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Insulin selectively stimulates nuclear phosphoinositide-specific phospholipase C (PI-PLC) β1 activity through a mitogen-activated protein (MAP) kinase-dependent serine phosphorylation

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Abstract Using NIH 3T3 cells, we have investigated nuclear phosphoinositide metabolism in response to insulin, a molecule which acts as a proliferating factor for this cell line and which is known as a powerful activator of the mitogen-activated protein (MAP) kinase pathway. Insulin stimulated inositol lipid metabolism in the nucleus, as demonstrated by measurement of the diacylglycerol mass produced in vivo and by in vitro nuclear phosphoinositide-specific phospholipase C (PI-PLC) activity assay. Despite the fact that nuclei of NIH 3T3 cells contained all of the four isozymes of the β family of PI-PLC (i.e. β1, β2, β3, and β4), insulin only activated the β1 isoform. Insulin also induced nuclear translocation of MAP kinase, as demonstrated by Western blotting analysis, enzyme activity assays, and immunofluorescence staining, and this translocation was blocked by the specific MAP kinase kinase inhibitor PD98059. By means of both a monoclonal antibody recognizing phosphoserine and in vivo labeling with [^{32}P]orthophosphate, we ascertained that nuclear PI-PLC-β1 (and in particular the b subtype) was phosphorylated on serine residues in response to insulin. Both phosphorylation and activation of nuclear PI-PLC-β1 were substantially reduced by PD98059. Our results conclusively demonstrate that activation of nuclear PI-PLC-β1 strictly depends on its phosphorylation which is mediated through the MAP kinase pathway.

Key words: Nucleus; Phosphoinositide; Phospholipase C; Mitogen-activated protein kinase; Phosphorylation; Serine

1. Introduction

Previous work from several independent laboratories has established the presence of a nuclear inositol lipid metabolism which is involved in the control of cell proliferation and differentiation [1–3]. The evidence supporting the existence within the nucleus of a polyphosphoinositide cycle derives from the use of different methodologies, ranging from enzymatic activity measurements in various nuclear subfractions to immunoelectron microscopy and, more recently, to molecular biology techniques (e.g. [4–8]). One of the most intriguing aspects of the nuclear polyphosphoinositide metabolism is that it is clearly distinct from the classic inositol lipid cycle located at the plasma membrane level. Therefore, there are in the literature numerous examples of stimuli that selectively activate the nuclear cycle only (e.g. [2]).

In the nuclear inositol lipid cycle a pivotal role is played by phosphoinositide-specific phospholipase C (PI-PLC) β1, the enzyme that hydrolyzes phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) yielding the two second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3). Nuclear PI-PLC-β1 is activated in response to different stimuli such as insulin-like growth factor-I (IGF-I) and interleukin 1α [9,10]. In IGF-I-treated Swiss 3T3 cells this activation resulted in increased intranuclear levels of DAG which are essential for attracting to this organelle the α isoform of protein kinase C [11]. Very recent data coming from our laboratory have highlighted the fact that nuclear PI-PLC-β1 controls cell cycle progression conceivably through mechanisms involving cyclin D3 and its kinase cdk4 [12]. Despite the fact that intranuclear phosphoinositide metabolism was first described 13 years ago [13], our knowledge of the mechanisms which control it is extremely limited. As a first step in elucidating how nuclear PI-PLC-β1 activity could be regulated, we have recently demonstrated that, in Swiss 3T3 fibroblasts, IGF-I-elicited activation of PI-PLC-β1 is dependent on a phosphorylation of the phospholipase, possibly mediated by mitogen-activated protein (MAP) kinase [14].

It should be remembered that members of the β family of PI-PLC (β1, β2, β3, and β4) possess a unique COOH-terminal domain downstream of their catalytic domain. This domain is highly enriched in lysine and arginine residues, which are con-

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Abbreviations: bFGF, basic fibroblast growth factor; 5′-BrdU, 5′-bromodeoxyuridine; BSA, bovine serum albumin; DAG, diacylglycerol; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; FITC, fluorescein isothiocyanate; IGF-I, insulin-like growth factor-I; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase kinase; PBS, phosphate-buffered saline; PI-PLC, phosphoinositide-specific phospholipase C; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate

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served in all the four members of the β family. These basic residues are very important in determining the nuclear localization of PI-PLC-β1 [15]. Consistently with their molecular structure, also the β2, β3, and β4 isoforms are present in the nucleus of NIH 3T3 fibroblasts [16]. NIH 3T3 cells represent an interesting model to study the pathways which lead to cell proliferation. Some clones of NIH 3T3 cells respond to insulin alone [17]. Insulin is known to induce an intranuclear migration of MAP kinase (e.g. [18]). Therefore, it could be considered to be an activator of nuclear PI-PLC-β1.

