-PP vs C, 1.17 ± 0.05 vs 1.06 ± 0.03).

The association of the regulatory subunit of PI3K (p85) with tyrosine-phosphorylated IRS-1 was also significantly higher (76.5%; $P < 0.05$) in islets cultured with INGAP-PP (Fig. 2H).

## Effect of PI3K Inhibitors on Insulin Secretion and GK Protein Levels

Addition of 2 PI3-K/Akt pathway inhibitors to the culture medium, either Wortmannin or LY294002, significantly decreased (in a dose-dependent manner) the amount of insulin released by the islets in response to 16.7 mM glucose in either C or INGAPPP-exposed islets (Fig. 3A and C). Both inhibitors blocked INGAP-PP effect upon insulin secretion at all concentrations tested, resulting in the same amount of insulin in C and INGAP-PP.

Under the abovementioned conditions, Wortmannin and LY294002 also decreased GK protein concentration in both C and INGAP-PP–exposed islets (Fig. 3B and D). Additionally, the inhibitors lowered the INGAP-PP–induced increase of GK protein concentration to levels similar to those measured in C islets.

## DISCUSSION

The current results confirm the in vitro enhancing effect of INGAP-PP on glucose-induced insulin secretion.[12–14] This effect would be specific, because it was not reproduced using a scrambled pentadecapeptide, the present work.[9,12]

It has been widely demonstrated that glucose metabolism in the β cell is one of the main mechanisms involved in the regulation of insulin secretion.[21] β-cell exposure to high glucose puts forward a complex mechanism that begins with the acceleration of glucose metabolism and ends with an increase in the β-cell cytosolic $Ca^{2+}$ concentration that triggers insulin release. In this process, GK is the rate-limiting step of glucose metabolism and plays a pivotal role in linking glucose metabolism with insulin secretion.[22,23]

On account of this important role of glucose on β-cell function, the question was whether INGAP-PP could also affect both GK activity/content and glucose metabolism in these cells. Despite the initial failure to demonstrate such effect,[13] we repeated the study, making some adjustments in the methodology to improve its sensitivity and reproducibility. By doing so, we have currently demonstrated that the increased insulin secretion induced by INGAP-PP in cultured islets was closely associated to a significant increase of GK activity—mainly due to an increase in its protein concentration rather than to its intracellular compartmentalization—and a concomitant increase of glucose metabolism.

Regarding the molecular mechanism of this effect, we have previously shown that INGAP-PP increased the protein expression of the 2 ATP-sensitive potassium channel ($K_{ATP}$-channels) subunits, with the consequent increment of the cytosolic $Ca^{2+}$ concentration in isolated islets[13] and an insulin-producing cell line (MIN6).[15] Complementarily, INGAP-PP increased the protein expression of M3 muscarinic receptors and phospholipase C-β2 in neonatal islets. In the treated islets, the cholinergic agonist

