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INGAP-PP up-regulates the expression of genes and proteins related to K^+_{ATP} channels and ameliorates Ca^{2+} handling in cultured adult rat islets

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ABSTRACT

Islet Neogenesis Associated Protein (INGAP) increases pancreatic β-cell mass and potentiates glucose-induced insulin secretion. Here, we investigated the effects of the pentadecapeptide INGAP-PP in adult cultured rat islets upon the expression of proteins constitutive of the K^+_{ATP} channel, Ca^{2+} handling, and insulin secretion. The islets were cultured in RPMI medium with or without INGAP-PP for four days. Thereafter, gene (RT-PCR) and protein expression (Western blotting) of Foxa2, SUR1 and Kir6.2, cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$), static and dynamic insulin secretion, and ^{86}Rb efflux were measured. INGAP-PP increased the expression levels of Kir6.2, SUR1 and Foxa2 genes, and SUR1 and Foxa2 proteins. INGAP-PP cultured islets released significantly more insulin in response to 40 mM KCl and 100 μM tolbutamide. INGAP-PP shifted to the left the dose-response curve of insulin secretion to increasing concentrations of glucose (EC_{50} of 10.0 ± 0.4 vs. 13.7 ± 1.5 mM glucose of the controls). It also increased the first phase of insulin secretion elicited by either 22.2 mM glucose or 100 μM tolbutamide and accelerated the velocity of glucose-induced reduction of ^{86}Rb efflux in perifused islets. These effects were accompanied by a significant increase in $[Ca^{2+}]_i$ and the maintenance of a considerable degree of $[Ca^{2+}]_i$ oscillations. These results confirm that the enhancing effect of INGAP-PP upon insulin release, elicited by different secretagogues, is due to an improvement of the secretory function in cultured islets. Such improvement is due, at least partly, to an increased K^+_{ATP} channel protein expression and/or changing in the kinetic properties of these channels and augmented $[Ca^{2+}]_i$ response. Accordingly, INGAP-PP could potentially be used to maintain the functional integrity of cultured islets and eventually, for the prevention and treatment of diabetes.

1. Introduction

INGAP (Islet Neogenesis Associated Protein) was identified as the active part of a pancreatic protein complex (Ilotropin), isolated from normal hamsters, whose pancreas heads were previously wrapped in cellophane [1]. The INGAP gene originates a transcript of 850 bp, forming a protein of 19,940 Da [2] and seems to be a member of the Reg family with great homology to Reg IIIδ, but with different sites of regulation and distribution within islet cells [3,4].

INGAP is apparently one of the factors that initiates during intrauterine development pancreatic β-cell differentiation [5] and presents some characteristics that make it an interesting tool for the study and induction of islet-cells differentiation. In fact, INGAP is normally produced only in the pancreas (islets, duct and exocrine cells), is detected at the onset of pancreatic cell differentiation, and possesses promoter regions distinct from those observed for the Reg IIIδ gene, which is sometimes expresses in response to inflammatory process [3,5–7].

A pentadecapeptide with the 104-108 aminoacid sequence of INGAP (INGAP-PP) increased ^3H-timidine incorporation into both pancreatic duct cells and a duct-cell line [1,2], and its administration to normal hamsters and dogs as well as to diabetic mice enhanced islet neogenesis [8,9]. Injection of INGAP-PP into streptozotocin-induced diabetic mice produced a simultaneous decrease in blood glucose levels and increased the rate of islet neogenesis [9]. Further, the increase in β-cell mass observed in hamsters with insulin resistance induced by a sucrose-rich diet (SRD), was accompanied by an increase in the number of INGAP-positive cells in the exocrine, endocrine and duct pancreatic subsectors [4]. Seven-day old pups from dams treated with SRD during pregnancy showed lower glycemia, higher β-cell mass, increased β-cell replication rate and islet neogenesis, together with a decreased rate of β-cell apoptosis. These animals also had duct-, islet- and acinar-cells that co-expressed INGAP/PDX1, which did not react with antibodies against islet hormones and had a high replication rate [10]. Since PDX1 is a transcription factor essential for the development of the pancreas and

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is considered a marker for β-cell precursors in pancreas from adult mammals, the presence of a large number of INGAP/PDX1 positive cells suggests the importance of this cell population for the development of new insulin-producing cells [10].

We have recently shown that β-cells of neonatal and adult normal rat islets cultured with INGAP-PP increased their size and released significantly more insulin in response to glucose and other secretagogues [11,12]. Using macroarray analysis, we also observed that INGAP-PP up-regulated several genes involved in islet metabolism, activation of insulin secretion machinery, and regulation of β-cell mass and islet neogenesis. Some of these genes were related to the K^+_{ATP} channels that play a crucial role in the mechanism of insulin secretion [12]. Furthermore, we have also observed that the addition of INGAP-PP to the culture medium enhances tolbutamide-induced insulin secretion in neonatal islets (unpublished data).

