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**Protein inhibitor of a neuronal nitric oxide synthase (PIN) is a new
regulator of glucose induced-insulin secretion**

Running title: PIN, a new regulator of insulin secretion

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ABSTRACT

We previously showed that pancreatic β -cells express a neuronal isoform of nitric oxide synthase (nNOS) that controls insulin secretion through two catalytic activities: NO production and cytochrome c reductase activity. We now provide evidence that the endogenous protein inhibitor of nNOS, called PIN, is expressed in rat pancreatic islets and INS-1 cells and displays 100% homology with brain PIN. Double immunofluorescence studies showed a colocalization of PIN and nNOS in insulin-secreting β -cells. Electron microscopy studies confirmed that PIN is mainly associated with insulin secretory granules and colocalized with nNOS in the latter. In addition, PIN overexpression in INS-1 cells enhanced glucose-induced insulin secretion, which is only partly reversed by addition of an NO donor, sodium nitroprusside (SNP), and unaffected by the inhibitor of cytochrome c reductase activity, miconazole. In contrast, the pharmacological inhibitor of nNOS, *N* ω -nitro-L-arginine methyl ester (L-NAME), amplified glucose-induced insulin secretion, an effect insensitive to SNP but completely normalized by addition of miconazole. In conclusion, PIN is implicated in the physiological regulation of glucose-induced insulin secretion, a role that might occur at the level of the exocytotic machinery, as a component of the molecular motors dynein and myosin V.

INTRODUCTION

The second messenger nitric oxide (NO) is synthesized from L-arginine by a family of enzymes known as NO synthases (NOSs). Three NOS isoforms have been identified, including the constitutive neuronal (nNOS) and endothelial NOS (eNOS) and the cytokine-inducible NOS (iNOS) (1). Constitutive NOSs produce small amounts of NO, implicated in physiological functions such as synaptic transmission (2) and vascular tone (3), whereas iNOS produces high amounts of NO, acting as a cytotoxic mediator in immune response (4). In addition to these beneficial roles, NO exerts deleterious effects as a result of its overproduction, leading to neuronal death during brain ischemia and certain neurodegenerative pathologies (5) and to autoimmune diseases (6). For these reasons, a tight regulation of NOSs catalytic activity is essential for its physiological function. If iNOS is regulated by cytokines at the transcriptional level, constitutive NOSs catalytic activity is, in contrast, closely modulated by different mechanisms. The enzymes activity is first controlled by the reversible binding of Ca^{2+} -calmodulin (7) that allows the transfer of NADPH-derived electrons from the reductase to the oxygenase domain of the enzyme (8). It is also positively or negatively regulated through phosphorylation by various protein kinases such as PKA and PKC (9, 10). In addition to the role of intracellular Ca^{2+} levels and protein kinases, constitutive NOSs are also regulated via protein-protein interactions. Thus, ENAP-1 (endothelial NOS-associated protein-1) (1), NOSIP (endothelial NOS interacting protein) (12), and caveolins (13) are known to modulate eNOS activity. Concerning nNOS, Jaffrey and Snyder identified in rat brain a 10-kDa protein, PIN (protein inhibitor of neuronal NOS), that interacts with the N-terminal part of the enzyme (14). Binding of PIN to nNOS prevents homodimerization of the enzyme with as a result an inhibition of NO production (14). PIN is a ubiquitous protein expressed in rats and humans, to an extent depending on the tissue studied (14, 15). Thus, PIN

is largely present in testis, moderately in different brain areas, kidney, and ventilary muscles and faintly in other tissues (16–18). Furthermore, PIN has also been identified as the light chain of cytoplasmic and flagellar dyneins and myosin V, two cytoskeletal components involved in the traffic of intracellular organelles and flagellar movements (19, 20). In addition, PIN is also able to interact with other partners as diverse as Bim (a proapoptotic factor of the Bcl-2 family) (21), I κ B (the cytoplasmic inhibitor of NF κ B) (22), DAP1 α (an NMDA receptor scaffolding protein) (23), NRF-1 (a transcriptional activator) (24), Swallow (an mRNA localization protein) (25), and gephyrin (a GABA or glycine scaffolding protein) (26), suggesting an interplay of PIN in a number of biological functions.

In previous studies, we showed that pancreatic β -cells express a neuronal isoform of NOS that displays three mutations when compared with cerebellar nNOS (27). Pancreatic β -cell nNOS is mainly localized in insulin secretory granules but also in mitochondria and nucleus. The enzyme is implicated in the control of insulin secretion and, as previously shown for brain NOS, is able to exert two catalytic activities: NO production and a cytochrome c reductase activity (27, 28). Furthermore, we could show that a normal balance between the two catalytic activities is essential for β -cell function, but the mechanisms that could regulate nNOS catalytic activities in pancreatic β -cells remain to be investigated. In an attempt to get insight into β -cell nNOS regulation, we hypothesized that PIN could be one of the factors implicated in nNOS regulation. This prompted us, first, to investigate if PIN is expressed in rat pancreatic β -cells; second, to study its subcellular localization; and finally to determine, by using the insulin-secreting cell line, INS-1, whether PIN could play a role in the regulation on glucose-induced insulin secretion.

RESEARCH DESIGN AND METHODS

Cell culture and isolation of islets. The insulin-secreting cell line INS-1 (a generous gift from Professor C. B. Wollheim) was cultured as previously described by Asfari et al. (29). Islets were isolated from adult male Wistar rats (Iffa Credo, Lyon, France) using collagenase digestion and separation on a Ficoll density gradient (30). For electron microscopy, islets were then incubated for 1 h at 37°C in Krebs Ringer Bicarbonate (KRB) buffer (108 mM NaCl; 1.19 mM KH₂PO₄; 4.74 mM KCl; 2.54 mM CaCl₂; 1.19 mM MgSO₄·7H₂O and 18 mM NaHCO₃), pH 7.4, supplemented with 1 g/l BSA and 2.8 mM glucose. For immunofluorescence studies, isolated cells from islets were obtained after dissociation with 0.025% trypsin/0.27 mM EDTA and then cultured in complete RPMI-1640 containing 5 mM glucose for one week.

RNA isolation and RT-PCR. Total RNA from rat isolated islets and INS-1 cells was extracted with the TRIzol™ reagent (Invitrogen, Carlsbad, CA). First strand cDNA was obtained from 10 µg of total RNA using 3 µg of random hexanucleotide primers (Invitrogen), 1 µg of oligo(dT) (Invitrogen), and Superscript II RNase H⁻ Reverse Transcriptase (Invitrogen). PCR was then performed with specific primers for PIN, 5'-TTGAGCGGCGCCAGCACCTTCCCT-3' and 5'-CGAGGTGTTCCCTTAGCAAGGCTG-3' and for β2-microglobulin, 5'-ATCTTTCTGGTGCTTGTCTC-3' and 5'-AGTGTGAGCCAGGATGTAG-3' for 40 cycles (denaturation 94°C, 30 s; annealing 60°C, 1 min; elongation 72°C, 1 min). PCR products were finally separated on 1.5% agarose gel and visualized by ethidium bromide staining.

