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Phosphoinositide-Specific Phospholipase Cβ1 Expression is Not Linked to Nerve Growth Factor-Induced Differentiation, Cell Survival or Cell Cycle Control in PC12 Rat Pheocromocytoma Cells

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Abstract Recent reports have highlighted that phosphoinositide-specific phospholipase Cβ1 expression is linked to neuronal differentiation in different experimental models. We sought to determine whether or not this is also true for nerve growth factor (NGF)-induced neuronal differentiation of rat PC12 cells. However, we did not find differences in the expression of both the forms of phosphoinositide-specific phospholipase Cβ1 (a and b) during sympathetic differentiation of these cells. Also, PC12 cell clones stably overexpressing phosphoinositide-specific phospholipase Cβ1 were not more susceptible to the differentiating effect of NGF. Furthermore, since it is well established that phosphoinositide-specific phospholipase Cβ1 affects cell proliferation, we investigated whether or not PC12 cell clones stably overexpressing phosphoinositide-specific phospholipase Cβ1 showed differences in survival to serum deprivation and cell cycle, when compared to wild type cells. Nevertheless, we did not find any differences in these parameters between wild type cells and the overexpressing clones. Interestingly, in PC12 cells the overexpressed phosphoinositide-specific phospholipase Cβ1 did not localize to the nucleus, but by immunofluorescence analysis, was detected in the cytoplasm. Therefore, our findings may represent another important clue to the fact that only when it is located within the nucleus phosphoinositide-specific phospholipase Cβ1 is able to influence cell proliferation. J. Cell. Biochem. 84: 56–67, 2002.

Key words: cell proliferation; apoptosis; phosphoinositide hydrolysis; nucleus; localization

Phosphoinositide-specific phospholipase Cs (PI-PLCs) are the enzymes that hydrolyze phosphatidylinositol 4,5-biphosphate (PtdIns $(4,5)P_2$) to yield the two second messengers, inositol 1,4,5 trisphosphate ($Ins(1,4,5)P_3$) and diacylglycerol (DAG) [Singer et al., 1997]. PI-PLCs are involved in several transmembrane signal transduction networks that regulate a variety of cellular functions [Noh et al., 1995]. Three classes of PI-PLCs have been identified in mammals: β, γ, and δ. Each class is composed of more than an isozyme (β, 1–4; γ, 1–2; and δ, 1–4) [Katan, 1998]. The three classes of PI-PLCs are linked to receptors by distinct mechanisms: the β isozymes are activated by the α subunits of the Gq subfamily of heterotrimeric G proteins as well as by the Gβγ dimer, whereas PLCγ isozymes are activated by both receptor and nonreceptor type protein kinases

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through tyrosine phosphorylation. The regulation of the δ isozymes is not well understood [Rhee and Bae, 1997]. Very recently it has been reported the identification of a novel class of PI-PLC, named PLCε, which is targeted to membranes by Ras oncoprotein [Song et al., 2000]. Since the β isozymes of PI-PLC are coupled to G proteins, they are activated in response to hormones, neurotransmitters, and sensory inputs [Kim et al., 1997; Adamski et al., 1999], and are enriched in the central nervous system [Min et al., 2000]. As far as PI-PLCβ1 is concerned, it is now established that two forms of this isozyme, generated by alternative splicing, exist [Bahk et al., 1994]. They are referred to as PI-PLCβ1a and PI-PLCβ1b, differing at their carboxyl-terminal sequence of 43 amino acids which is present only in the PI-PLCβ1a form. Lately, a report suggested that PI-PLCβ1 expression correlates with neuronal differentiation and synaptic plasticity in rat somatosensory cortex [Hannan et al., 1998]. Moreover, Novak et al. [2000] have demonstrated an increased expression of PI-PLCβ1 following retinoic acid-mediated differentiation of NT2 stem nervous cells. Subsequently, Hannan et al. [2001] have shown that during development of the cerebral cortex, the invasion of thalamic axons triggers a critical signaling mechanism involving the activation of PI-PLCβ1 and metabotropic glutamate receptors in the cortical neurons. Homozygous null mutation of PI-PLCβ1 dramatically disrupted the cytoarchitectural differentiation of barrels in the mouse somatosensory cortex, thus indicating that PI-PLCβ1 activation is critical for the coordinated development of the neocortex.