On account of the key participation of K^+_{ATP} channels in the regulation of both $[Ca^{2+}]i$ concentrations and insulin release, we have currently investigated the possible mechanism by which INGAP-PP enhances insulin secretion in cultured adult rat islets. Our results showed that addition of INGAP-PP to these islets, cultured for 4 days, renders them more sensitive to glucose, KCl and tolbutamide stimuli. This effect is, at least in part, due to an increase in protein expression of the two components of the K^+_{ATP} channels and of their transcription factor Foxa2 [13,14], together with an improvement of Ca^{2+} handling by the islet-cells.

2. Materials and methods

2.1. Animals and islets

Islets of adult Wistar rats were isolated by collagenase digestion and cultured in RPMI-1640 medium supplemented with 5% fetal bovine serum, 11 mM glucose, 100 IU of penicillin/mL, 100 μg of streptomycin/mL at 37 °C in a 5% CO_2/air atmosphere for 4 days. At day 0 of culture, we added 10 μg/mL of INGAP-PP to half of the plates, using the remaining plates as controls (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. Static insulin secretion

Cultured adult isolated islets were rinsed in Krebs-bicarbonate buffer, pH 7.4, previously gassed with a mixture of CO_2/O_2 (5/95%), and preincubated in 1 mL of KREBS-bicarbonate buffer containing 3% (w/v) BSA and 5.6 mM glucose at 37 °C for 30 min. After this period, groups of 5 islets were incubated for 1 h in 1 mL of the same buffer with the addition of increasing concentrations of glucose (2.8 to 22.2 mM), 40 mM KCl, or 100 μM tolbutamide. At the end of the incubation period, aliquots of the medium were collected for insulin determination by radioimmunoassay.

2.3. Dynamic insulin secretion and ^{86}Rb efflux

Cultured adult islets were perifused in the presence of 2.8 or 5.6 mM glucose [baseline secretion]. A 30-min adaptation period was allowed before the first sample was taken for insulin assay and was followed by a further 15 min control period before changing the glucose concentration in the perifusate or before tolbutamide addition. Pancreatic effluents were collected every min during the first 5 min, immediately before and 10 min after the beginning of the stimuli. Effluents were collected in plastic tubes, immediately frozen and maintained at −20 °C for insulin radioimmunoassay measurement.

Cultured adult islets were preincubated for 90 min in Krebs-bicarbonate buffer containing $^{86}RbCl$ (1.0–2.0 MBq/mL) and 8.3 mM glucose, washed 3 times, and transferred to the perifusion system. After an adaptation period of 25 min, the effluent medium was collected every 2 min and the ^{86}Rb efflux was expressed as a functional outflow rate (% instantaneous islet content/min). Different concentrations of glucose were introduced in the perifusion medium, as shown in Fig. 6.

2.4. Glucose oxidation

Groups of 15 cultured islets each were placed in wells containing 100 μL of KREBS-bicarbonate buffer supplemented with trace amounts of D–[U–^{14}C] glucose (0.1–0.2 MBq/mL), plus non-radioactive glucose to a final concentration of 11.1 mM. The wells were maintained in empty 20 mL scintillation vials, which were gassed with 5% O_2 and 95% CO_2 and capped airtight with rubber membranes. The vials were shaken continuously for 2 h at 37 °C in a water bath. After incubation, 0.1 mL HCl (0.2 mM) and 0.2 mL hyamine hydroxide was injected through the rubber cap into the glass cup containing the incubation medium and into the counting vial, respectively. After 1 h at room temperature, 2 mL of scintillation fluid was added to the hyamine and the radioactivity was counted. The rate of glucose oxidation was expressed as pmol/islet.h.

2.5. RT-PCR analysis

Semi-quantitative RT-PCR was performed using specific primers to confirm the differential expression of three genes. Reverse transcription was carried out with 3 μg of total RNA using Moloney murine leukemia virus-reverse transcriptase (Superscript II) and random hexamers, according to manufacturer's instructions (Invitrogen, CA, USA). RT-PCR assays were performed in quadruplicate using recombinant *Taq* DNA polymerase (Invitrogen, CA, USA) and 10 pmol of each primer in a master mix of 50 μl. The primer sets used in RT-PCR analysis were: Kir6.2: sense 5′TTAGCGCCACCATTCATATG3′, antisense 5′TCCGGAAGATGCTAAACTTG3′, 31 cycles, 62 °C; SUR1: sense 5′ TTCCACATCCTGGTCACACCG3′, antisense: 5′AGAAGGAGCGAGGACTT-GCCAC3′, 29 cycles, 62 °C; Foxa2: sense: 5′CTGAGTGGAAACATTG-GGG3′, antisense: 5′GATTTGTGGAACTCTGGCCA3′; 29 cycles, 62 °C; RPS29: sense: 5′AGGCAAGATGGGTCACCAGC3′, antisense: 5′AGTC-GAATCATCCATTCAGGTCG3′, 29 cycles, 54 °C. 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, BioRad) followed by quantification using Scion Image analysis software (Scion Corp., Frerderick, MD). The results were expressed as a ratio of target to RPS-29 signals. The RNAs used for RT-PCR analysis were obtained from five sets of experiments.