Western blotting. Isolated islets, INS-1 cells, testis, and brain were homogenized in 20 mM Tris lysis buffer, pH 7.4, containing 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, and a cocktail of protease inhibitors (Roche Applied Science, Mannheim, Germany). Insoluble

materials were removed by centrifugation. The proteins (75 µg) were fractionated on a 14% tricine polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking with 5% dried skim milk, filters were then incubated overnight with a monoclonal anti-PIN antibody (diluted 1:500, Transduction Laboratories, Lexington, KY). After three washings, the membrane was finally incubated with a horseradish peroxidase-conjugated anti-mouse antibody (diluted 1:3000, Sigma-Aldrich, Steinheim, Germany). Immunoreactivity was detected using an enhanced chemiluminescence reaction (Amersham Biosciences, Little Chalfont, U.K).

Immunofluorescence. INS-1 and islet cells were seeded on poly-L-lysine (Sigma-Aldrich) coated Lab-Tek® Chamber Slide™ System. After fixation with 2% paraformaldehyde for 20 min, the cells were permeabilized 5 min in 0.1% Triton X-100 and saturated in 2% BSA. A combination of the following antibodies was then applied to the cells overnight: a monoclonal anti-PIN antibody (diluted 1:25, Transduction Laboratories), a goat anti-PIN antibody (diluted 1:25, Santa Cruz Biotechnology, Santa Cruz, CA), a rabbit anti-nNOS antibody (diluted 1:100, Euro-Diagnostica, Malmö, Sweden) and a guinea pig anti-insulin antibody (diluted 1:300, ICN Biomedicals, Irvine, CA). After several washings, the cells were incubated for one hour with either an FITC-conjugated anti-guinea pig antibody (diluted 1:100, Vector Laboratories, Burlingame, CA), an FITC-conjugated anti-mouse antibody ((diluted 1:100, Vector Laboratories), an FITC-conjugated anti-goat antibody (diluted 1:100, Vector Laboratories), a Texas Red-conjugated anti-mouse antibody (diluted 1:100, Vector Laboratories), a Texas Red-conjugated anti-goat antibody (diluted 1:100, Vector Laboratories), or a Texas Red-conjugated anti-rabbit antibody (diluted 1:100, Vector Laboratories). After three additional washings, the cells were finally mounted in Citifluor (Citifluor LTD, London, UK) and observed with the BioRad confocal microscope equipped with an argon-krypton laser, using the facilities of the Centre Régional d'Imagerie Cellulaire

(CRIC, Montpellier, France). The negative control was performed by incubating the cells with only the secondary antibody.

Electron microscopy. Two hundred freshly isolated islets were fixed in a solution of 100 mM phosphate buffer, pH 7, containing 2.5% paraformaldehyde and 0.1% glutaraldehyde for one hour at room temperature. After several washings in PBS-50 mM NH₄Cl, the islets were post-fixed in 1% osmium tetroxide for 2 minutes. The islets were then dehydrated in an ascending series of ethanol and routinely embedded in LR White (Electron Microscopy Sciences, Fort Washington, PA). Ultrathin sections of 60 nm were cut using a Reichert ultramicrotome (Ultracut S, Vienna, Austria) and deposited on gold grids. Sections were first saturated with 10% fetal calf serum and then incubated overnight with either the monoclonal anti-PIN antibody (diluted 1:25), the goat anti-PIN antibody (diluted 1:25), the rabbit anti-nNOS antibody (diluted 1:100), or the guinea pig anti-insulin antibody (diluted 1:500). After several washings, anti-mouse, anti-goat, anti-rabbit and anti-guinea pig antibodies labeled respectively with 10-nm, 15-nm, 10-nm and 5-nm gold particles (diluted 1:25, British Biocell, Cardiff, UK) were applied to the sections for 1 h at room temperature. Sections were rinsed in deionized water, stained with 2% uranyl acetate for 20 min, and then observed with a transmission electron microscope (Hitachi H-7100, Düsseldorf, Germany). The specificity of the immune reaction was tested by incubating the sections with only the secondary antibody.

Transfection and incubation experiments. INS-1 cells were seeded on poly-L-lysine (Sigma-Aldrich)-coated 24-well plates at a density of 4×10^5 cells per well for cells non transfected and at 3.5×10^5 cells per well for transfected cells. After 3 days of culture, cells were transfected with a eukaryote expression vector (pCR3.1, Invitrogen) containing the cDNA of rat PIN using Lipofectamine Plus™ Reagent (Invitrogen), according to the manufacturer's protocol. Control cells were also obtained by transfection with the empty vector under the same conditions. Forty eight hours after transfection, surexpression of PIN

was estimated by RT-PCR, as described above. At this time, the cells transfected or not were washed and pre-incubated for 1 h at 37°C in KRB buffer containing 2 g/l BSA in the absence of glucose. After removal of the medium, the cells were incubated for another hour at 37°C in the same buffer in the presence of 5 mM glucose with or without *N*ω-nitro-L-arginine methyl ester (L-NAME), miconazole nitrate salt, and sodium nitroprusside dihydrate (Sigma-Aldrich). At the end of the incubation period, the medium was collected and insulin was measured by the radioimmunological method of Herbert et al. (31) with rat insulin (Linco Research, Inc., St. Charles, MI) as the standard, allowing a sensitivity of 0.06 ng/ml.

Nitric oxide synthase assay. nNOS catalytic activity has been estimated in INS-1 extracts by measuring the production of citrulline from arginine in the presence of the enzyme cofactors, i.e., BH₄, FAD, FMN, NADPH, Ca²⁺, and calmodulin. After transfection and incubation as described above, INS-1 cells were harvested and lysed in homogenization buffer containing 25 mM Tris-HCl, 1 mM EDTA, and 1 mM EGTA, according to the manufacturer's recommendation (Nitric Oxide Synthase Assay Kit, Calbiochem, San Diego, CA). NOS activity was measured after a 1-h reaction at 37°C in the presence of 3 μM BH₄, 1 μM FAD, 1 μM FMN, 25 μM NADPH, 15 μM CaCl₂ and 10 μCi L-[2,3,4,5-³H]arginine (MP Biomedicals, Irvine, CA) and separation of labeled citrulline from arginine by affinity chromatography.

Statistical analysis. Insulin secretion values are plotted on the graphs as means ± SEM. Each value represents the mean of n experiments always performed in quadruplicates. The data were analyzed using a Student's *t* test.