In this paper, we have investigated whether or not changes in nuclear polyphosphoinositide metabolism took place in NIH 3T3 cells stimulated with insulin, and we have sought to determine if PI-PLC β family isozymes other than β1 could be activated at the nuclear level by the growth factor. Moreover, we have ascertained whether MAP kinase could be involved in the regulation of the nuclear inositol lipid cycle in response to insulin.

2. Materials and methods

2.1. Materials

ET-18-OCH_3, PD98059, and protein G plus/protein A agarose were from Calbiochem (La Jolla, CA, USA). The p42/p44 MAP kinase enzyme assay system, [^{3}H]PtdIns(4,5)P_2, [^{32}P]orthophosphate, [γ-^{32}P]ATP, fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG, peroxidase-conjugated anti-rabbit, anti-mouse IgG, and anti-mouse IgM, and the enhanced chemiluminescence (ECL) detection kit were from Amersham Pharmacia Biotech (Uppsala, Sweden). The protein assay kit (detergent-compatible) was from Bio-Rad (Hercules, CA, USA). Monoclonal antibody to 5′-bromodeoxyuridine (5′-BrdU) was from Becton Dickinson (Palo Alto, CA, USA). Affinity-purified rabbit polyclonal antibodies to all PI-PLC isoforms except β1 were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Also from Santa Cruz was a rabbit polyclonal antibody to MAP kinase. A monoclonal antibody recognizing the N-terminus of PI-PLC-β1 was obtained from Transduction Laboratories, Lexington, KY, USA. Monoclonal antibody to phosphoserine (clone 16B4, an IgM) was purchased form Alexis Biochemicals, Laufelfingen, Switzerland. Monoclonal antibody recognizing the phosphorylated (Thr202/Tyr204) form of p42/44 MAP kinase was from New England BioLabs Inc., Beverly, MA, USA. Insulin, epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF) were obtained from Roche Molecular Biochemicals, Milan, Italy. Monoclonal antibody to β-tubulin and all of the other reagents of highest purity grade were from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Cell culture

NIH 3T3 fibroblasts (ATCC CRL 1658) were cultured in Dulbecco's minimum essential medium (D-MEM) containing 10% fetal calf serum. Prior to stimulation, cells were subcultured at a density of 10^4/cm^2, and incubated until they became confluent (6 days). They were then cultured for an additional 24 h in serum-free medium containing 0.5% bovine serum albumin (BSA). Quiescent cultures were washed twice with serum-free medium containing 0.2% BSA, then incubated in the same medium for the indicated times in the presence of insulin (1 μg/ml), bFGF (2 ng/ml), or EGF (10 ng/ml). The ET-18-OCH_3 PI-PLC inhibitor (100 μM) was present starting 5 min prior to stimulation with insulin. For experiments performed in the presence of PD98059, the compound was present at 20 μM for 1 h prior to stimulation.

2.3. Cell cycle analysis

This was accomplished as reported elsewhere [6]. Briefly, cells were labeled for 60 min with 100 μM 5′-BrdU, fixed in ice-cold 70% ethanol, incubated for 30 min in 4 N HCl, and then reacted with a FITC-conjugated monoclonal antibody to 5′-BrdU for 30 min at 4°C, followed by propidium iodide (5 μg/ml) counterstaining. Samples were analyzed by means of a FACStar Plus flow cytometer (Becton Dickinson, Palo Alto, CA, USA).

2.4. Preparation of isolated nuclei and of cytoplasmic fraction

The preparation of nuclei stripped of their envelopes was accomplished as previously reported [9,14,16]. Briefly, cells (5×10^6) were suspended in 500 μl of 10 mM Tris–HCl, pH 7.8, 1% Nonidet P-40, 10 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml of leupeptin and aprotinin for 2 min at 0°C. Then 500 μl of double-distilled H_2O was added and the cells allowed to swell for 2 min. Cells were sheared by 10 passages through a 22-gauge needle. Nuclei were recovered by centrifugation at $400\times g$ for 6 min and washed once in 10 mM Tris–HCl, pH 7.4, 2 mM $MgCl_2$, plus protease inhibitors as above. Preparation of nuclei retaining the envelopes was performed in the absence of Nonidet P-40, by leaving the cells to swell after the addition of double-distilled H_2O for 5 min [16]. To obtain the cytoplasmic fraction, cells were resuspended in 10 mM Tris–HCl, pH 7.8, 2 mM $MgCl_2$ and protease inhibitors as for nuclei, then homogenized with 20 strokes in a Dounce homogenizer. Nuclei were sedimented at $400\times g$ for 6 min.

2.5. Electron microscopy

Pelleted NIH 3T3 cell nuclei obtained with the two different methods reported above were processed for transmission electron microscope analysis. Briefly, they were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 35 min, postfixed in 1% osmium tetroxide and embedded in Araldite. Thin sections were stained with uranyl acetate and lead citrate and observed with a Philips CM10 electron microscope.

