Characterization of a G Protein-activated Phosphoinositide 3-Kinase in Vascular Smooth Muscle Cell Nuclei*

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Recent studies highlight the existence of an autonomous nuclear polyphosphoinositide metabolism related to cellular proliferation and differentiation. However, only a few data document the nuclear production of the putative second messengers, the 3-phosphorylated phosphoinositides, by the phosphoinositide 3-kinase (PI3K). In the present paper, we examine whether GTP-binding proteins can directly modulate 3-phosphorylated phosphoinositide metabolism in membrane-free nuclei isolated from pig aorta smooth muscle cells (VSMCs). In vitro PI3K assays performed without the addition of any exogenous substrates revealed that guanosine 5'-S-(β-thio)triphosphate (GTPγS) specifically stimulated the nuclear synthesis of phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3), whereas guanosine 5'-S-(β-thio)diphosphate was ineffective. PI3K inhibitors wortmannin and LY294002 prevented GTPγS-induced PtdIns(3,4,5)P3 synthesis. Moreover, pertussis toxin inhibited partially PtdIns(3,4,5)P3 accumulation, suggesting that nuclear Gαi/Go proteins are involved in the activation of PI3K. Immunoblot experiments showed the presence of Gαo proteins in VSMC nuclei. In contrast with previous reports, immunoblots and indirect immunofluorescence failed to detect the p85α subunit of the heterodimeric PI3K within VSMC nuclei. By contrast, we have detected the presence of a 117-kDa protein immunologically related to the PI3K. These results indicate the existence of a G protein-activated PI3K inside VSMC nucleus that might be involved in the control of VSMC proliferation and in the pathogenesis of vascular proliferative disorders.

Vascular smooth muscle cells (VSMCs) play a central role in the fibroproliferative response during the development of atherosclerosis and of restenosis after angioplasty (1, 2). Recently, we have demonstrated that phosphoinositide 3-kinase (PI3K) was essential for the progression of VSMCs throughout the G1 phase of the cell cycle (3), implying that a better understanding of the PI3K signaling pathway might be of pathophysiological relevance. PI3K phosphorylates the D3 position of the inositol phosphoinositides (PI) to generate the putative second messengers PtdIns(3,4)P2 and PtdIns(3,4,5)P3 (4). In addition to proliferation, the PI3K products have also been involved in cell transformation, apoptosis, vesicle trafficking, and cytoskeleton organization (5, 6).

Three distinct classes of PI3Ks have now been identified on the basis of their in vitro substrate specificity, structure, and mode of regulation (7, 8). The most studied are class I PI3Ks, which in vitro phosphorylate PtdIns, PtdIns(4)P, and PtdIns(4,5)P2, and display a preference for PtdIns(4,5)P2 in vivo. Class I PI3Ks form a heterodimeric complex and are subdivided according to the adaptor protein associated with the catalytic subunit. Class IA PI3Ks consist of a 110-kDa catalytic subunit (p110α, β, δ) (9) and a 85-kDa adaptor protein (p85α, β, δ) (10) containing Src homology 2 (SH2) domains that link them to tyrosine kinase signaling. In contrast, class IB PI3K or PI3Kβ defines a G protein-coupled receptor-regulated PI3K (11). It is made of a p110γ catalytic subunit and a p101 regulatory subunit unrelated to p85 (12). The p110γ can be activated in vitro by both the α and βγ subunits of heterotrimeric G proteins (11–13). This stimulation is considerably enhanced by the p101 adaptor (12).