RESULTS

PIN is expressed in rat pancreatic islets and INS-1 cells. To investigate whether the endogenous protein inhibitor of nNOS, PIN, previously identified in rat brain (14), is, as nNOS, also present in rat pancreatic β -cells (27), we used the RT-PCR approach with primers based on the sequence of rat brain PIN (accession number U66461). As shown in Fig. 1A, a single band at the predicted size was obtained in islets of Langerhans as well as in INS-1 cells. Sequencing of this fragment revealed complete analogy with the sequence of rat brain PIN (data not shown). This result is consistent with the fact that PIN has been shown to be a highly conserved protein: a 90% sequence homology is observed between *Chlamydomonas*, *Caenorhabditis elegans*, *Drosophila*, and humans.

Expression of the PIN protein was confirmed in islets and INS-1 cells extracts by Western blotting with a monoclonal anti-PIN antibody (Fig. 1B). We identified a 10-kDa protein, displaying a molecular mass identical to that observed in brain and testis. Nevertheless, the brain PIN band appeared slightly higher than that in testis and islets, suggesting that different posttranslational modifications of the protein occurs in these tissues. The level of PIN expression in the islets and INS-1 cells is intermediate and lower than in testis and brain. These experiments provide evidence that pancreatic islets and INS-1 cells produce the PIN protein.

PIN is localized in insulin-secreting β -cells in association with nNOS. To confirm the presence of PIN in pancreatic β -cells, we performed double-immunofluorescence studies on isolated cells obtained from rat islets and on INS-1 cells using a monoclonal (Fig. 2A–C and G–I) and a polyclonal (Fig. 2D–F and J–L) anti-PIN antibody in combination with an anti-insulin antibody. These experiments clearly show that PIN is expressed in islet β -cells (Fig. 2A–F) as well as in INS-1 cells (Fig. 2G–L). PIN is also present in glucagon-secreting α -cells

and to a lesser extent in somatostatin-secreting δ -cells (data not shown). PIN staining was found to be cytoplasmic and displayed a punctuated aspect, identical for the two antibodies used. Furthermore, PIN was strongly colocalized with insulin, as shown by the appearance of a yellow color after merging the two fluorescences (Fig. 2C, F, I, and L). This strong colocalization suggests that PIN is associated with insulin secretory granules, as previously demonstrated for nNOS (27).

As PIN is able to interact with the N-terminal part of nNOS (14), we also studied the colocalization of the two proteins in islet β -cells (Fig. 3A–F) and in INS-1 cells (Fig. 3G–L). PIN staining was strongly associated with nNOS when using the monoclonal (Fig. 3A–C and G–I) or the polyclonal (Fig. 3D–F and J–L) antibodies, whereas nNOS was not exclusively colocalized with PIN (Fig. 3). This difference is due to the fact that nNOS is also strongly present in mitochondria (27). These results suggest that PIN is associated with nNOS at the level of the secretory granules.

To determine the subcellular localization of PIN, we performed electron microscopy on sections of pancreatic islets using the same set of anti-PIN antibodies (Fig. 4). A similar location was observed with the monoclonal (Fig. 4A and B) and the polyclonal (Fig. 4C–H) antibodies. In β -cells, PIN was mainly found to be associated with mature insulin secretory granules at the level of the dense core (large arrow head), the halo (thin arrow), and the membrane of the granules (large arrow) (Fig. 4A–C). Weak staining also appeared in less mature granules but only in association with the dense core (Fig. 4A, D, and E; empty arrow). A small part of PIN was also present in the cytoplasm (Fig. 4A; small arrow head), as well as in the mitochondria (data not shown). Interestingly, dimers of PIN could also be detected in the dense core (Fig. 4D; large arrow head), the halo (Fig. 4F; thin arrow), and the membrane (Fig. 4E; large arrow) of insulin secretory granules, suggesting that dimerization of PIN

occurs in β -cells as previously shown in the brain (19). Double staining of PIN and nNOS confirmed colocalization of both proteins at the level of insulin granules (Fig. 4G and H).

PIN overexpression induced an enhanced glucose-induced insulin secretion in INS-1 cells. To assess the physiological function of PIN on insulin secretion, we performed overexpression experiments in INS-1 cells. After 48 hours of transfection, we observed a clear increase in PIN mRNA by RT-PCR (Fig. 5A). In the presence of 5 mM glucose, insulin secretion was increased by 33% in cells overexpressing PIN as compared with cells transfected with the control vector ($P < 0.001$, $n = 6$) (Fig. 5B). To understand the mechanism by which PIN overexpression enhanced insulin secretion and as PIN has been shown to inhibit NO production (14), we first tested the effects of an NO donor, sodium nitroprusside (SNP) on β -cell function. NO is known to drastically decrease glucose-induced insulin secretion in the isolated perfused rat pancreas (32) through inhibition of key glycolytic (33, 34) and TCA cycle (35) enzymes. We first determined the dose of SNP able to decrease glucose-induced insulin secretion in INS-1 cells. The NO donor, at 3 and 30 nM, decreased insulin secretion by 21% ($P < 0.01$ versus glucose alone, $n = 3$) and 14% ($P < 0.05$ versus glucose alone, $n = 3$), respectively, an inhibition no longer observed for higher SNP concentrations (Fig. 5C). In PIN overexpressing cells, SNP (3 nM) failed to bring insulin secretion back to the level achieved with SNP in control cells ($P < 0.01$, $n = 3$) (Fig. 5B). We then investigated whether the PIN effect could also result from an increase in cytochrome c reductase activity of nNOS, previously shown to occur in pancreatic β -cells in the isolated perfused rat pancreas and to account for part of the stimulation of insulin secretion after pharmacological inhibition of nNOS by L-NAME (27). An inhibitor of nNOS-induced cytochrome c reductase activity, miconazole, at a dose of 10 μ M ($n = 6$), ineffective under control conditions (27), only slightly and non significantly reduced the increased secretion observed after overexpression of PIN (Fig. 5B).

To compare the effect of the protein inhibitor PIN with that of the pharmacological inhibition of nNOS on insulin secretion, we used L-NAME at a dose of 5 mM, previously shown not to produce non-specific depolarizing effects (36). Addition of L-NAME to 5 mM glucose induced a 65% increase in insulin release ($P < 0.001$ versus glucose alone, $n = 3$) (Fig. 6A). This effect was not reversed by the addition of increasing concentrations of SNP (3 nM to 300 μ M, $n = 3$) (Fig. 6A), but was drastically reduced by 95% in the presence of miconazole (10 μ M) ($P < 0.001$, $n = 3$) (Fig. 6B). The secretory effect induced by blockade of nNOS with L-NAME is thus related to an increased cytochrome c reductase activity of the enzyme, which, in contrast, accounts for only a minor part of the positive insulinotropic effect of PIN, suggesting that the nNOS inhibitors act through different mechanisms.