Furthermore, PI-PLCβ1 is likely to be involved in the transformed growth of small cell lung carcinoma [Beckman et al., 1998; Strassheim et al., 2000]. Other than at the plasma membrane, PI-PLCβ1 has been found in the nucleus, where it plays an important role in the control of cell proliferation in response to insulin-like growth factor-I (IGF-I) and in the cell cycle progression and differentiation of mouse erythroleukemia (MEL) cells [reviewed in D'Santos et al., 1998; Martelli et al., 1999a; Cocco et al., 2001].

With the above rationale in mind, we decided to investigate whether or not also in nerve growth factor (NGF)-exposed PC12 cells, neuronal differentiation was accompanied by an enhanced expression of PI-PLCβ1. However, our results demonstrated this not be the case. Moreover, the stable overexpression of PI-PLCβ1 forms did not increase the number of PC12 cells that differentiated into sympathetic neurons in response to NGF, indicating that such a PI-PLC isozyme is not linked to this type of differentiation. Interestingly, in PC12 cells overexpressed PI-PLCβ1 did not localize to the nucleus, but was found by immunofluorescence staining in the cytoplasm. No changes in survival to serum deprivation or in the cell cycle progression were also observed in the stably transfected clones when compared to wild type cells. These results might be another clue to the fact that only when present in the nucleus, PI-PLCβ1 is capable of affecting cell proliferation.

MATERIALS AND METHODS

Cell Culture, Differentiation, and Transfection

PC12 cells (pheocromocytoma cells of rat adrenal medulla origin) were grown in Dulbecco's Modified Minimum Essential medium (D-MEM) supplemented with horse serum (10%) and fetal calf serum (5%) at 37°C in the presence of 5% CO_2. For neuronal differentiation, cells were incubated for 4 days in the presence of 50 ng/ml NGF (2.4S, from male mouse submaxillary gland, Upstate Biotechnology, Lake Placid, NY) in medium containing 1% horse serum. For serum deprivation, cells were washed five times by centrifugation in serum-free D-MEM and incubated in D-MEM for the indicated times. For obtaining stable cell clones overexpressing either PI-PLCβ1a or β1b, 5×10^4 cells were seeded in a well of a 6-well plate, and incubated overnight in serum-free medium with a mixture of 1 μg cDNA and 10 μl of Lipofectamine (Life Technologies Italia, Milan, Italy). Both PI-PLCβ1a and β1b cDNA were cloned in the pRc/CMV eukaryotic expression vector. Control cells were transfected with the empty vector (mock-transfected). Cells were then washed twice with serum-free medium, then seeded in a 10 cm Petri dish. After 48 h, clonal selection was started with 750 μg/ml of G418. Overexpressing clones were identified by both fluorescence immunocytochemistry and immunoblotting employing a monoclonal antibody which recognizes the two PI-PLCβ1 forms (see later). For evaluation of neuronal differentiation, cells were seeded onto rat tail collagen-coated coverslips and treated with

50 ng/ml NGF for 4 days. They were then scored for neurite outgrowth. The percentage of cells that possessed neurites was determined by counting treatments in triplicate. A minimum of 500 cells were counted within a treatment group [Wooten et al., 2000].

Source of Antibodies

Two different types of antibodies of PI-PLCβ1 were employed during this study. A monoclonal antibody raised to the N-terminus of PI-PLCβ1, which recognizes both the subtypes of the enzyme, was purchased from Transduction Laboratories (Lexington, KY). A rabbit polyclonal antibody (G-12) directed to an epitope exclusively mapping to the C-terminus of PI-PLCβ1a was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies to PI-PLCβ2, β3, and β4 were from Santa Cruz Biotechnology.