Moreover, there is now considerable evidence that a nuclear PI cycle, apart from that occurring in the plasma membrane, is involved in the regulation of nuclear functions (14). Indeed, it has been demonstrated that nuclei contain almost all the enzymes involved in the classical PI cycle, including kinases required for the synthesis of PtdIns(4,5)P2, phospholipases C, and diacylglycerol kinase (15, 16). Furthermore, specific changes in the nuclear levels of PI have been implicated in both cell growth and differentiation (17–19). To date, information concerning the role and the regulation of nuclear PI3Ks are still very limited. Immunocytochemical and biochemical analyses demonstrate the presence of the p85a regulatory subunit in the nuclei of rat and human cells (20–22) and the growth factor-dependent nuclear translocation of the p110β catalytic subunit in osteoblast-like cells (23), suggesting that class IA PI3Ks exist in the nucleus. Recently, a study based on the immunolocalization of epitope-tagged p110y in HepG2 cells reported that PI3Kγ translocates to the nucleus after serum stimulation (24). Since a nuclear G protein-regulated PI3K activity has not yet been demonstrated, we investigated whether GTP-binding proteins directly modulate 3-phosphorylated phosphoinositide (3-PI) metabolism in membrane-free nuclei isolated from pig...
aorta VSMCs. Our data provide the first evidence for the existence of a pertussis toxin (PTX)-sensitive PI3K inside VSMC nucleus. This enzyme, which might be related to PI3Kγ, phosphorylates an intranuclear pool of PtdIns(4,5)P_2, suggesting that nuclear PtdIns(3,4,5)P_2 may regulate protein kinases in vascular proliferative disorders.

EXPERIMENTAL PROCEDURES

Chemicals and Antibodies—All culture reagents were obtained from Life Technologies Inc. U73122, wortmannin, and LY294002 were obtained from Biomol (Plymouth Meeting, PA). GTPγS, GDPβS, and RNase-free DNase I were from Boehringer-Mannheim. [γ-32P]ATP (3000 Ci/mmol) was purchased from PerkinElmer Life Sciences. Monovalent anti-p110γ, fluorescein isothiocyanate-conjugated anti-rabbit antibodies, and the enhanced chemiluminescence (ECL) system were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Polyclonal anti-p110γ was kindly provided by S. Roche. Specific polyclonal anti-Gαo antibodies directed against the last 10 amino acids of the common carboxyl-terminal sequence of the Gαo and G Gα(o)-C(ter) or against the amino acid 291–302 of the α subunit of Gαo (anti-Gαoα) were obtained and characterized as previously described (25). Horseradish peroxidase-conjugated anti-rabbit/mouse antibodies and polyclonal anti-p85α antibody were, respectively, sc-18 and sc-208 obtained from Biodesign International. GoQαo antisera was a generous gift of A. Yart and F. Raynal.

Cell Culture and Isolation of VSMC Nuclei—VSMCs were prepared from 6-week-old pig thoracic aorta using the explant technique (26) and cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, as previously described (27). For all the experiments, VSMCs were used from the third to the sixth passage.

Growing VSMCs were washed twice with ice-cold calcium- and magnesium-free PBS and once with a hypotonic buffer containing 5 mM Tris-HCl, 1.5 mM KCl, 2.5 mM MgCl_2, pH 7.4. All subsequent procedures were carried out at 4 °C. Medium was then switched to hypotonic buffer supplemented with 200 mM Na_3VO_4, 1 mM NaF, 1 mM EDTA, 1 mM EGTA, 100 μM phenylmethylsulfonylfluoride, 10 μg/ml each aprotinin, benzamidin, and leupeptin for 1 min, and VSMCs were lyzed by the addition of 1% Nonidet-P40 and 1% deoxycholic acid. Cells were allowed to swell for 1 min and were sheared by three passages through a 25-gauge needle. The cell lysate was layered over a 0.3M/2M sucrose discontinuous gradient and was centrifuged at 500g_25 for 1 min, and were sonicated (10 kHz for 30 s) using an ultrasonic cell disrupter (Branson Sonifier 250) and treated for 1 h at 4 °C with 10 units/1.5 ml of RNase-free DNase I, for 15 min in 0.1 M PBS containing 100 μM GTPγS, 100 μM GDPβS, and 10 mM dithiothreitol, 50 mM NaCl, 4 mM MgCl_2, 200 μM Na_3VO_4, 1 mM NaF, and proteases inhibitors, as above. As to the yield of the nuclear isolation, an average of 0.5 × 10^6 nuclei were obtained from 1 × 10^6 cells. 1 × 10^6 and 1 × 10^5 nuclei contained 300 μg and 30 μg of proteins, respectively.