Therefore, we also studied the combined effects of PIN overexpression and L-NAME treatment in the presence of 5 mM glucose. In control cells, L-NAME again stimulated insulin secretion ($P < 0.001$ versus glucose alone, $n = 6$) and the inhibitor produced, in PIN overexpressing cells, a 38% increase ($P < 0.001$ versus glucose alone) (Fig. 7A). Thus, L-NAME remains able to amplify insulin release in cells overexpressing PIN (19% versus control cells with L-NAME, $P < 0.05$), bringing additional evidence that PIN and L-NAME exert their positive effects on insulin secretion through different mechanisms. To ascertain whether the two inhibitors target nNOS, we measured the effects of PIN overexpression and L-NAME either alone or when combined on nNOS catalytic activity in the presence of exogenous labeled arginine. PIN overexpression only slightly reduced (-10%, non significant, $n = 4$) nNOS activity, whereas a 31% inhibition was observed with L-NAME in control cells ($P < 0.01$) versus 48% in PIN transfected cells ($P < 0.01$) (Fig. 7B).

DISCUSSION

Our data clearly show that PIN, the protein inhibitor of nNOS, is expressed in rat pancreatic β -cells, as previously shown for nNOS (27), and positively modulates insulin secretion. Coincidence of nNOS and PIN also occurs in other tissues and has been reported in brain (16), kidney (18), and ventilatory muscles (17). nNOS is always coexpressed with its endogenous inhibitor, but, conversely, PIN, which is moderately expressed in all rat and human tissues (14, 15), does not necessarily colocalizes with nNOS. The relative level of expression of the two proteins differs depending on both tissues and species. In brain, nNOS is mainly expressed in the cerebellum, whereas PIN is essentially found in the cortex, the medulla, and the hippocampus (16). Likewise, nNOS expression varies among species, which is not the case for PIN, found to be relatively constant (17). Therefore, it might be assumed that the magnitude of nNOS catalytic activity in a tissue, is the result of the relative level of expression of both proteins.

In addition to coincidence of both proteins in pancreatic β -cells, immunofluorescence studies showed that PIN was strongly colocalized with nNOS, whereas the enzyme was not exclusively associated with the inhibitor. This discordance probably results from the fact that a part of nNOS is present in β -cell mitochondria, where PIN is only faintly expressed. In other tissues, PIN often colocalizes with nNOS in the intracellular compartments, as shown in kidney, where PIN is present in the apical membranes of the inner medullary collecting duct, a site where nNOS is strongly expressed (18). Colocalization of PIN and nNOS in pancreatic β -cells makes it possible for them to interact at the major site of nNOS localization, i.e., insulin secretory granules. This assumption is strongly supported by electron microscopy studies which showed that PIN, similarly to nNOS, is mainly associated with insulin secretory granules and that PIN-nNOS association is detected at the levels of the vesicles membrane

and the dense core as well. Concerning its membrane association, we hypothesize that PIN localizes outside the secretory granules, as PIN is also known to be the light chain of dyneins and myosin V (19, 20). Myosin Va has recently been shown to be expressed in pancreatic β -cells, where it is implicated in the transport of dense core secretory vesicles (37). Thus, it is tempting to assume that PIN is associated on the one hand with myosin Va and on the other hand with nNOS at the level of insulin secretory granules.

The physiological role played by PIN is yet unknown. One function is the modulation of nNOS catalytic activity to prevent deleterious overproduction of NO. Indeed, it has been reported that a cyclooxygenase-induced increase of PIN expression is, through an inhibition of the production of superoxide and NO by nNOS, able to prevent nerve growth factor-deprived PC12 cells from apoptosis (38). Concerning pancreatic β -cells, PIN overexpression resulted in a clear increase in insulin secretion, bearing evidence that this highly conserved protein is involved in the stimulus-secretion coupling of β -cell. The mechanism involved is, however, less tightly coupled to nNOS catalytic activity. Indeed, the NO donor SNP reduced insulin secretion in control cells but only poorly overcame the effect of PIN overexpression. Furthermore, *in vitro*, a strong increase in PIN mRNA did not significantly affect nNOS catalytic activity. Taken together, these data suggest that the effect of PIN might occur at a more distal level. Interestingly, PIN is also present in dyneins and myosin V, that are respectively microtubules and microfilaments-based molecular motors, and, as such, is involved in the trafficking of organelles and the association of intracellular proteins with the cytoskeleton. Thus, PIN has been shown to be essential for the retrograde particle movement of intraflagellar transport in *Chlamydomonas reinhardtii* (39). In addition, I κ B, the inhibitor of the transcription factor NF κ B, binds to PIN, allowing cytosolic retention of NF κ B until a signal dissociates the complex, enabling NF κ B nuclear translocation (22). Likewise, association of the complex between PIN and 53BP1 (the p53 binding protein 1) with dynein

prevents dynein-dependent nuclear translocation of p53 induced by DNA damage (40). Moreover, microtubule-associated PIN interacts with the 3'-terminal part of parathyroid hormone mRNA, thus targeting mRNA to specific cytoplasmic areas, with as a result, a cellular asymmetric expression of the protein (41). In pancreatic β -cells, PIN could thus be involved in the migration of insulin granules as suggested by expression of myosin Va and by data from our electron microscopic studies.

Additional evidence for the role of PIN in intracellular trafficking events also finds support in data we obtained using L-NAME. In previous studies, we could show that the non-metabolizable analog is able to increase the molecular interaction between nNOS and cytochrome c as well as nNOS-related cytochrome c reductase activity. We now confirm such an effect in INS-1 cells using miconazole, the inhibitor of nNOS-related cytochrome c reductase activity. Reduction of cytochrome c by nNOS, that results from a direct interaction between the two proteins, has been shown to be KCN insensitive (42), which is in line with the inability of SNP to affect L-NAME insulinotropic effect. As reduced cytochrome c is a well known electron transporter for mitochondrial ATP production, it might be assumed that the effect of L-NAME could be triggered by ATP generated through a mechanism not directly related to glucose metabolism. Increased ATP generation could thus be involved in the reinforcement by L-NAME of the PIN overexpression effect, as a fuel for ATP-driven molecular motors. Such an assumption is also supported by the inability of the natural analog of L-NAME, L-arginine, to strengthen the PIN effect (data not shown), as the positively charged amino acid is known to depolarize pancreatic β -cells via biophysical events not related to ATP production (43).

In summary, taken together, the data we obtained by immunofluorescence and electron microscopic studies as well as in functional experiments clearly point to an effect of PIN in the intracellular trafficking of insulin secretory granules. As a matter of fact, PIN and nNOS

clearly interact in the insulin secretory granules, and such events also occur at the vesicle membrane. Since PIN is the light chain of dynein and myosin V, it is tempting to assume that PIN and nNOS, as two specific interacting partners, interfere with other multiprotein complexes of the exocytotic machinery.