Preparations of Whole Cell Homogenate and Protein Assay

Cells were sedimented at 1,000*g* for 10 min and washed twice in phosphate buffered saline, pH 7.4 (PBS), then homogenized by 10 passages through a 25-gauge needle in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.1% sodium dodecylsulfate (SDS), 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and aprotinin, 40 μg/ml bestatin, 10 μg/ml E-64, 17 μg/ml calpain inhibitor I, and 7 μg/ml calpain inhibitor II. Protein assay was performed according to the instruction of the manufacturer using the Bio-Rad (Hercules, CA) Protein Assay (detergent compatible).

Immunoblotting

Whole cell homogenates were mixed with an equal volume of 2× boiling lysis buffer, containing 125 mM Tris-HCl, pH 6.8, 4% SDS, glycerol, 10% 2-mercaptoethanol, and the protease inhibitor cocktail. Samples were then briefly sonicated to shear DNA and reduce viscosity, boiled for 5 min to solubilize protein, and stored at −80°C until required. Protein (typically 70–80 μg/lane) was separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) on 6% gels, and electrophoretically transferred to nitrocellulose sheets using a semi-dry blotting apparatus (Hoefer/Pharmacia Biotech, Uppsala, Sweden). Sheets were saturated for 60 min at 37°C in blocking buffer, then incubated overnight at 4°C in blocking buffer containing the primary antibodies. Blocking buffer was 4% bovine serum albumin (BSA), 3% normal goat serum (NGS). After four washes in PBS containing 0.1% Tween 20, sheets were incubated for 30 min at room temperature with peroxidase-conjugated secondary antibodies (from Sigma Chemical Co., St. Louis, MO), diluted 1:5,000 in PBS-Tween 20, and washed as above. Bands were visualized by the enhanced chemiluminescence (ECL) method using Lumi-Light^{Plus} (Roche Molecular Biochemicals, Milan, Italy).

Immunofluorescence Staining

Cultures of PC12 cells, grown on glass coverslips, were washed twice in PBS, fixed with freshly prepared 4% paraformaldehyde (30 min at room temperature) and permeabilized with 0.4% Triton X-100 in PBS (10 min). Samples were reacted for 3 h with the monoclonal antibody (diluted 1:100 in 2% BSA, 3% NGS) to PI-PLCβ1. The secondary antibody was a fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (from Sigma), diluted 1:200 as above. All incubations were carried out at 37°C. Samples were subsequently washed three times in PBS, counterstained for DNA with 0.01 μg/ml 4′-6-diamidino-2-phenylindole (DAPI) in PBS and mounted in 20 mM Tris-HCl, pH 8.2, 90% glycerol containing 2.3% of the antifading agent 1,4-diazobicyclo-[2.2.2]-octane (BDH, Poole, UK). Slides were observed and photographed using a Zeiss Axiophot epifluorescence microscope.

Measurement of Phosphoinositide Hydrolysis in Intact PC12 Cells

Cells were pre-labeled for 72 h at 37°C in D-MEM containing 1/3 of normal inositol concentration supplemented with 20 μCi/ml of [^{3}H] inositol (Amersham/Pharmacia Biotech, Uppsala, Sweden), by which time labeling of inositol lipids had achieved isotopic equilibrium [Novak et al., 2000]. Cells adherent to 6-cm dishes were rinsed with 2 ml of buffer A (142 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 5.6 mM D-glucose, 30 mM HEPES, pH 7.4) and incubated for 15 min at 37°C in 1.5 ml of the same buffer containing 1 μM ionomycin. Reactions were terminated by rapid aspiration of the buffer and the addition of 2 × 1 ml of 6% (w/v) ice-cold trichloroacetic acid. Cell lysates were scraped