Lipid Kinase Assay—Nuclei were disrupted by sonication (10 kHz for 3 × 1 s) using an ultrasonic cell disrupter (Branson Sonifier 250) and treated for 1 h at 4 °C with 10 units/L/10^6 nuclei RNase-free DNase I. All assays were conducted in a final volume of 100 μl of assay buffer containing 5 × 10^6 nuclei, 1 μM thapsigargin (ATPase inhibitor), and 5 μM U73122 (phospholipase C inhibitor). For experiments in the presence of PI3K inhibitors and a Gαo/γ inhibitor, nuclei were reincubated with 20 nM wortmannin or 10 μM LY294002 and 5 μg/ml pertussis toxin, respectively, for 15 min on ice. When indicated, 100 μM GTPγS or 100 μM GDPβS was added for another 15 min. The assays were then started by the addition of 1 μM ATP (10 μl) containing 65 μCi of [γ-32P]ATP, and the 32P incorporation was allowed for 15 min at 30 °C under shaking. For exogenous lipid phosphorylation, 30 μl of lipid vesicles containing 100 μM PtdIns and 200 μM phosphatidylserine were added 5 min before starting the assay. Reaction was stopped by addition 1360 μl of 1 N HCl, 0.5% Triton X-100, 300 μl of 2 N HCl, and 280 μl of 200 mM EDTA. Lipids were immediately extracted after the modified procedure of Bligh and Dyer (28). Lipids were then analyzed either on oxalate-coated thin-layer chromatography plates (Silica Gel 60, Merck) developed in isopropanol:acetic acid:H_2O (65:13.4) or by HPLC on a Partisphere SAX column (Whatman International Ltd, U. K.) after deacetylation, as previously described (29). The synthesis of radioactive standard PtdIns(3)P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3 was performed from specific anti-phosphatidic acid, suggesting a residual PLC activity, but we never detected radiolabeled 3-PI. To look for the possible presence of a G protein-regulated PI3K (11–13), we next tested whether 100 μM GTPγS, a nonhydrolyzable GTP analogue, could trigger the production of 3-PI in isolated nuclei.

RESULTS

Purity of VSMC Nuclear Preparations—Membrane-depleted nuclei from pig aorta VSMCs were isolated by hypotonic shock combined with detergents. The purity of nuclear preparations was evaluated by biochemical and immunochemical analyses. Lactate dehydrogenase and 5'-nucleotidase activities, recognized as markers for cytoplasm and plasma membrane were, respectively, found to be 0.29 ± 0.12% (n = 3) and 0.18 ± 0.08% (n = 3) of the activity in the total homogenate. Furthermore, Western blot analysis using anti-tubulin antibody showed the absence of immunoreactivity to the cytoskeletal proteins in the purified nuclei (Fig. 1A). In addition, the nuclear fraction was highly enriched with phosphatidylinositol phospholipase C (Fig. 1B). Finally, electron microscopy analysis confirmed that the isolation procedure yielded nuclei of high purity (Fig. 1C). No appreciable morphological change of the nuclei was noted during the isolation procedure, the nuclear structure was maintained, and the lysis procedure completely removed the nuclear envelope to leave the naked lamellar structure and nuclear pore remnants (Fig. 1C, right panel).

VSMC Nuclei Contain a GTP-dependent PI3K Activity—Previous studies suggest the existence of a nuclear PI3K pathway (20–24), but the intranuclear location and regulation of a PI3K activity has not clearly been demonstrated. Therefore, we first investigated whether membrane-free nuclei were able to produce 3-PI from endogenous precursors. We assessed nuclear PI3K activity in vitro by phosphate incorporation from [γ-32P]ATP into inositol lipids. All assays were conducted in the presence of 2 mM EGTA and 5 μM U73122, a phospholipase C (PLC) inhibitor, so that nuclear PLC activity would not interfere with PI3K activity.