2.6. 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, 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 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 BioRad miniature slab gel apparatus. The electrotransfer of proteins from the gel to nitrocellulose was carried out 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, Foxa2 (1:500)

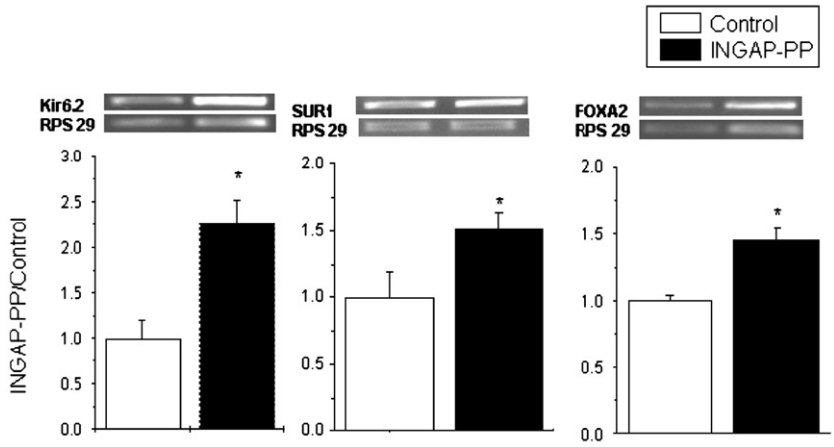

Fig. 1. Semiquantitative RT-PCR analysis of Kir6.2, SUR1 and Foxa2 gene expressions in adult rat islets cultured for 4 days with or without 10 μg/ml of INGAP-PP. The bars represent the means ± SEM of five experiments performed with specific sets of primers [see Methods section] and normalized against RPS-29. *$P<0.05$ for control vs. INGAP-PP.

and SUR1 (1:500) (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).

2.7. Measurement of intracellular Ca^{2+} ($[Ca^{2+}]_i$)

Groups of cultured control and INGAP-PP-treated islets were transferred to plates containing Krebs-bicarbonate buffer with 5.6 mM glucose and 1% albumin, continuously gassed with 95% O_2 and 5% CO_2 and incubated for 1 h at 37 °C. Thereafter, 5 μM fura2/AM (Invitrogen, CA, USA) and pluronic F-127 (Invitrogen, CA, USA), in a ratio of 1:1, were added and the islets incubated for an additional period of 2 h. Islets were then transferred to an open chamber thermostatically regulated (37 °C), placed on the stage of an inverted microscope (Nikon UK, Kingston, UK), and perifused with Krebs-bicarbonate buffer without albumin at flow rate of 1.5 ml/min. The solution was continuously gassed with 95% O_2 and 5% CO_2 to maintain pH at 7.4. A ratio image was acquired approximately at every 5 s with an ORCA-100 CCD camera (Hammamatsu Photonics Iberica, Barcelona, Spain), in conjunction with a Lambda-10-CS dual filter wheel (Sutter Instrument Company, CA, USA), equipped with 340 and 380 nm, 10 nm bandpass filters, and a range of neutral density filters (Omega opticals, Stanmore, UK). Data were obtained using the ImageMaster3 software (Photon Technology International, NJ, USA).

2.8. Statistical analysis

Results are expressed as means ± SEM. Statistical comparisons between INGAP-treated islets and the respective control groups were made using the Student's unpaired *t*-test or ANOVA. *P* values <0.05 indicate a significant difference.

3. Results

3.1. Effects of INGAP-PP on gene and protein expressions

Fig. 1 shows that INGAP-PP increased the transcription of Kir6.2 and SUR1 genes that encode the two protein components of the K^+_{ATP} channels in pancreatic islets (2.3- and 1.5-fold increase vs. control, respectively; $P<0.05$). INGAP-PP also increased the expression of Foxa2 gene, which encodes an important transcription factor that controls the expression of SUR1 and Kir6.2 genes (1.5-fold increased vs. control islets; $P<0.05$). Western blot analysis also showed a 2.8- and 1.5-fold increase in the protein expressions of SUR1 and Foxa2, respectively, in INGAP-PP-treated islets compared with controls (Fig. 2).