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FIGURE LEGENDS

FIG. 1. Analysis of PIN expression in rat islets and INS-1 cells by RT-PCR and Western blotting. (A) Total RNA from islets and INS-1 cells was isolated, and cDNA was amplified with primers based on the sequence of the PIN. (-RT) is a negative control for PCR preparation performed without cDNA. β_2 -microglobulin (β_2m) primers were used as positive controls. Molecular markers (MM) show bands corresponding to fragments of 2000, 1200, 800, 400, 200, and 100 bp. (B) Proteins from a testis-, an INS-1 cell-, an islet-, and a brain extract were separated by 14% tricine polyacrylamide gel, transferred to a nitrocellulose membrane, and incubated with a monoclonal anti-PIN antibody. Immunoreactivity was detected by a chemiluminescence kit.

FIG. 2. Localization of PIN in isolated β -cells from rat islets and in INS-1 cells by double immunofluorescence. Isolated β (A–F) and INS-1 cells (G–L) were double labeled with an anti-insulin and a monoclonal (A–C; G–I) or a polyclonal (D–F; J–L) anti-PIN antibody, and fluorescence was analyzed by dual-channel confocal microscopy. Insulin and PIN stainings appear respectively as green (A, D, G, and J) and red (B, E, H, and K) fluorescence. Coincidence of both fluorescences results in the appearance of a yellow color (C, F, I, and L). The scale bars are 10 μ m.

FIG. 3. Localization of PIN and nNOS in isolated β -cells from rat islets and in INS-1 cells by double immunofluorescence. Isolated β (A–F) and INS-1 cells (G–L) were double labeled with an anti-nNOS and a monoclonal (A–C; G–I) or a polyclonal (D–F; J–L) anti-PIN antibody and fluorescence was analyzed by dual-channel confocal microscopy. PIN and nNOS stainings appear respectively as green (A, D, G, and J) and red (B, E, H, and K)

fluorescence. Coincidence of both fluorescences results in the appearance of a yellow color (*C, F, I, and L*). The scale bars are 10 μm .

FIG. 4. Subcellular localization of PIN in β -cells by electron microscopy performed on sections of isolated rat islets. *A to C* shows part of a β -cell rich in insulin secretory granules at different stages of maturation labeled with an anti-insulin antibody coupled to 5-nm gold particles and the monoclonal (*A, B*) and polyclonal (*C*) antibodies coupled to 10-nm (*A*) or 15-nm (*B, C*) gold particles. PIN staining is associated with mature insulin secretory granules at the level of the dense core (large arrow head), the halo (thin arrow), and the membrane of the granule (large arrow). PIN is also present in the cytoplasm (*A*; small arrow head) and in the dense core of less mature granules (*A, D, and E*; empty arrow). *D to F* shows dimers of PIN detected at the levels of the dense core (*D*; large arrow head), the halo (*F*; thin arrow), and the membrane (*E*; large arrow) of the granules. (*G, H*) shows double staining of PIN (15-nm particles) and nNOS (10-nm particles). Colocalization of both proteins occurs at the level of the dense core (*G*; large arrow head) and the membrane (*H*; large arrow) of the granules. Magnification is $\times 60,000$.

FIG. 5. Effect of PIN overexpression on glucose-induced insulin secretion in INS-1 cells. (*A*) Analysis of PIN overexpression by RT-PCR after a 48-hour transfection of INS-1 cells with an expression vector containing or not PIN cDNA (PIN/Control). (-RT) is a negative control for PCR preparation performed without cDNA. β_2 -microglobulin ($\beta_2\text{m}$) primers were used as positive controls. Molecular markers (MM) show bands corresponding to fragments of 2000, 1200, 800, 400, 200, and 100 bp. (*B*) Effect of PIN overexpression on glucose-induced insulin secretion in the presence or absence of an NO donor, sodium nitroprusside (SNP) or the inhibitor of cytochrome c reductase activity, miconazole, in INS-1 cells. Insulin secretion was

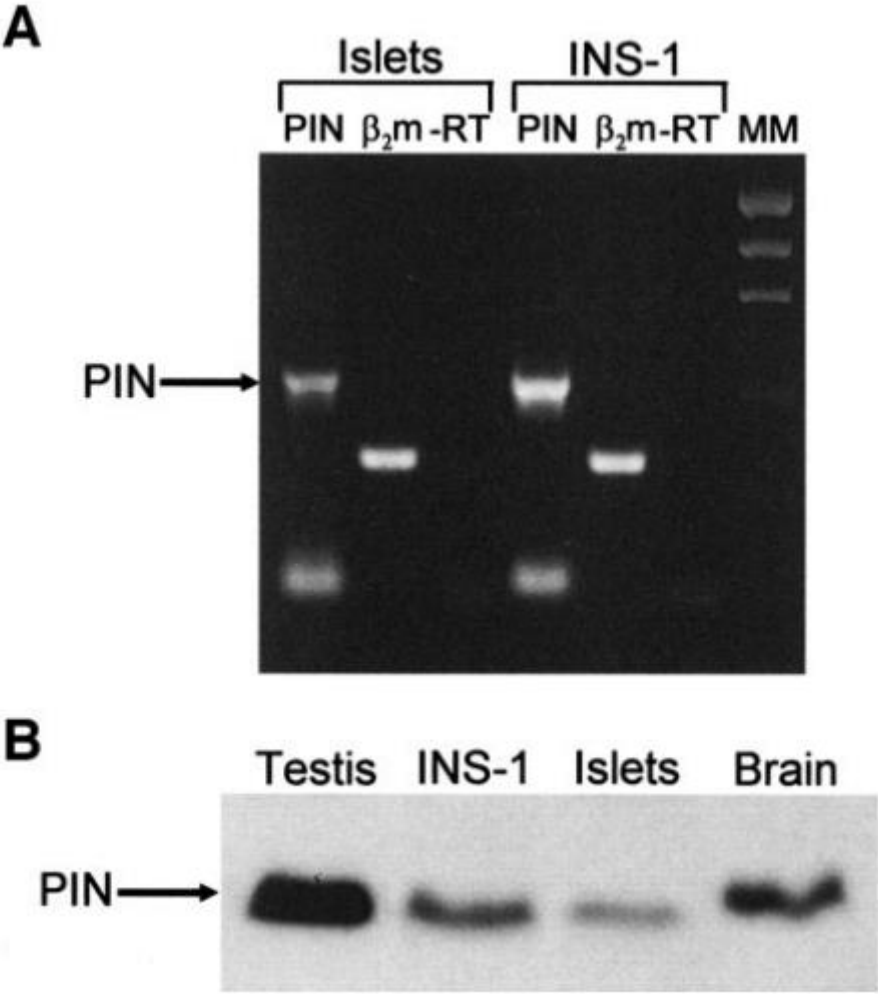
measured after incubation of transfected INS-1 cells (with PIN cDNA (PIN) or the empty vector (Control)) for 1 h in the presence of 5 mM glucose (G5) alone or in combination with 3 nM SNP or 10 μ M miconazole (Mico). (C) Effect of increasing concentrations of SNP on glucose-induced insulin secretion. Insulin secretion was measured after incubation of non-transfected INS-1 cells for 1 h in the presence of 5 mM glucose (G5) with or without SNP (3 nM – 300 μ M). (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, and NS non significant).