As shown in Fig. 2A (upper panel), HPLC analyses revealed a peak of (32P)phosphatidic acid, suggesting a residual PLC activity, but we never detected radiolabeled 3-PI. To look for the possible presence of a G protein-regulated PI3K (11–13), we next tested whether 100 μM GTPγS, a nonhydrolyzable GTP analogue, could trigger the production of 3-PI in isolated nuclei.

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(Fig. 2A, middle panel). Under this condition, we detected the synthesis of only one PI3K product, the PtdIns(3,4,5)P3, and the incorporation into [32P]PtdIns(3,4,5)P3 was 49073 ± 2000 cpm/107 nuclei (n = 3) (Fig. 3A). This peak coincided with the HPLC profile of pure [32P]PtdIns(3,4,5)P3 used as a control (Fig. 2B). In addition, the [32P]phosphatidic acid peak was always present, and we never observed the production of any other inositol lipids, especially PtdIns(3)P and PtdIns(3,4)P2. These data strongly suggest that a nuclear GTP-dependent PI3K is selective for nuclear PtdIns(4,5)P2 or that PtdIns and PtdIns(4)P present in the nuclear membrane were removed during nuclei isolation. To address this question, vesicles containing PtdIns/phosphatidylycerine (100 µM/200 µM, final concentrations) were added to nuclear PI3K assays, and the formation of [32P]PtdIns(3,4,5)P3 synthesis was [117-kDa PI3K but Not p85α Is Expressed Inside VSMC Nuclei—We next performed immunoblot experiments to iden-
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Fig. 3. Effects of PI3K inhibitors on GTPγS-induced nuclear PtdIns(3,4,5)P3 synthesis. Membrane-depleted nuclei were isolated, and nuclear lipid kinase activity was assayed as described in Fig. 2. Nuclei were pretreated for 15 min with 20 nM wortmannin or 10 μM LY294002 before incubation with 100 μM GTPγS. A, lipids were extracted and analyzed by HPLC. Quantification of [32P]PtdIns(3,4,5)P3 synthesis is expressed as cpm/10^7 nuclei. Data for GTPγS alone are the mean ± S.E. from three independent experiments. B, lipids were extracted and analyzed by TLC. The data shown are representative of two different experiments. The position of standard radioactive 3-PI is shown as the control. P13P, PtdIns(3)P; P13,4,P2, PtdIns(3,4)P2; P1P, PtdIns(4)P; P13,4,5,P3, PtdIns(3,4,5)P3.

Fig. 4. Exogenous PtdIns phosphorylation by nuclear GTP-dependent PI3K. Membrane-depleted nuclei were isolated, and nuclear lipid kinase activity was assayed in the presence of exogenous lipids. Nuclei (N) were preincubated in the absence (A, upper panel, and B) or in the presence (A, lower panel, and B) of 100 μM GTPγS. Vesicles of PtdIns-phosphatidylserine at the final concentrations 100 μg/200 μg were added 5 min before starting the reaction. A, representative HPLC profiles of three different experiments. B, quantification of [32P]PtdIns(3)P synthesis is expressed as cpm/10^7 nuclei. Data are the mean ± S.E. from three independent experiments. The asterisk indicates significant difference using the Student’s t test (p < 0.01). PA, phosphatidic acid; PIP, inorganic phosphate; P13P, PtdIns(3)P; P14P, PtdIns(4)P; P15P, PtdIns(3,4,5)P3.

Nuclei were pretreated for 15 min with 20 nM wortmannin or 10 μM LY294002 before incubation with 100 μM GTPγS. A, lipids were extracted and analyzed by HPLC. Quantification of [32P]PtdIns(3,4,5)P3 synthesis is expressed as cpm/10^7 nuclei. Data for GTPγS alone are the mean ± S.E. from three independent experiments. B, lipids were extracted and analyzed by TLC. The data shown are representative of two different experiments. The position of standard radioactive 3-PI is shown as the control. PI3P, PtdIns(3)P; PI3,4,P2, PtdIns(3,4)P2; PI1P, PtdIns(4)P; PI3,4,5,P3, PtdIns(3,4,5)P3.