2.6. Measurement of DAG mass produced in vivo, PI-PLC and MAP kinase activity assays

The DAG levels were measured according to Divecha et al. [4], using DAG kinase enzyme purified from rat brain. For the PI-PLC activity assay, the procedure outlined by Martelli et al. [9] was followed. MAP kinase activity was analyzed by using the Biotrak MAP kinase assay according to the manufacturer's instructions.

2.7. Solubilization of nuclear PI-PLC isoforms and immunoprecipitation

Membrane-deprived nuclei were resuspended at 1 mg DNA/ml in 5 mM Tris–HCl, pH 8.0, 0.1 mM EDTA and allowed to swell at 4°C for 10 min before rupturing by 40 passages through a 25-gauge hypodermic needle. The lysate was centrifuged for 10 min at $48\,000\times g$. 5 μg of protein from nuclear lysates was incubated under constant agitation for 1 h at 4°C in the presence of 1.25 μg of antibody to the various PI-PLC-β isoforms. Protein G plus/protein A agarose was then added to 10% (w/v) and incubation proceeded for an additional 60 min. Immune complexes were collected by centrifugation, and the supernatant was assayed for residual PI-PLC activity [10].

2.8. Western blotting analysis

80 μg of nuclear or cytoplasmic protein, separated on 7.0% SDS–PAGE, was transferred to nitrocellulose sheets using a semi-dry blotting apparatus. Sheets were saturated in phosphate-buffered saline, pH 7.4 (PBS), containing 5% normal goat serum and 4% BSA for 1 h at 37°C (blocking buffer), then incubated overnight at 4°C in blocking buffer containing primary antibodies. After four washes in PBS containing 0.1% Tween-20, samples were incubated for 30 min at room temperature with peroxidase-conjugated secondary antibodies, diluted 1:5000 in PBS–Tween-20, and washed as above. Bands were visualized by the ECL method. When required, densitometric analysis was performed on the Molecular Analyst GS670 (Bio-Rad).

2.9. In vivo labeling with [^{32}P]orthophosphate

Cells were labeled for 6 h in phosphate-free D-MEM in the presence of 200 μCi/ml [^{32}P]orthophosphate. Nuclei (from 2×10^7 cells) were then isolated from control and insulin-treated cells in buffers containing 2.0 mM Na_3VO_4 and 5 mM NaF, lysed and immunoprecipitation was carried out with anti-PLC-β1 antibodies, as detailed above. Samples were then electrophoresed, blotted, and autoradiographed.

2.10. Immunofluorescence staining

Cells growing on coverslips were fixed with freshly made 4% paraformaldehyde in PBS for 30 min at room temperature, permeabilized with 0.2% Triton X-100 for 10 min, then incubated for 3 h at 37°C with a monoclonal antibody (diluted 1:30) recognizing activated

(phosphorylated) MAP kinase. The secondary antibody was a FITC-conjugated anti-mouse IgG, diluted 1:200. Samples were photographed using a Zeiss Axiophot epifluorescence microscope.

3. Results

3.1. Cell proliferation

The effects of insulin, bFGF, and EGF on cell proliferation were studied by flow cytometry of 5′-BrdU-labeled cells. All of these growth factors are known to activate MAP kinase [8,19,20]. As shown in Fig. 1, insulin (1 μg/ml) induced DNA replication in approximately 18 ± 2% of the cells. In contrast, neither bFGF nor EGF had any significant effect on cell proliferation. Because of these results, we decided to restrict any further analysis to insulin-stimulated cells alone.

3.2. Purity of nuclear fractions

As shown in Fig. 2A, nuclei obtained without detergent retained the nuclear envelope, whereas if Nonidet P-40 was present during the preparations, the envelope was completely stripped (Fig. 2B). As demonstrated by Western blotting analysis (Fig. 2C), nuclei retaining the envelope were contaminated by β-tubulin, which, on the other hand, was completely absent from preparations of membrane-free nuclei.

3.3. DAG mass levels and PI-PLC activity in subcellular fractions

We first measured in vivo DAG mass levels following stimulation with insulin. As shown in Table 1, a 2 min incubation with insulin caused a 32% rise in DAG mass in membrane-stripped nuclei only. At 60 min of stimulation this value had almost completely returned to basal levels. We next assayed PI-PLC activity in samples treated with insulin. As presented in Table 1, stimulation of quiescent NIH 3T3 cells for 2 or 60 min did not significantly affect PI-PLC activity in either cytoplasmic fraction or nuclei retaining their envelope. In contrast, in preparations of envelope-stripped nuclei, PI-PLC activity rose about two-fold over basal levels (from 10.5 ± 2.1 to 21.47 ± 3.1 nmol Ins(1,4,5)P_3/mg protein/30 min of incubation) at 2 min of stimulation, while at 60 min it was reduced to 12.5 ± 2.9 nmol Ins(1,4,5)P_3/mg protein/30 min of incubation. Pretreatment of cells with a pharmacological inhibitor of PI-PLC (ET-18-OCH_3, employed at 100 μM) almost completely abolished the activity in all the subcellular fractions prepared from either quiescent or growth factor-stimulated cells.