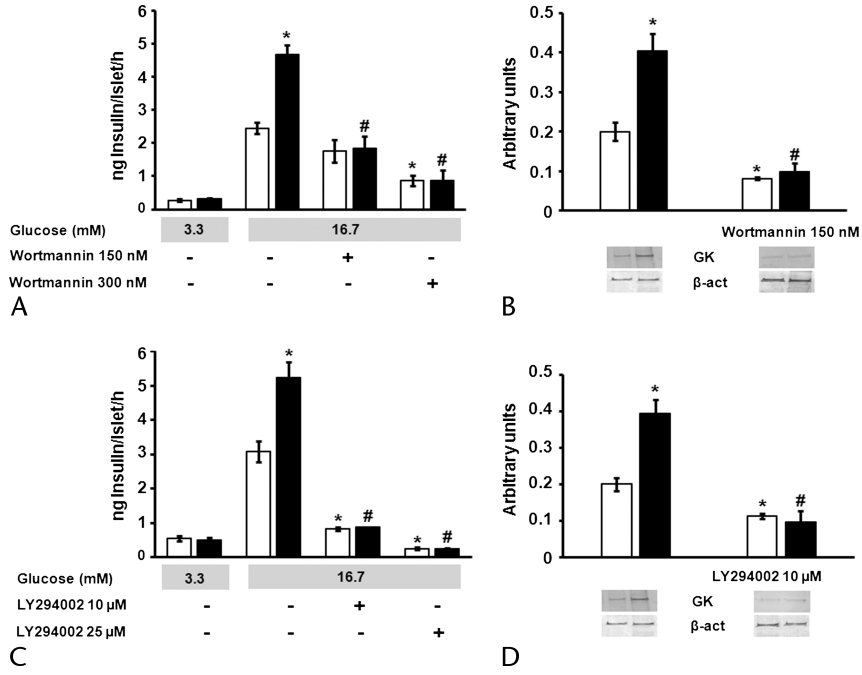


**FIGURE 3.** Effect of Wortmannin and LY294002 on insulin secretion and GK protein levels. (A and C) Glucose-induced insulin secretion in the presence of 150 and 300 nM Wortmannin or 10 μM and 25 μM LY294002. (B and D) GK protein levels measured in the presence of 150 nM Wortmannin or 10 μM LY294002. β-actin was used as housekeeping protein. In all cases, bars represent means ± SEM from four different experiments performed in islets cultured for 4 days in the absence (C, white bars) or presence of 10 μg/mL INGAP-PP (black bars). $^{*}P < 0.05$ compared to C bars, $^{\#}P < 0.05$ compared to INGAP-PP bars.

carbachol enhanced both the insulin release and the phosphorylation rate of P70S6K$^{-Thr389}$ and MAPK3/1.[18] Because these 2 compounds are downstream targets of PI3K and MAPK, respectively, we assumed and ascribed the enhancing INGAP-PP effects to an increase of PI3K, even though we did not directly measure it.

It has been shown that GK exerts a beneficial effect on β-cell mass[24] and survival.[25] Thus, the reported beneficial effect of INGAP-PP on β-cell viability and proliferation rate[8,11,15,26] could be partially ascribed to the peptide-induced increase of GK protein content. Because INGAP-PP did not modify GK mRNA levels, we could assume that the increased protein mass of the enzyme could be ascribed to changes at posttranscriptional level. This latter effect was not completely unexpected because increased GK protein expression without a concomitant increase in its mRNA had been reported.[23,27] In our in vitro model, INGAP-PP also increased IR, IRS-2, and PI3K protein concentration but not IRS-1 content. This cascade of insulin intracellular mediators in the islets and their stimulation by glucose had been earlier reported by Velloso et al[28] and published evidence supports its modulatory role on insulin secretion/synthesis[29] as well as on β-cell growth and survival.[30] Phosphorylation on tyrosine residues of IR, IRS-2 and IRS-1, was significantly higher in islets exposed to INGAP-PP. Because the tyrosine-phosphorylated IRS-1/PI3K (p85) complex activates the catalytic subunit of PI3K (p110), its increased level in treated islets represents an objective evidence of the stimulatory effect of INGAP-PP on PI3K activity. This activation cascade might play an important role in the mechanism by which INGAP-PP stimulates glucose metabolism and insulin secretion because such effect was blunted by 2 different PI3K inhibitors. Thus, these data provide the first direct evidence of the effect of INGAP-PP on the early step of intracellular insulin pathway, namely, PI3K protein expression and its upstream activators. Such INGAP-PP-induced effect on PI3K activity can also explain why this peptide activates other downstream mediators of the enzyme in neonate cultured islets[18] and in highly proliferative duct-like structures obtained from quiescent adult human islets.[31]

PI3K blockers also decreased significantly GK protein concentration in control islets and blunted the INGAP-PP stimulation on this enzyme, suggesting that the PI3K pathway might also regulate GK concentration/activity, a key step in the mechanism by which INGAP-PP enhances insulin secretion. Several facts support this concept: (a) the PI3K/Akt/FoxO1 cascade contributes to the regulation of GK gene expression in response to IGF-I stimulation in INS-1 cells;[32] (b) knockdown of PI3K-C2 expression and subsequent reduction of protein kinase B/Akt1 activity in the β cell impairs glucose-stimulated insulin release, partly due to the reduction of GK expression;[33] and (c) the over expression of protein kinase B/Akt in β-cells greatly expands islet mass and increases insulin secretion.[34]

The increased effect of INGAP-PP on IR protein expression and phosphorylation could also participate in the mechanism by which the peptide enhances insulin secretion. In fact, insulin activates the transcription of its own gene, stimulates islet GK transcription via insulin receptor B type,[35] and compartmentalization,[36] as well as glucose metabolism and insulin secretion.[37] Impairment of this insulin-positive feedback effect significantly decreases islet glucose metabolism and insulin release in animals with genetic or dietary-induced insulin resistance.[38] Further studies are required to demonstrate the relative weight of this insulin positive feedback within the complex mechanism by which INGAP-PP enhances insulin secretion.