FIG. 6. Effect of L-NAME on glucose-induced insulin secretion in INS-1 cells. (A) Effect of L-NAME on glucose-induced insulin secretion in the absence or presence of increasing concentrations of the NO donor, sodium nitroprusside (SNP). Insulin secretion was measured after incubation of non-transfected INS-1 cells for 1 h in the presence of 5 mM glucose (G5) with or without 5 mM L-NAME \pm SNP (3 nM – 300 μ M). (B) Effect of the inhibitor of cytochrome c reductase activity, miconazole, on the potentiation of glucose-induced insulin provoked by L-NAME. Insulin secretion was measured after incubation of non-transfected INS-1 cells for 1 h in the presence of 5 mM glucose (G5) with or without 5 mM L-NAME and 10 μ M miconazole (Mico). (*** $P < 0.001$).

FIG. 7. Combined effect of PIN overexpression and L-NAME on glucose-induced insulin secretion in INS-1 cells and on nNOS catalytic activity. (A) Effect of PIN overexpression on insulin secretion induced by glucose alone or in combination with L-NAME. Insulin secretion was measured after incubation of transfected INS-1 cells (with PIN cDNA (PIN) or the empty vector (Control)) for 1 h in the presence of 5 mM glucose (G5) and 5 mM L-NAME. (B) Effect of PIN overexpression with or without L-NAME on nNOS catalytic activity. nNOS catalytic activity was measured for 1 h on INS-1 extracts obtained after incubation for 1 h of transfected cells (with PIN cDNA (PIN) or the empty vector (Control)) in the presence of 5

mM glucose (G5) with or without 5 mM L-NAME. (** $P < 0.001$, * $P < 0.01$, * $P < 0.05$, and NS non significant).