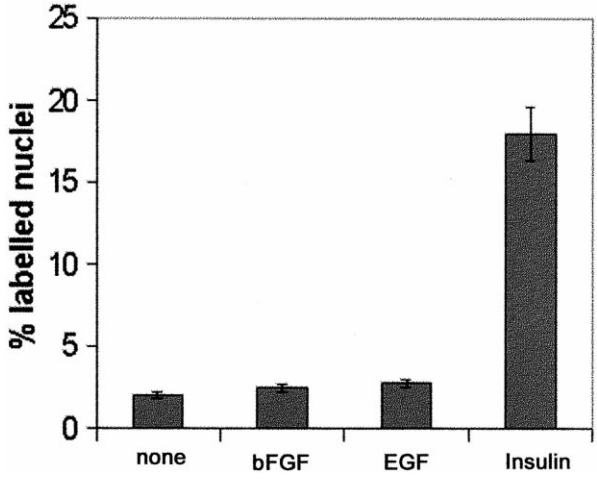

Fig. 1. Effect of insulin, bFGF, and EGF on the proliferation of NIH 3T3 cells. 20 h after exposure to the growth factors cells were incubated for 60 min with 100 μM 5′-BrdU, labeled with an anti-5′-BrdU antibody, and then analyzed by flow cytometry. The histograms show the percentage of labeled nuclei. The data represent the mean from three different experiments ± S.D.

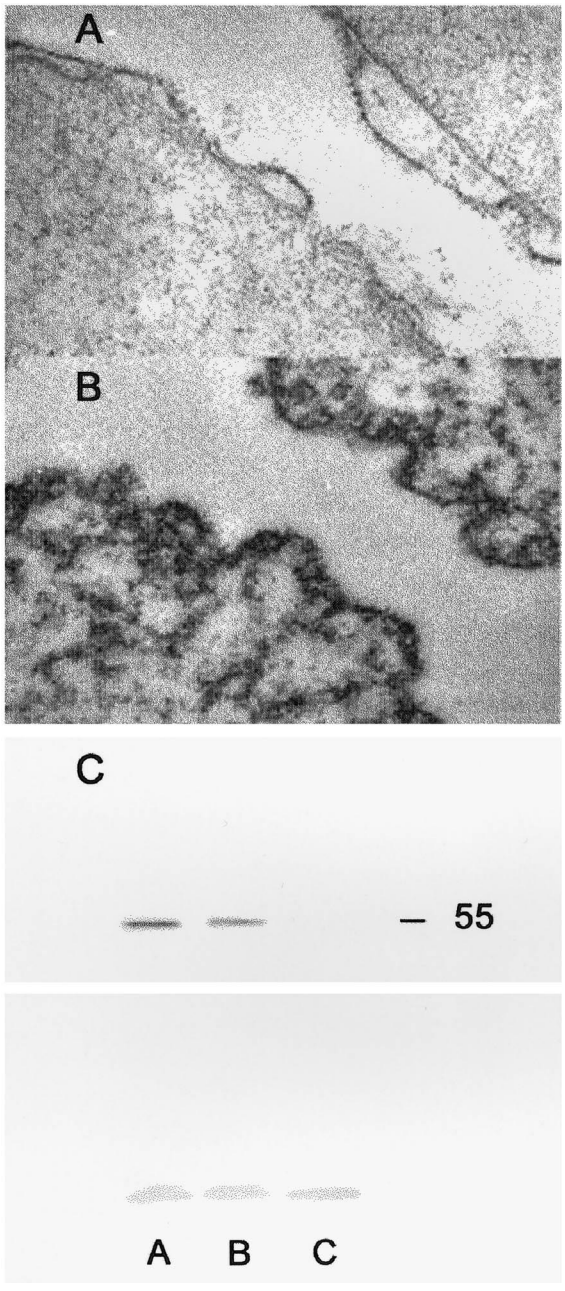

Fig. 2. A,B: Electron microscopy pictures of nuclei isolated from NIH 3T3 cells by means of two different techniques. A: Nuclei isolated in the absence of 1% Nonidet P-40 still retain the envelope. B: Nuclei isolated in the presence of the detergent are completely stripped of the envelope. C: Western blotting analysis with monoclonal antibody to β-tubulin. Lane 1: cytoplasmic fraction; lane 2: intact nuclei; lane 3: membrane-deprived nuclei. Bands with a M_r of 55 kDa were detected.