3.2. Effect of INGAP-PP on insulin secretion

Pancreatic islets cultured with INGAP-PP and then incubated for 1 h with increasing concentrations of glucose (2.8–22.2 mM), released

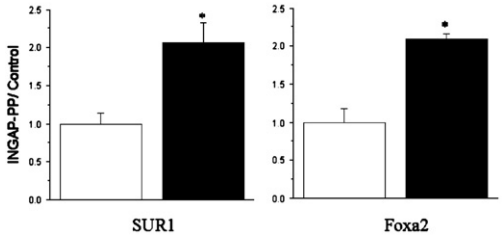

Fig. 2. SUR1 and Foxa2 protein expression in adult rat islets cultured for 4 days in the absence or presence of 10 μg/ml of INGAP-PP. Equal amounts of protein extracts were submitted to SDS-PAGE electrophoresis, transferred to a nitrocellulose membrane and then incubated with specific anti-SUR1 and anti-Foxa2 antibodies. The normalized values represent the means ± SEM of three experiments. *$P<0.05$ for control vs. INGAP-PP-treated islets.

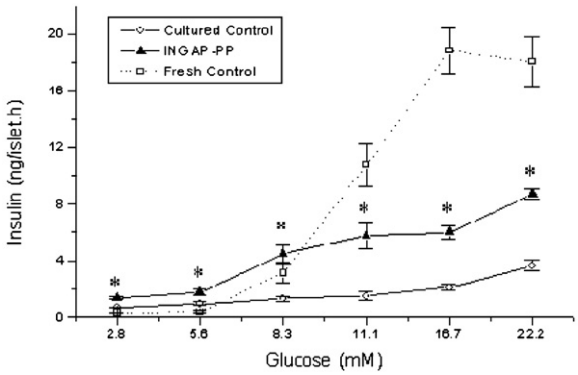

Fig. 3. Effects of INGAP-PP-treatment upon insulin secretion. Isolated adult rat islets were cultured for 4 days with or without 10 μg/ml of INGAP-PP, and then incubated for 1 h with increasing concentrations of glucose [2.8 to 22.2 mM]. Values represent the means ± SEM of 6 cases from two different experiments. *$P<0.05$ for control vs. INGAP-PP-treated islets. For comparison, a dose-response curve of insulin secretion to glucose, obtained in fresh isolated islets, is also shown [dashed line].

significantly more insulin at each glucose concentration tested than those cultured without the pentadecapeptide (Fig. 3). As a consequence, the INGAP-PP curve was shifted to the left showing an EC_{50} of 10.0±0.4 vs. 13.7±1.5 mM of the control islets ($P<0.05$). For comparison, Fig. 3 also shows a dose-response curve to glucose, obtained with freshly-isolated islets. At low and intermediate concentrations of glucose (2.8–8.3 mM), the insulin secretion from freshly isolated and INGAP-PP-treated islets was similar, but, the secretion was significantly greater in the former in response to higher glucose concentrations (11.1–22.2 mM glucose) ($P<0.05$). Depolarizing concentrations of KCl also induced a greater insulin response in INGAP-PP-treated islets compared to controls (Fig. 4A). At 2.8 mM glucose, INGAP-PP rendered the islets more sensitive to tolbutamide (100 μM); however, this enhancing effect of INGAP-PP upon tolbutamide-induced insulin secretion was no longer observed at 22.2 mM glucose (Fig. 4B).

Dynamic secretion studies revealed that, in the presence of 2.8 mM glucose (min 30–45), insulin secretion was slightly higher but not statistically significant, in INGAP-PP-treated islets compared with controls. Addition of 22.2 mM glucose to the perifusate (min 46) induced a significant increase in the biphasic release of insulin (Fig. 5A) in both groups of islets. During the first phase (min 58), insulin secretion reached a peak of 196±66 and 81±30 pg·islet^{-1} in INGAP-PP-treated and control islets, respectively ($P<0.05$). The area under the curve (AUC) (min 55 to 68) was also significantly higher in INGAP-PP-treated islets compared to control islets (1400±100 vs. 700±60 ng/islets.10 min, respectively; $P<0.05$).

In the presence of 5.6 mM glucose, tolbutamide (100 μM) induced a biphasic release of insulin in control islets, characterized by a short first phase followed by another short second phase. The first phase of the tolbutamide-induced secretion (min 55–60) was significantly greater in INGAP-PP-treated islets compared to control islets with an integrated secretion of 34±1.0 and 15±0.4 pg/islets/min for INGAP-PP and control islets, respectively ($P<0.05$). Conversely, the second phase of insulin release (min 61–68) was greater in control than in INGAP-PP-treated islets ($P<0.05$) (Fig. 5B).