Figure 1



Figurte 2

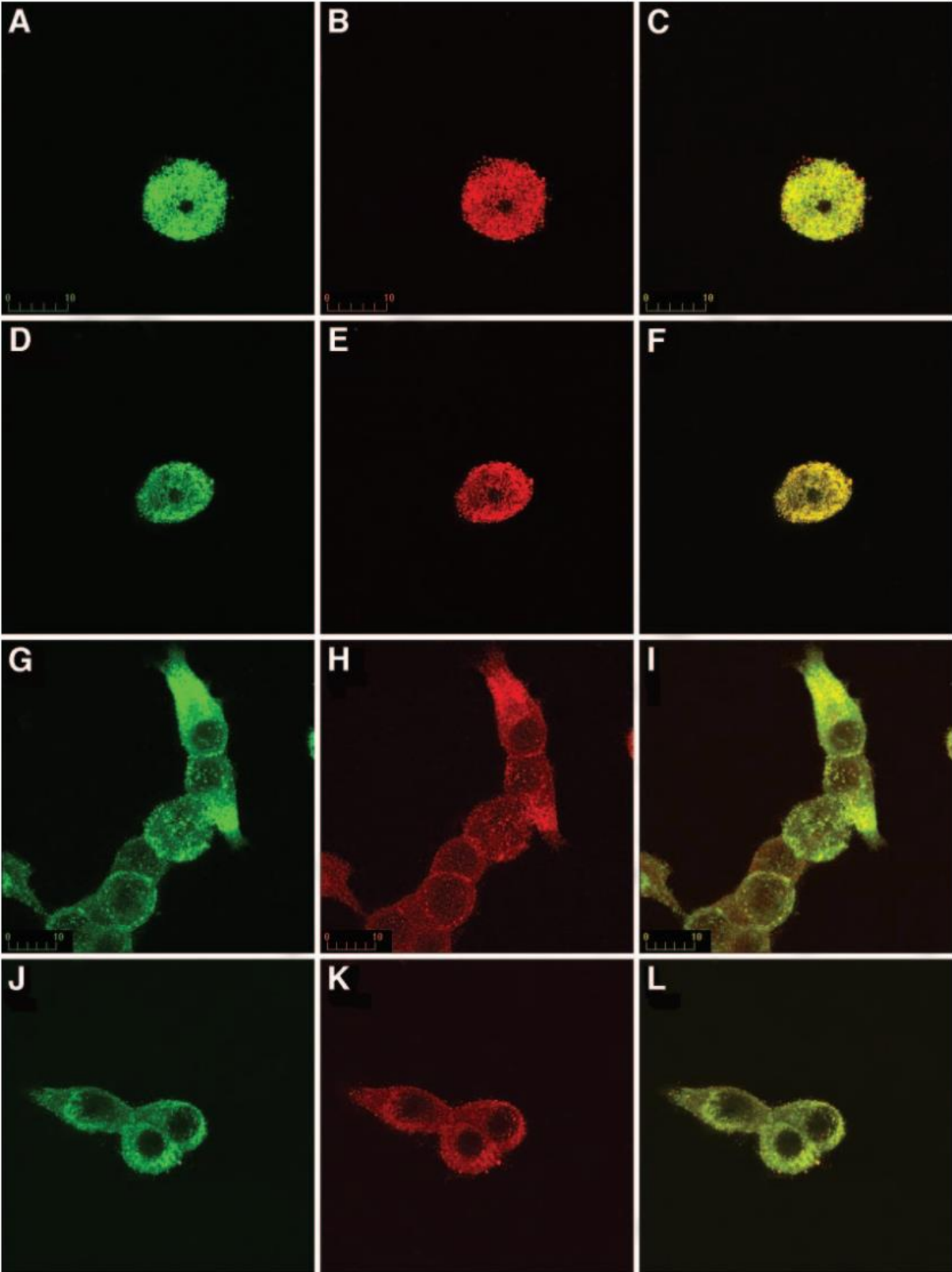


Figure 3

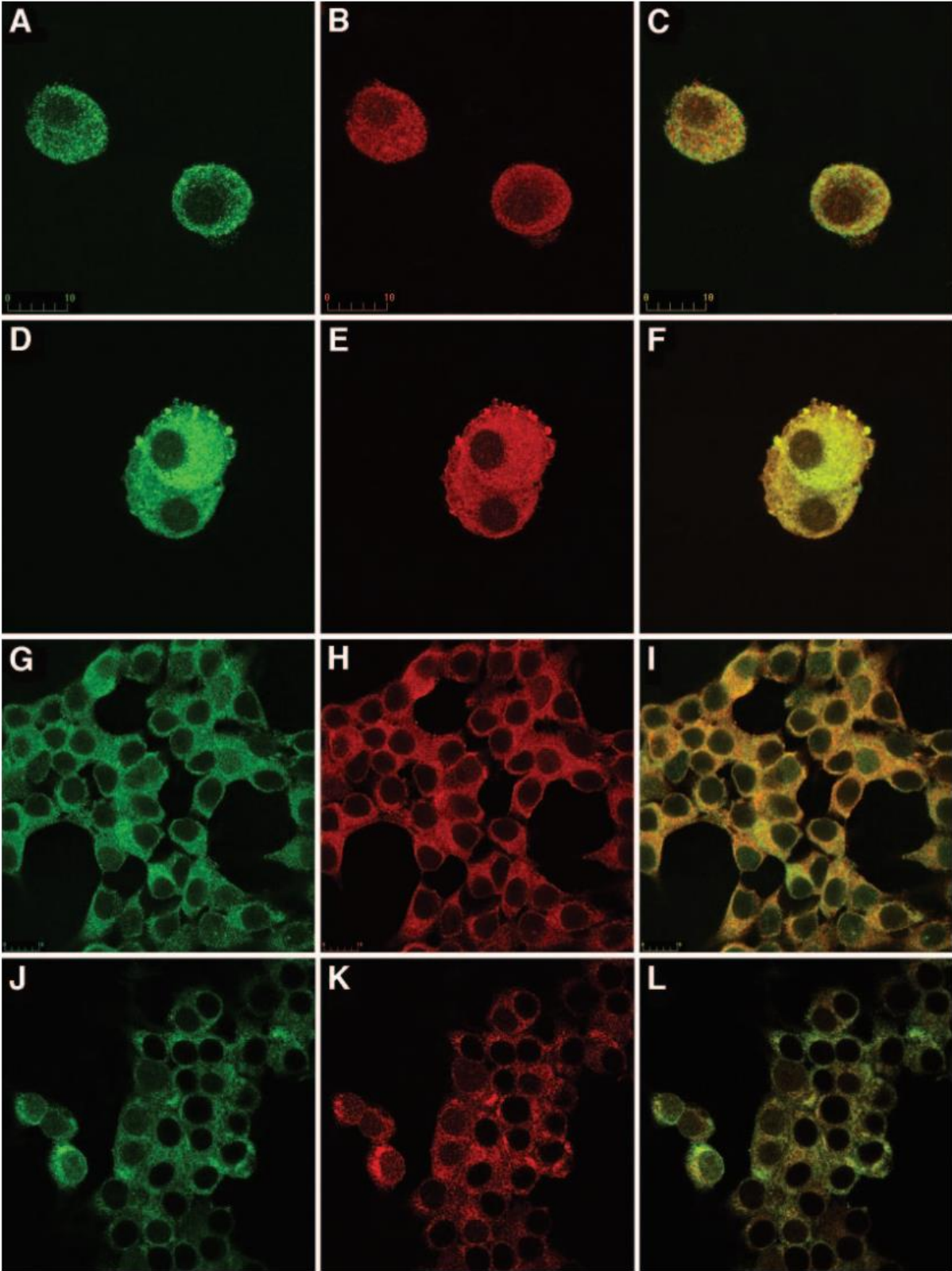


Figure 4