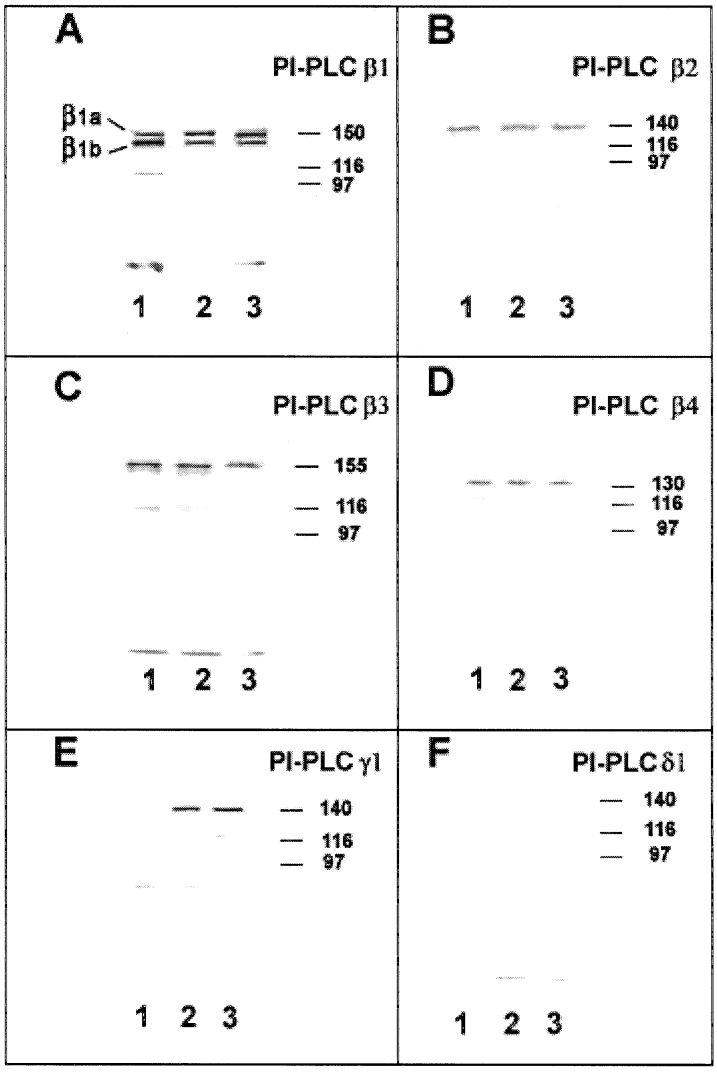

Fig. 3. Western blotting analysis of PI-PLC isozymes in subcellular fractions of NIH 3T3 cells. Lane 1: envelope-stripped nuclei. Lane 2: nuclei retaining the envelope. Lane 3: cytoplasmic fraction. 80 μg of protein was blotted to each lane.

3.4. PI-PLC isoforms present in subcellular fractions of NIH 3T3 cells

Recent results from our laboratory [16] have demonstrated the existence, in the nucleus of NIH 3T3 cells, of all the members of the β family of PI-PLC: β1, β2, β3, and β4. In Fig. 3 we confirm these data by Western blotting analysis. However, since for these experiments we employed a monoclonal antibody to the N-terminus of PI-PLC-β1, we were able to detect that in membrane-stripped nuclei the b subtype of this isoform is more represented, while in both membrane-retaining nuclei and cytoplasm the a subtype of PI-PLC-β1 predominates. In addition, we show that PLC-γ1 was also present in the cytoplasmic fraction and in preparations of

Table 1
In vivo DAG mass levels and in vitro PI-PLC activity in NIH 3T3 cell subcellular fractions after stimulation with 1 μg/ml insulin

Condition	DAG (pmol/mg protein)			PI-PLC (nmol Ins(1,4,5)P_3 liberated/mg protein/30 min incubation)		
	Cytoplasmic fraction	Nuclei with envelope	Membrane-stripped nuclei	Cytoplasmic fraction	Nuclei with envelope	Membrane-stripped nuclei
Control	313.6 ± 27.7	187.0 ± 15.7	99.1 ± 7.7	114.6 ± 18.1	20.4 ± 3.1	10.5 ± 2.1
Insulin 2 min	321.1 ± 27.3	195.8 ± 21.2	131.8 ± 15*	122.9 ± 23.4	23.6 ± 3.7	21.5 ± 3.1*
Insulin 60 min	329.8 ± 34.9	199.3 ± 18.0	106.6 ± 12.3	119.7 ± 20.4	21.6 ± 3.8	12.5 ± 2.9
Control, plus ET-18-OCH_3	ND	ND	ND	9.3 ± 1.5	2.9 ± 0.8	1.6 ± 0.9
Insulin 2 min, plus ET-18-OCH_3	ND	ND	ND	10.1 ± 1.7	2.7 ± 0.6	1.8 ± 1.1

The data represent the mean from three different experiments ± S.D. The asterisks indicate significant differences ($P < 0.01$) in a Student's paired *t*-test. ND, not determined.