Altogether, the current study objectively shows for the first time that INGAP-PP enhances glucose-induced insulin secretion acting at multiple β-cell targets: it increases protein concentration and tyrosine phosphorylation of 2 key intracellular insulin mediators (IRS-2 and PI3K), GK protein concentration and activity, and glucose metabolism. The PI3K/AKT pathway would participate in the mechanism by which INGAP-PP regulates GK protein

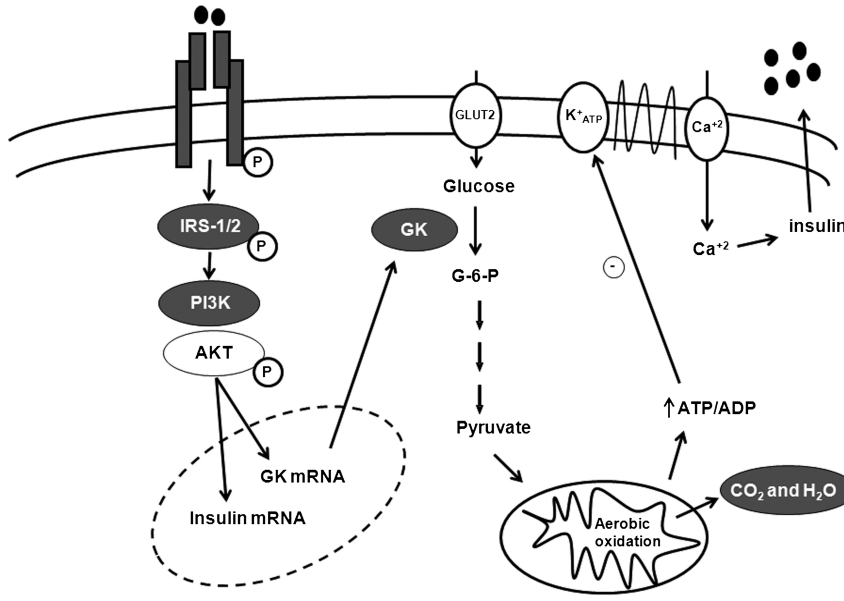


**FIGURE 4.** Mechanisms involved in the enhancing effect of INGAP-PP upon glucose-induced insulin secretion. Glucose enters the β-cell via glucose transporter 2 (GLUT2) and is phosphorylated by GK, the rate-limiting enzyme of glycolysis, that is upregulated by insulin through the PI3K/AKT pathway. The increased rate of β-cell glycolysis and mitochondrial metabolism enhances the cytosolic ATP/ADP ratio, which closes the $K_{ATP}$-channels that induces a plasma membrane depolarization. The latter opens the voltage-gated calcium channel rising the cytoplasmic $Ca^{2+}$concentration that finally triggers insulin release. INGAP-PP would enhance glucose-induced insulin secretion by increasing glucose phosphorylation rate and its metabolism as well as the intracellular insulin mediators (steps shown in gray).

expression/activity and consequently insulin release. Intracellular insulin mediators and processes affected by INGAP-PP are represented in Figure 4. These INGAP-PP–mediated changes would result in a more robust β cell to release insulin and to react against the deleterious effect of streptozotocin,[9,26] oxidative stress,[39] and endoplasmic reticulum stress.[40]

Recently, it has been demonstrated that INGAP-PP promoted the differentiation of human umbilical cord mesenchymal stem cells into insulin-producing cells,[41] as well as the viability, differentiation, and regeneration of islets before transplantation.[42]

On account of these results, the potential use of INGAP-PP to maintain/enhance the secretory capacity of cultured islets previous to their transplant might be worth exploring, together with the promising use of INGAP-PP for the treatment of people with diabetes,[16] considering the additional low risk of hypoglycemia associated with its use because its effect on insulin secretion is glucose concentration-dependent.

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