Having shown that a nuclear PI3K phosphorylates exogenous PtdIns in the absence of GTPγS and considering that p85α has been reported in rat liver nuclei (20), we checked whether class IA PI3Ks would also be present in VSMC nuclei (Fig. 6). We used an anti-p85α antibody to detect this adaptor protein and loaded the same amount of proteins (15 μg) from cell homogenate or from purified nuclei (Fig. 6A). In contrast to previous reports, p85α was undetectable in VSMC nuclei when compared with total cell homogenates, although 10 times as much nuclei were assayed. The absence of nuclear p85α expression was further confirmed by indirect immunofluorescence microscopy. As shown in Fig. 6B, the p85α labeling was evident as a ring at the perinuclear region and as fluorescent dots in the cytoplasm and the plasma membrane, whereas the nuclear interior remained unstained. These results strongly suggest that the heterodimeric PI3K p85α/p110 is absent from our nuclear preparations but could be present in the nuclear envelope, whereas a 117-kDa PI3K γ-like kinase is, significantly, located inside VSMC nuclei.

The Nuclear GTP-dependent PI3K Could Be Regulated by G Proteins—We next sought to determine whether nuclear heterotrimeric G protein Gαi proteins could be responsible for the nuclear PI3K activation by using PTX. Pretreatment of isolated nuclei with 5 ng/ml PTX inhibited about 50% of the GTPγS-induced PI3K activity (Fig. 7A). Moreover, to identify PTX-sensitive G proteins inside VSMC nuclei, we performed immunoblots using specific antibodies directed against both Gαi1/Gαi2 subunits (anti-Gαi1(C-ter)) or against the α subunit of Gαi3 (anti-Gαi3). As shown in Fig. 7B, a protein of 42–43 kDa is recognized by both antibodies in our nuclear preparations. These results suggest...
that PTX-sensitive G proteins are present in VSMC nuclei and could be involved in nuclear PI3K activation.

**DISCUSSION**

In the present study, we provide the first evidence that a GTP-dependent PI3K generates the second messenger PtdIns(3,4,5)P3 from a pre-existing nuclear pool of PtdIns(4,5)P2, directly within VSMC nucleus. Furthermore, we showed that this PI3K activity, which could be related to a PI3Kγ, is coupled to nuclear G\(_{i}\)/G\(_{0}\) heterotrimeric G proteins.

**Fig. 5. Expression of PI3K\(_{\gamma}\) in VSMCs.** A, proteins from \(1 \times 10^5\) whole VSMCs (lane 1) and \(1 \times 10^5\) purified nuclei (lane 2) were fractionated on 7.5% SDS-PAGE, transferred, and probed with monoclonal anti-p110\(_{\gamma}\) antibody. As a positive control, proteins from \(1 \times 10^5\) COS-7 cells overexpressing human p110\(_{\gamma}\) were also probed (lane 3). The specificity was tested by probing proteins from \(1 \times 10^5\) COS-7 cells overexpressing human p110\(_{\alpha}\) (lane 4) and proteins from \(1 \times 10^5\) VSMC nuclei without the primary antibody incubation (lane 5). B, proteins from VSMC purified nuclei (1 \(\times 10^5\) to 5 \(\times 10^5\) nuclei) were immunoblotted with polyclonal anti-p110\(_{\gamma}\) antibody. The data are from a single experiment representative of at least three others.

**Fig. 6. Expression of p85\(_{\alpha}\) in VSMCs.** A, Western blotting analysis of p85\(_{\alpha}\). 15 \(\mu\)g of proteins from 5 \(\times 10^6\) cells and 5 \(\times 10^6\) nuclei were fractionated on 7.5% SDS-PAGE, transferred, and immunoblotted with polyclonal anti-p85\(_{\alpha}\) antibody. The data are from a single experiment representative of at least three others. B, p85\(_{\alpha}\) subcellular localization by immunofluorescence. Cells were plated on glass coverslips, fixed, permeabilized, and incubated with polyclonal anti-p85\(_{\alpha}\) antibody. The bar represents the width of 10 \(\mu\)m.