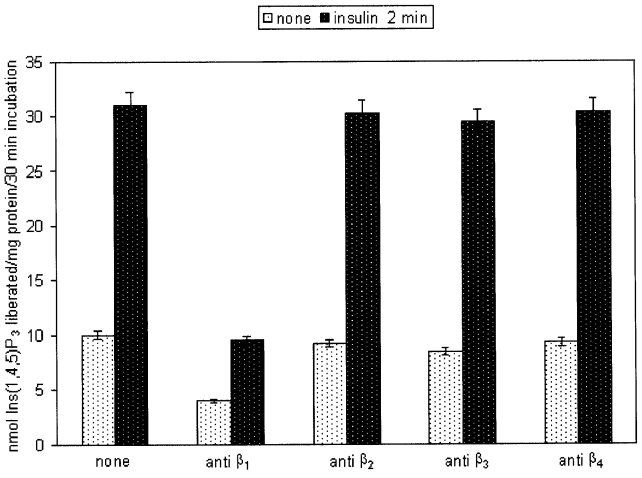

Fig. 4. Residual soluble nuclear PI-PLC activity following immunoprecipitation with antibodies to the different isozymes of the β family. Data are expressed as mean from three different experiments ± S.D.

nuclei retaining the envelope, but not in membrane-stripped nuclei. In contrast, PLC-δ1 was not expressed at all in these cells.

3.5. Insulin selectively stimulates nuclear PI-PLC-β1 activity

Since membrane-stripped nuclei retain all of the four members of the β family of PI-PLC, it was very important to determine which (if any) of these isoforms was activated in response to insulin. To this end, nuclei were lysed and the lysates were subjected to immunoprecipitation with antibodies specific for the various PI-PLC-β isozymes. Then, residual PI-PLC activity was assayed in the supernatant of the immunoprecipitates. As shown in Fig. 4, only the antibody to PI-PLC-β1 was capable of reducing in a significant manner PI-PLC activity which remained in the supernatant of the lysates after immunoprecipitation, both in control nuclei (from 9.8 ± 2.0 to 4.7 ± 0.8 nmol Ins(1,4,5)P_3/mg protein/30 min of incubation) and in nuclei prepared from growth factor-stimulated cells (from 21.2 ± 3.4 to 7.4 ± 1.8 nmol Ins(1,4,5)P_3/mg protein/30 min of incubation).

3.6. Insulin induces translocation to the nucleus of MAP kinase and serine phosphorylation of nuclear PI-PLC-β1

Since our previous results suggested (but did not prove) that phosphorylation of nuclear PI-PLC-β1 was dependent on MAP kinase [14], we decided to verify this issue in NIH 3T3 cells. As shown in Fig. 5A, some nuclear immunoreactivity for MAP kinase (both the p42 and the p44 isoform) was detected in control nuclei. Insulin was capable of inducing a marked nuclear translocation of MAP kinase already after 1 min of stimulation. The amount of intranuclear MAP kinase further increased at 2 min after the beginning of stimulation with the growth factor. In vitro assays also revealed a marked increase in intranuclear activity of MAP kinase already after 1 min of stimulation with insulin (Fig. 5B). Furthermore, immunofluorescence staining of insulin-stimulated NIH 3T3 cells with a monoclonal antibody which specifically reacts with activated (phosphorylated) MAP kinase showed the enzyme to be present within the nucleus after 1 min of treatment with the growth factor (Fig. 5C).

The overall amount of intranuclear PI-PLC-β1 recovered by immunoprecipitation (both the a and b subtypes) did not change in response to insulin, nor it was affected by incubation with PD98059, a selective pharmacological inhibitor of MAP kinase kinase (MEK) (Fig. 6A). The blots from the immunoprecipitates shown in Fig. 6A were then stripped and reprobed with an antibody to phosphoserine. As presented in Fig. 6B, anti-phosphoserine antibody barely stained the immunoprecipitates from control cells. In response to a 2 min stimulation with insulin, we detected a marked increase in the immunoreactivity, especially regarding the b subtype of PI-PLC-β1. The increase was reduced if the cells had been incubated for 1 h with PD98059 prior to insulin stimulation. Indeed, in this case, the densitometric analysis revealed a 63% decrease in the intensity of the immunoreactive bands.