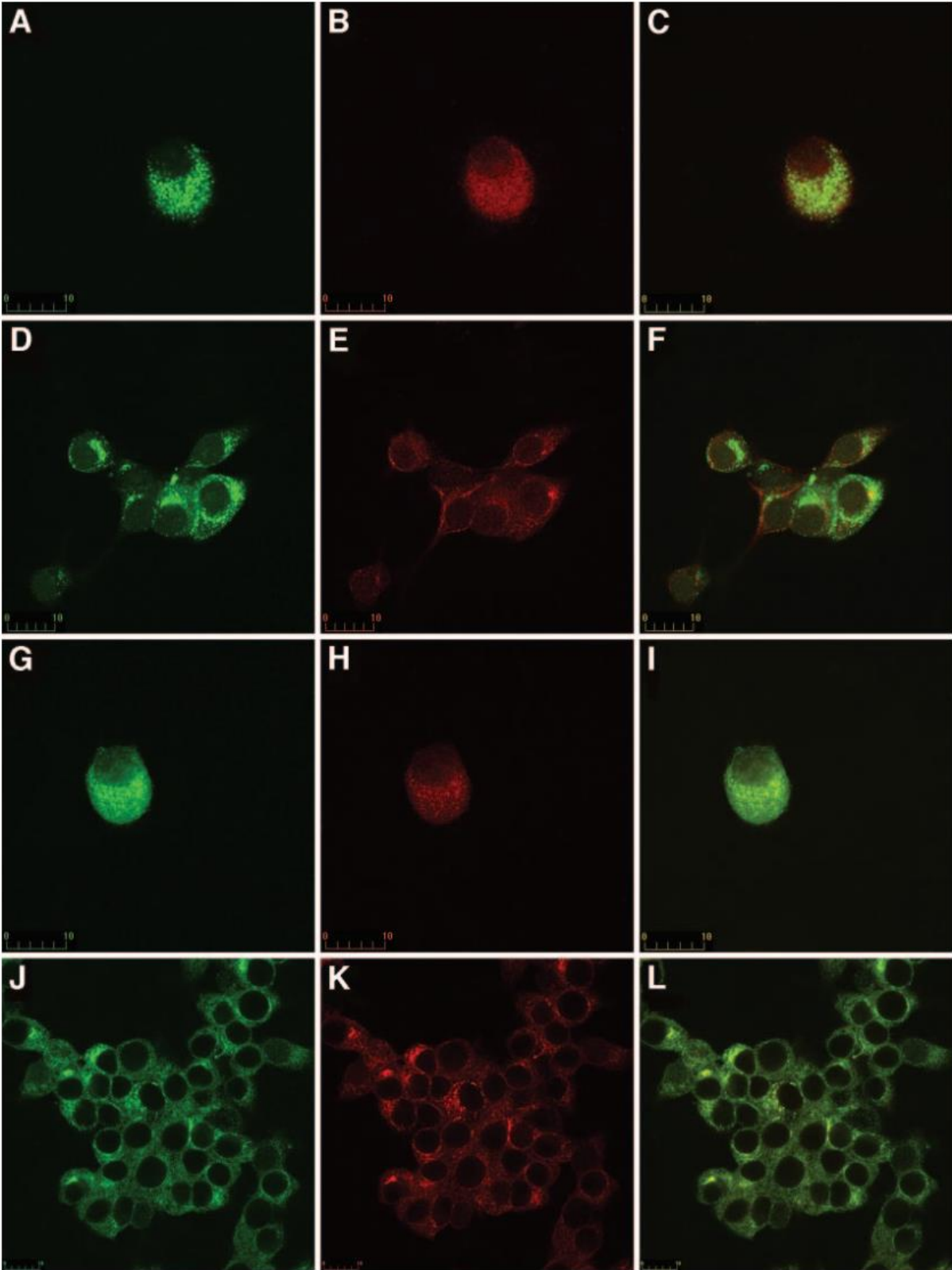


Figure 5

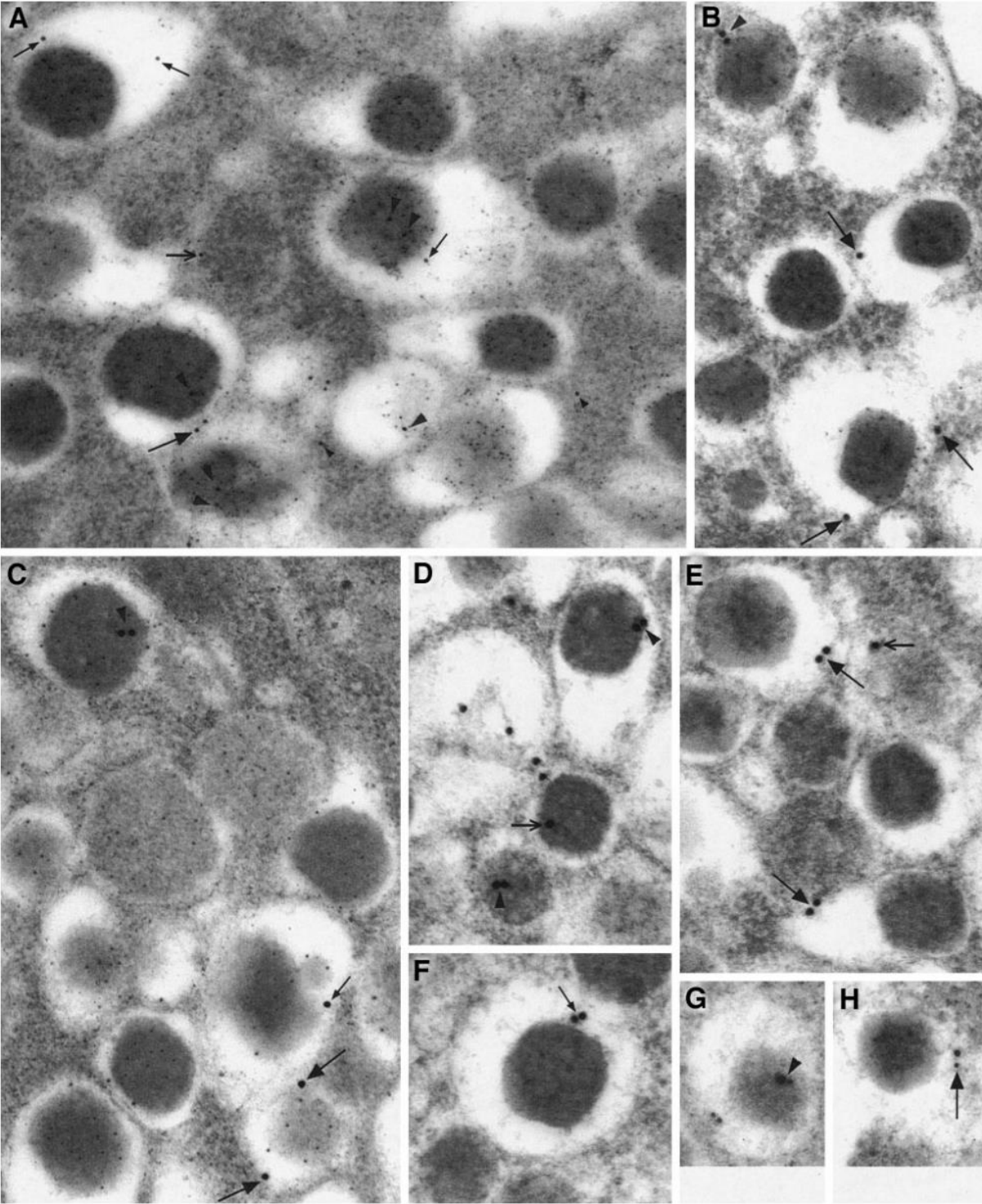


Figure 6

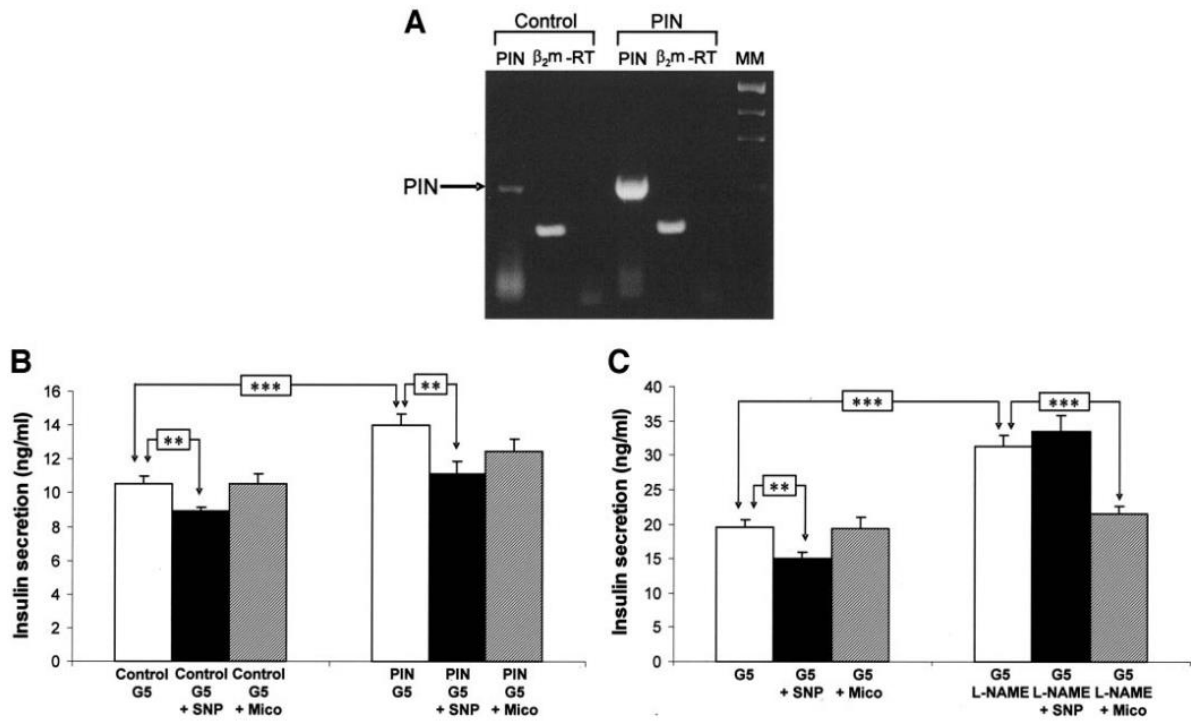


Figure 7