**Fig. 7. Effects of PTX on GTP\(_{\gamma}\)S-induced nuclear PtdIns(3,4,5)P\(_3\) synthesis and expression of G\(_{i}\) proteins in VSMCs.** Membrane-depleted nuclei were isolated, and nuclear lipid kinase activity was assayed as described in Fig. 2. A, nuclei were pretreated for 15 min with 5 ng/ml pertussis toxin before incubation with 100 \(\mu\)M GTP\(_{\gamma}\)S. Lipids were extracted and analyzed by HPLC. Quantification of \([^{32}P]PtdIns(3,4,5)P_3\) synthesis is expressed as cpm/10\(^7\) nuclei. Data for GTP\(_{\gamma}\)S alone are the mean \pm S.E. from three independent experiments. Data for PTX represent the mean of two independent experiments. B, Western blotting analysis of heterotrimeric G\(_{i}\) protein. Protein from \(1 \times 10^5\) whole cells or \(5 \times 10^5\) purified nuclei were fractionated on 10% SDS-PAGE, transferred, and probed with polyclonal anti-G\(_{i}\)(C-ter) or anti-G\(_{i}\) antibodies. Experiments illustrated are representative of at least three distinct experiments. PIP\(_3\), PtdIns(3,4,5)P\(_3\).
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The PtdIns(3,4,5)P$_3$-dependent signaling pathways in the nucleus are still unknown. However, PtdIns(3,4,5)P$_3$ synthesis was associated with activation of the serine/threonine kinases, protein kinase C$_z$, and Akt/protein kinase B (34, 35), and the nuclear translocation of these two PtdIns(3,4,5)P$_3$ effectors was demonstrated upon mitogenic stimulation (36, 37). More recently, Neri et al. (38) show that nuclear PtdIns(3,4,5)P$_3$ production correlates both with p85a/p110 PI3K and protein kinase C$_z$ translocations to the nucleus of nerve growth factor-treated PC12 cells. Thus, the G protein-activated PI3K could produce PtdIns(3,4,5)P$_3$ inside VSMC nuclei to recruit and/or activate downstream effectors. In this respect, we also observed the presence of protein kinase C$_z$ and active Akt/protein kinase B in VSMC nuclei (data not shown). The nuclear targets of protein kinase C$_z$ and Akt/protein kinase B only begin to be described. Indeed, a major component of the nucleolus, nucleolin, has been shown to be phosphorylated by protein kinase C$_z$ (36), and Akt/protein kinase B has been reported to promote phosphorylation of the nuclear transcription factors CREB (forkhead in rhabdomyosarcoma 1) (40). Finally, evidence has been provided that PIP3BP, a PtdIns(3,4,5)P$_3$-binding protein, is exported out of the nucleus by the expression of constitutively activated PI3K (41).

Nuclear GTP-dependent and -independent PI3Ks—In response to GTP$\gamma$S, we never detected nuclear PtdIns(3)P or PtdIns(3,4)P synthesis from endogenous substrates, suggesting that PtdIns and PtdIns(4)P, which have been reported during nuclei isolation. On the other hand, the GTP-dependent PI3K could produce PtdIns(3,4,5)P$_3$ inside VSMC nuclei to recruit and/or activate downstream effectors. In this respect, we also observed the presence of protein kinase C$_z$ and active Akt/protein kinase B in VSMC nuclei (data not shown). The nuclear targets of protein kinase C$_z$ and Akt/protein kinase B only begin to be described. Indeed, a major component of the nucleolus, nucleolin, has been shown to be phosphorylated by protein kinase C$_z$ (36), and Akt/protein kinase B has been reported to promote phosphorylation of the nuclear transcription factors CREB (forkhead in rhabdomyosarcoma 1) (40). Finally, evidence has been provided that PIP3BP, a PtdIns(3,4,5)P$_3$-binding protein, is exported out of the nucleus by the expression of constitutively activated PI3K (41).

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