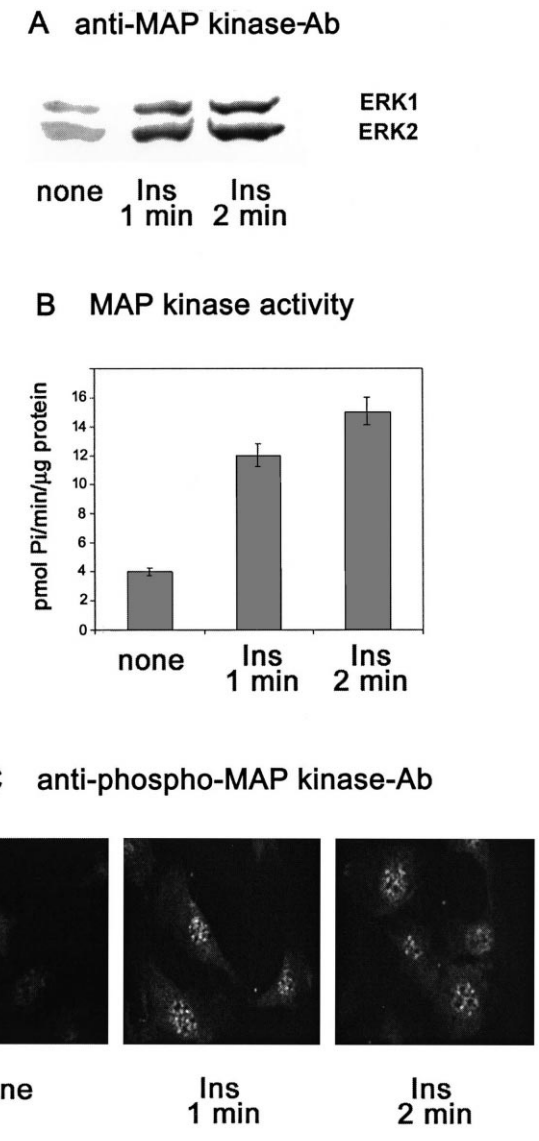

Fig. 5. Insulin (Ins) induces translocation to the nucleus of MAP kinase. A: Western blotting analysis with a polyclonal antibody showing intranuclear translocation of MAP kinase. B: Histograms showing enhanced nuclear MAP kinase activity in response to insulin. C: Immunofluorescence staining of NIH 3T3 cells (control and insulin-treated) using a monoclonal antibody to phosphorylated MAP kinase. In B, results represent the mean ± S.D. of triplicate determinations.

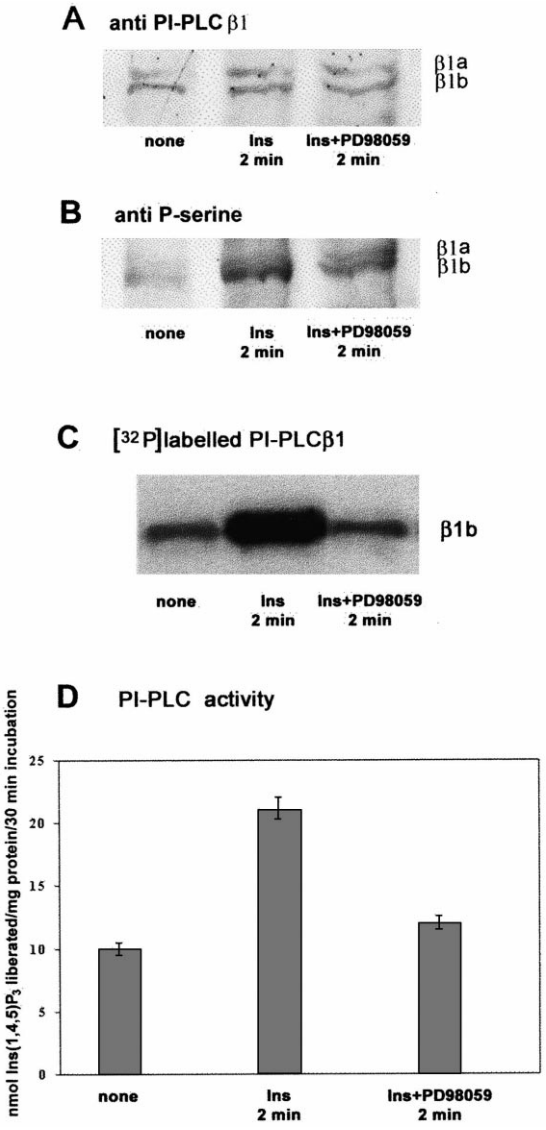

Fig. 6. Insulin (Ins) induces serine phosphorylation of nuclear PI-PLC-β1. A: Western blotting analysis showing immunoprecipitated nuclear PI-PLC-β1 from control and insulin-treated samples. B: The blot from A was stripped and then reprobed with a monoclonal antibody to phosphoserine. C: Autoradiogram of immunoprecipitated nuclear PI-PLC-β1 from [^{32}P]orthophosphate-labeled control and insulin-treated cells. D: PD98059 blocks the insulin-evoked rise in nuclear PI-PLC activity. In D, results represent the mean ± S.D. of triplicate determinations.