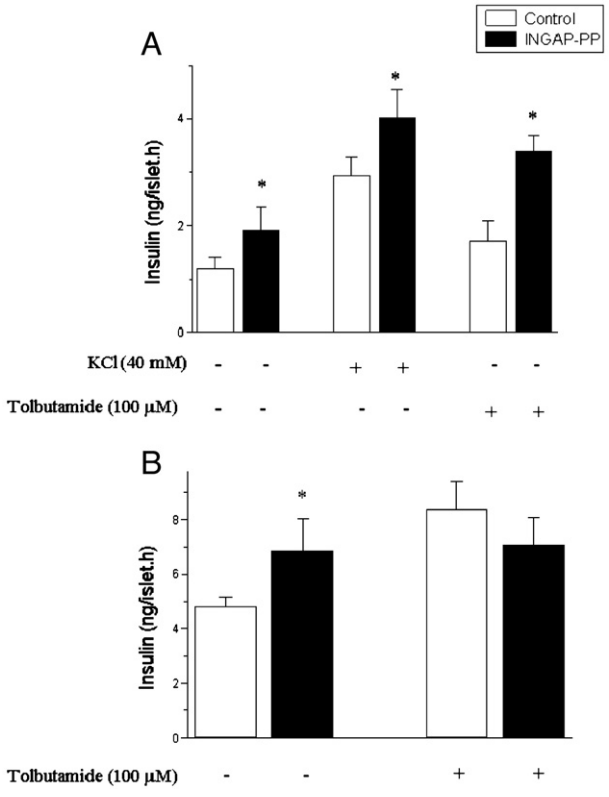

Fig. 4. Effects of 40 mM KCl and 100 μM tolbutamide on insulin secretion from INGAP-PP-treated and control islets. Isolated adult rat islets were cultured for 4 days with or without 10 μg/ml of INGAP-PP, and then incubated for 1 h with 2.8 [A] or 22.2 mM glucose [B]. Values represent the means±SEM of 6 cases from two different experiments. *$P<0.05$ for control vs. INGAP-PP-treated islets.

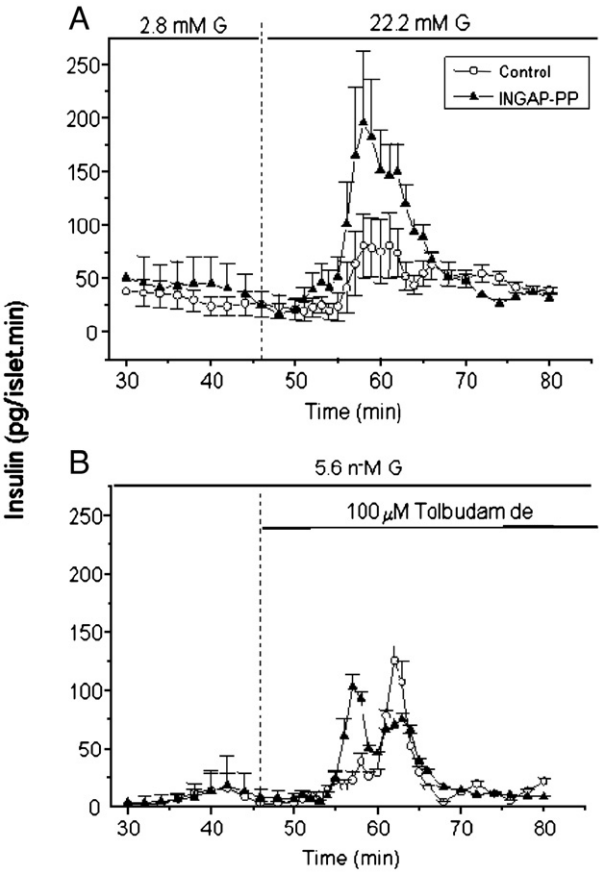

Fig. 5. Changes induced by INGAP-PP upon the dynamic pattern of insulin secretion elicited by 22.2 mM glucose and 100 μM tolbutamide. After 4 days in culture with or without 10 μg/ml of INGAP-PP, the islets were transferred to a chamber and perifused for 80 min. In A, 2.8 mM glucose was present from the onset of the experiment and was increased to 22.2 mM at between 45–70 min. In B, 5.6 mM glucose was present from the onset until the end of the perifusion period and tolbutamide [100 μM] was introduced at 46 min, remaining in the perifusate until 80 min. Batches of 100 islets were perifused in each experiment. Values are the means±SEM of four experiments.

3.3. Effect of INGAP-PP on ^{86}Rb efflux

The efflux rate of ^{86}Rb (used as a substitute for K^+) in the absence of glucose (min 26–40) was similar in both groups. Addition of 22.2 mM glucose to the perifusate induced a marked and comparable reduction of ^{86}Rb efflux in both INGAP-PP-treated and untreated islets. The effect of glucose was rapidly sustained but rapidly reversed following the withdrawal of the sugar from the perifusate (min 60 onward) (Fig. 6). However, the velocity of reduction in ^{86}Rb efflux rate, induced by 22.2 mM glucose (min 40–46), seems to be greater in INGAP-PP-treated than in control islets, as shown by the regression line (−13.1±3.7 for INGAP-PP vs. −6.6±0.3 for control islets; $P<0.05$) (inset Fig. 6), indicating possible changes in the kinetics properties of the channels and/or their sensitivity to glucose.