into centrifuge tubes on ice and centrifuged at 2,400*g* for 5 min at 4°C. The supernatants were washed with 5 × 2 ml of water-saturated diethyl ether and neutralized with $NaHCO_3$. Radioactivity associated with [^{3}H] inositol phosphates in the supernatant was quantified as previously reported [Fisher et al., 1984]. The cell pellets were analyzed to determine the incorporation of [^{3}H] inositol into phosphoinositides as described elsewhere [Novak et al., 1999]. Production of inositol phosphates was then normalized as the ratio of [^{3}H] inositol phosphates to total radioactivity (i.e., that incorporated in inositol phosphates and inositol lipids).

Cell Counts

PC12 cells were plated in equal numbers in 6-well plates in control or in experimental conditions for the indicated times. The culture medium was removed and reserved. The cells were removed from culture dishes by treatment with 0.1% trypsin Ca^{2+}/Mg^{2+}-free PBS. After trypsinization, cells were gently triturated to separate clumps and the original culture medium was added back. After brief centrifugation (1,000*g* for 5 min) to concentrate the cells, the supernatant fluid was removed and an equal volume of trypan blue solution (0.4% trypan blue, 0.81% NaCl, and 0.06% K_2HPO_4) was added. Triplicate samples (at least 500 cells/each) were counted using a hemocytometer [Mesner et al., 1992].

Cell Cycle Analysis

To evaluate the cell cycle, 1×10^6 cells were fixed in 1 ml cold 70% ethanol at 4°C for 1 h. The cells were centrifuged, washed in PBS, resuspended in 0.4 ml PBS, and treated with 0.5 μg/ml RNAse A (from Sigma) for 1 h at 37°C. After addition of 40 μg/ml of propidium iodide, samples were incubated in the dark at 4°C for 10 min and then analyzed by an EPICS XL flow cytometer (Coulter Immunology, Hialeah, FL), as previously described [Neri et al., 1999a].

RESULTS

Effects of NGF on PC12 Neuronal Differentiation

As presented in Figure 1, NGF (50 ng/ml) induced a progressive neuronal differentiation of PC12 cells, as exemplified by the number of the cells with dendrites and a single axon. The number of differentiated cells was in good agreement with the findings of others who have employed the same experimental conditions [e.g. Brodie et al., 1999]. In Figure 2, we show the morphology of NGF-treated PC12 cells at various stages of differentiation.

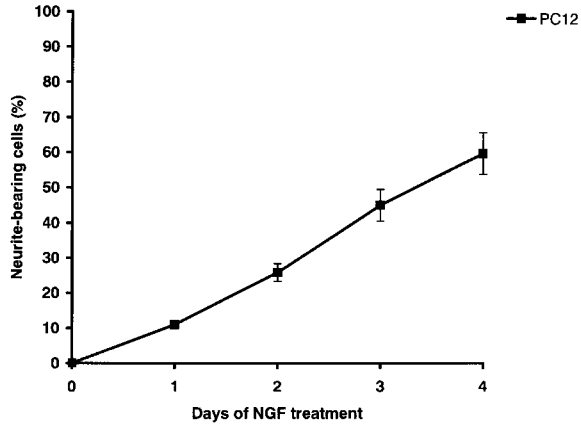

Fig. 1. NGF (50 ng/ml) induces neuronal differentiation of PC12 cells. The diagram shows the percentage of neurite-bearing cells at various times of NGF treatment. Results are the mean of three different experiments ± SD. A minimum of 500 cells were counted for each experiment.

Expression of PI-PLCβ1 Forms Upon NGF-Induced Differentiation

We next analyzed by immunoblotting the amount of PI-PLCβ1a and b along the differentiation process of PC12 cells. For the detection of the two forms we employed a monoclonal antibody which recognizes a common epitope present at the N-terminus of both the forms. As presented in Figure 3, no major differences were seen between control (untreated) and cells treated with NGF up to 4 days.