When nuclei prepared from in vivo [^{32}P]orthophosphate-labeled cells were lysed and immunoprecipitated using an antibody specific to the PI-PLC-β1 b splicing variant [21], it was possible to see a dramatic increase in the phosphorylation of the phospholipase in response to a 2 min stimulation with insulin. This increase was almost completely prevented by pretreatment of cells with PD98059 (Fig. 6C).

Finally, in Fig. 6D we demonstrate that also the insulin-elicited increase in nuclear PI-PLC-β1 activity was almost completely blocked by preincubation with PD98059.

4. Discussion

We have found that insulin stimulates the nuclear polyphosphoinositide cycle in NIH 3T3 cells, as demonstrated by DAG mass measurement and PI-PLC activity assays. The results we have obtained are superimposable on our own findings obtained with IGF-I-treated Swiss 3T3 cells [9,11]. As previously reported by Divecha et al. [4], insulin-induced changes in nuclear inositol metabolism were seen only in nuclei stripped of their envelopes by detergent treatment. This is highly suggestive of events taking place in the nuclear interior and not at the periphery of the organelle.

Moreover, we have demonstrated that, in response to insulin, only nuclear PI-PLC-β1 was activated, even though the nucleus of NIH 3T3 cells also contains the β2, β3, and β4 isozymes of PI-PLC. It should be remembered that our previous results have indicated that, all together, PI-PLC-β2, -β3, and -β4 represent less than 50% of the β family of PI-PLC in membrane-deprived nuclei of NIH 3T3 cells and that the activity due to these isoforms is about 35% of the total nuclear PI-PLC activity [16]. In our opinion, the fact that almost no activity was immunoprecipitated by the antibodies to these three isoforms indicates that they are not active in the nucleus upon insulin stimulation. The slight inhibition of the basal activity ascribed to the β2, β3, and β4 isozymes after immunoprecipitation of the nuclear lysates is compatible with the amount of these isoforms, also taking into account that the antibodies did not completely immunodeplete the lysates (data not shown).

Upon treatment with insulin, MAP kinase translocated to the nucleus, and PI-PLC-β1 was phosphorylated on serine residues, as indicated by the use of both the lysates from in vivo [^{32}P]orthophosphate-labeled cells and a monoclonal antibody raised against phosphoserine residues. In this context, it should be recalled that at amino acids 980–983 the phospholipase displays a typical MAP kinase consensus sequence, that is Pro-Ser-Ser-Pro [21,22]. It is of great interest that such a sequence does not exist in other isoforms of the β family of PI-PLC, and this is in agreement with the fact that insulin activates only nuclear PI-PLC-β1. MAP kinase translocation to the nucleus as well as PI-PLC-β1 phosphorylation and activation were markedly reduced by the specific MEK pharmacological inhibitor PD98059. Taken together, these findings strongly suggest that nuclear PI-PLC-β1 is activated upon its phosphorylation by MAP kinase. These results extend and support the data of a previous investigation, in which phosphorylation of nuclear PI-PLC-β1 was detected in cells that had been radiolabeled in vivo with [^{32}P]orthophosphate. If MAP kinase translocation to the nucleus was blocked by cytoskeleton depolymerization, no phosphorylation and activation of PI-PLC-β1 were detectable [14]. We would like to emphasize that our data show that particularly the b subtype of PI-PLC-β1 was hyperphosphorylated upon treatment of quiescent NIH 3T3 cells with insulin. It is now accepted that this subtype of PI-PLC-β1 is more abundantly expressed in the nucleus, while subtype a predominates in the cytoplasm [23]. These results appear intriguing, because so far serine phosphorylation of PLC-β isozymes by either protein kinase A or protein kinase C has been regarded as a mechanism to inhibit their GTP binding protein-dependent activation [24–26]. However, in these cases the serine residue involved is 1105. Moreover, our results establish yet another difference between the nuclear inositide cycle and the same cycle which operates at the plasma membrane level: membrane-associated PI-PLC-β isoforms are stimulated through interaction with

the $G_{\alpha q}$ class of heterotrimeric GTP binding proteins as well as by βγ subunits [27]. In contrast, either GTPγS or AlF_4 failed to stimulate PI-PLC-β1 activity in Swiss 3T3 cell isolated nuclei, thus ruling out the hypothesis that a GTP binding protein could be responsible for the activation of PI-PLC-β1 in this organelle [28].

In conclusion, we believe we have identified a novel mechanism which controls the activity of nuclear PI-PLC-β1, and we have also begun to clarify how a signal, generated at the plasma membrane, can reach the nucleus to stimulate the residing inositol lipid cycle. Future investigations will undoubtedly clarify how other steps of this cycle are regulated at the nuclear level.

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