3.4. Effect of INGAP-PP on glucose utilization

Glucose oxidation rate, measured as the $^{14}CO_2$ production from U [^{14}C]D-glucose, was not significantly different in INGAP-PP-treated and control islets (47±2.7 vs.45±2.8 pmol/islet.h), respectively.

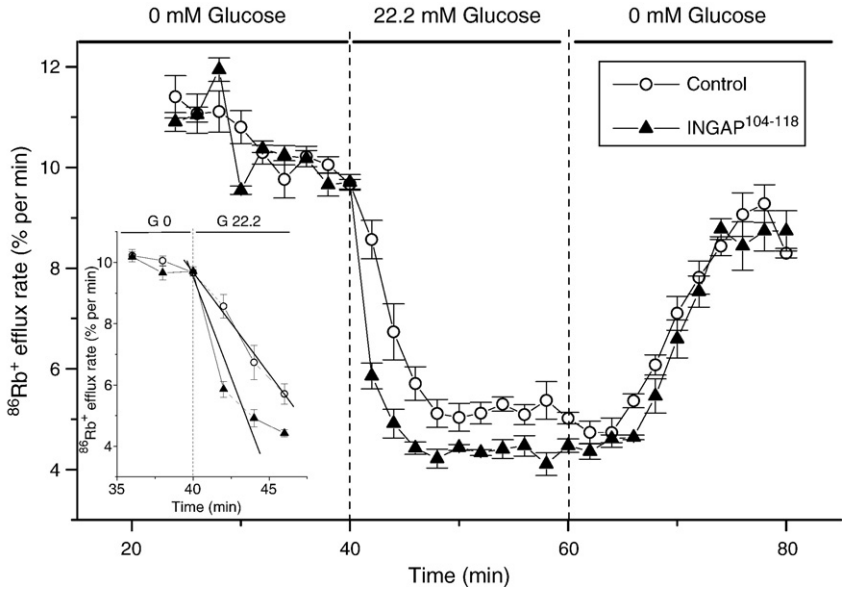

Fig. 6. Effect of 22.2 mM glucose on the ^{86}Rb efflux rate from control and INGAP-PP-treated islets. After 4 days in culture, with or without 10 μg/ml INGAP-PP, batches of 100 islets were pre-labeled with ^{86}RbCl [1–2 MBq/ml] in the presence of 16.7 mM glucose, washed 3 times, and transferred to the perifusion chamber. After an adaptation period of 25 min, the effluent medium was continuously collected every 2 min until the end of the perifusion [min 80]. Glucose [22.2 mM] was present in the medium from 40 min until 60 min. Values are the means ± SEM of four experiments. The inset of the figure illustrates the slope of the linear regression from each curve [min 40–46].

3.5. Effects of INGAP-PP on $[Ca^{2+}]_i$

Fig. 7 (A, B) shows that 11.1 mM glucose increased the cytoplasmic Ca^{2+} concentrations in both control and INGAP-PP islets. The increment in Ca^{2+} concentration was significantly higher in INGAP-PP islets as judged either by the maximal (ΔR) values reached in the presence of 11.1 mM glucose (peak values; Fig. 7C) or by the area under curve (AUC) measured during the glucose stimulation (Fig. 7D) ($P<0.05$). During the plateau phase, the majority of INGAP-PP, but not control, islets showed Ca^{2+} oscillations similar to those observed in freshly isolated islets (not shown). Fig. 7 also shows that INGAP-PP islets increased $[Ca^{2+}]_i$ peak and AUC during a 40 mM KCl pulse, compared to control islets (Fig. 7E and F, respectively; $P<0.05$). Finally, tolbutamide (100 μM) also produced an increase in cytoplasmic Ca^{2+} concentration of a similar magnitude in both groups of islets.

4. Discussion

Our current results show that INGAP-PP enhanced significantly the release of insulin in response to either metabolic (glucose) or non metabolic (40 mM KCL and 100 μM tolbutamide) stimuli of cultured normal isolated islets. These data suggest that the peptide would exert this enhancing effect by acting upon a common secretory pathway. Since all these stimuli share the common sequential steps of β-cell membrane depolarization, opening of voltage dependent Ca^{2+}-channels, and increase in cytosolic Ca^{2+} concentration that lead to the release of insulin [15], these paths become suitable targets for INGAP-PP effect. The lack of effect of INGAP-PP upon glucose oxidation rate, the enhanced expression of the two components (SUR1 and Kir6.2), and the changes in ^{86}Rb efflux kinetics and in $[Ca^{2+}]_i$ concentration support such assumption. Thus, we can conclude that addition of INGAP-PP to the culture medium preserves islet-cells function, particularly in relation to Ca^{2+} handling, turning the islets more sensitive to the stimulatory effect of different secretagogues.

The insulin-secretion enhancing effects of INGAP-PP show some characteristics that merit further analysis. Freshly isolated islets, incubated for 1 h, release more insulin than islets previously cultured with or without INGAP-PP in response to high (≥11.1 mM) glucose (Fig. 3, dashed line). Addition of INGAP-PP to the culture medium significantly enhanced the release of insulin at every glucose concentration tested and restored the insulin response to levels comparable to those measured in freshly-isolated islets when challenged with a glucose concentration comparable to the circulating levels measured in intact normal animals (5.6–8.3 mM). Thus, this effect might be a useful tool to maintain the glucose-induced insulin response of islets stored in culture for future transplants.

On the other hand, in the presence of low glucose, tolbutamide stimulated the release of insulin in precultured islets, but this release was significantly higher in islets cultured with INGAP-PP (Fig. 4A). This enhanced response to tolbutamide however, was no longer observed when tested in the presence of 22.2 mM glucose (Fig. 4B). Although we do not have a definitive explanation, this lack of effect may indicate that, at 22.2 mM glucose, the majority of K^+_{ATP} channels in the INGAP-PP treated islets were already closed.

INGAP-PP also significantly enhanced the glucose-stimulated secretion of insulin in a dynamic model. In islets perifused with 5.6 mM glucose, addition of tolbutamide induced a biphasic insulin response but of lower magnitude than that triggered by high glucose. Under these conditions, control islets released a tiny first phase and INGAP-PP treatment significantly increased its size, while it lowered the second phase. This effect of INGAP-PP upon the first phase of insulin secretion is particularly important because this phase is specifically decreased and blunted in people with impaired glucose tolerance and diabetes, respectively [16]. We are, thus, tempted to speculate that in these two clinical conditions, INGAP-PP could be an effective alternative to restore to normal the first phase of insulin response. The issue deserves the development of a more specific experimental design to test the consistence of such hypotheses.

Another interesting effect of INGAP-PP on the islet function is related to the cytoplasmic Ca^{2+}concentration. The increase in $[Ca^{2+}]_i$ and the maintenance of $[Ca^{2+}]_i$ oscillations (similar to those found in freshly isolated islets), observed in INGAP-PP islets challenged with 11.1 mM glucose or 100 μM tolbutamide, parallel the significantly higher release of insulin in response to these secretagogues. It is known that culture conditions such as duration and glucose concentrations impair the $[Ca^{2+}]_i$ oscillations in pancreatic islets [17–19]. Mice islets cultured for 4 days in the presence of 10 mM glucose exhibit a decreased frequency of $[Ca^{2+}]_i$ oscillations, increase of

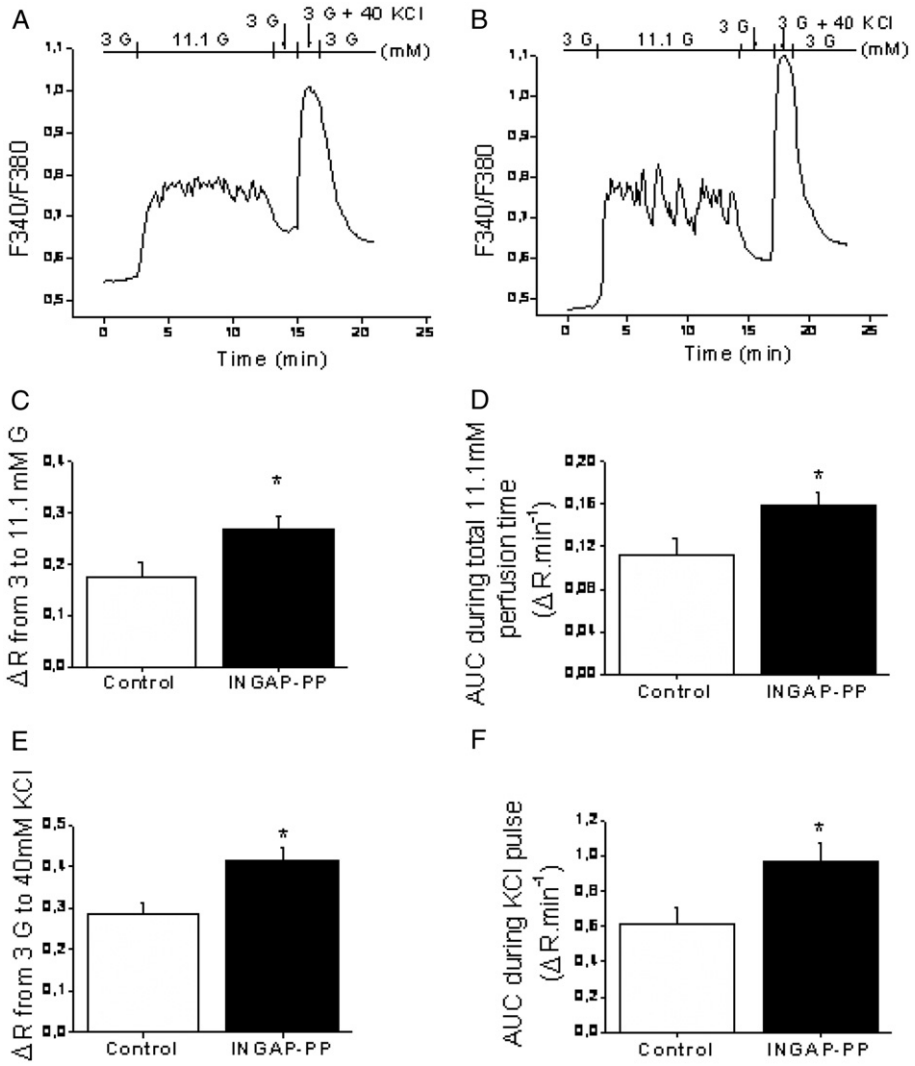

Fig. 7. Effects of 11.1 mM glucose and 40 mM KCl on $[Ca^{2+}]_i$ measurements in control and INGAP-PP islets. After 4 days in culture, with or without 10 μg/ml INGAP-PP, the islets were incubated in a KREBS-buffer containing 5.6 mM glucose, Fura2/AM and pluronic F-127 for 2 h. Then, the islets were transferred individually to a chamber adapted in an inverted microscope and continuously perfused with KREBS-buffer (1.5 ml/min at 37 °C). In A and B, representatives $[Ca^{2+}]_i$ recording from control and INGAPP-PP cultured rat islet under different glucose concentrations (G) and KCl, as indicated. The means ± SEM represent the maximum peak (C) and area-under-curve (AUC) (D) of $[Ca^{2+}]_i$ (ΔR) at 11.1 mM glucose, and the maximum peak (E) and AUC (F) of $[Ca^{2+}]_i$ (ΔR) at 40 mM KCl (n = 7–9 islets from three different donors, $P<0.05$). The recordings are representative for 7–9 with similar results.

duration of the oscillations and eventually their disappearance [17]. A recent study has demonstrated that the K^+_{ATP} channel plays a critical role in Ca^{2+} sequestration by non-endoplasmic reticulum (non-ER) compartments in mouse pancreatic β-cells that, in turn, modulate Ca^{2+} oscillations in these cells [20]. Thus, an increase in the proteins expression that form the K^+_{ATP} channels may favor the glucose-and tolbutamide-induced insulin secretion observed in INGAP-PP treated islets. We have to admit however, that the increased expression of these proteins did not justify *per se* the higher amount of Ca^{2+} observed when these islets were depolarized by 40 mM KCl. Since we have not addressed this issue in the present study, we can only speculate that these effects could be due to an overall improvement of the INGAP-PP islets function.

As occurs with neonatal islets [12], INGAP-PP induced a simultaneous increase in the expressions of SUR1 and Kir6.2 genes in adult rat islets, as well an increase in the expression of Foxa2 that controls the expression of these two proteins. Foxa2 also acts as a transcriptional activator, required for the maintenance of PDX1 transcription function and its deletion down regulates Pdx1 mRNA and protein levels in β-cells [14]. Thus, Foxa2 may play an important role in the control of β-cell function and mass [13,14,21], and its mutations in human beings might contribute to the development of impaired glucose homeostasis and diabetes [14].

We have previously reported that seven-day old pups from SDR dams present an increase in glucose-induced insulin secretion, in the β-cell mass, in the β-cell replication rate, in the islet neogenesis and a decrease in the rate of β-cell apoptosis. These animals also show a simultaneous increase in the number of cells located in duct-, islet- and acinar-compartments that co-express INGAP/PDX1 and have a high replication rate [10]. These evidences as well as the current data together with the reported effect of INGAP-PP upon the expression of different genes in the islets and its preventive effect upon streptozotocin-induced diabetes, strongly suggest that this peptide plays an important role of in the control of β-cell mass and function.

In conclusion, the current results confirm our previous report showing that INGAP-PP enhances the release of insulin in response to different stimuli [11,12]. They also provide new evidences that INGAP-PP exerts such an effect, at least in cultured islets, by enhancing the expression of constitutive proteins of the K^+_{ATP} channels and/or the kinetic properties of these channels and improving the Ca^{2+} handling by islet-cells. These results merits further studies to prove the potential usage of INGAP-PP to maintain/enhance the secretory capacity

of cultured islets previous to their transplant and also for the prevention and treatment of diabetes.

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