Expression of PI-PLCβ2 and β3 Upon NGF-Induced Differentiation

We also wanted to verify, by immunoblotting analysis, if other members of the β family of PI-PLC changed upon differentiation. However, as shown in Figure 4, no major changes were seen also for PI-PLCβ2 and β3 along the neuronal differentiative pathway. We did not detect PI-PLCβ4 in these cells (data not shown).

Overexpression of PI-PLCβ1 Forms in PC12 Cells

PC12 cells were transfected with constructs containing either rat brain PI-PLCβ1a or PI-PLCβ1b. Three clones for each construct were

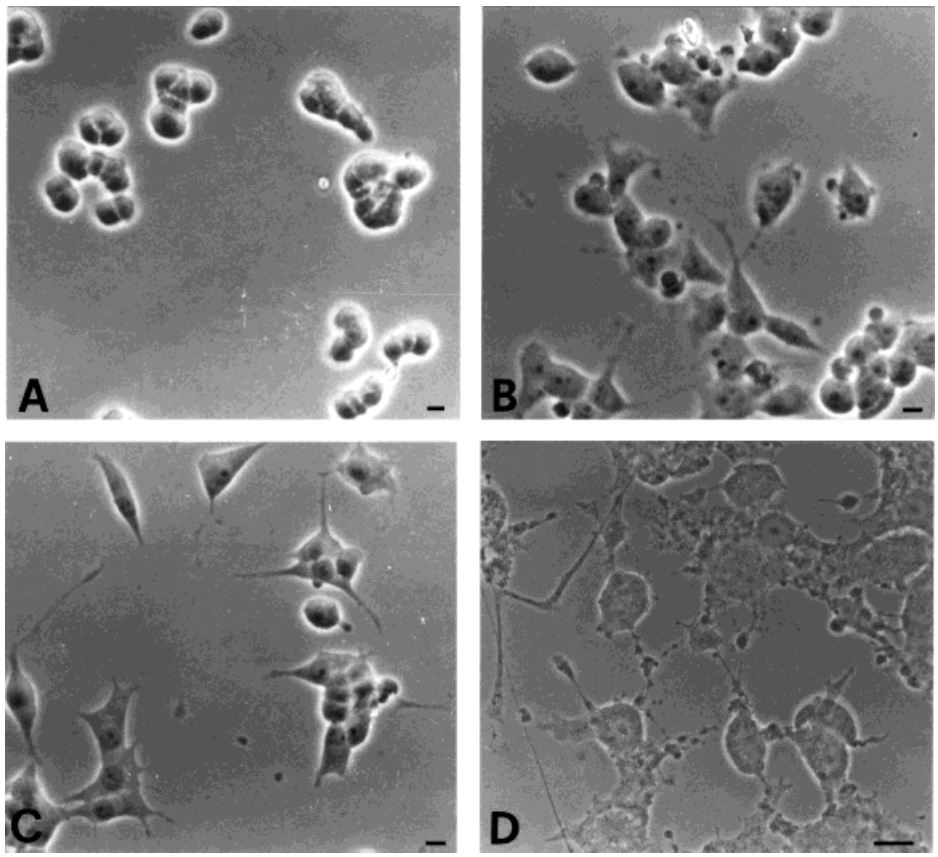

Fig. 2. Morphological assay of PC12 differentiation in response to NGF (50 ng/ml). **A:** control (untreated cells); **B**, **C**, **D:** cells treated with NGF for 24, 48, and 96 h, respectively. Scale bars: 10 μm.

obtained. The clones were analyzed by both immunoblotting (Fig. 5) and immunofluorescence staining (Fig. 6). For the detection of PI-PLCβ1a we employed a polyclonal antibody to an epitope present in C-terminus of this form. By immunoblotting (Fig. 5A) it was possible to see that the three clones overexpressed approximately the same amount of the protein of interest. Mock-transfected cells did not show any increase in the amount of PI-PLCβ1a when compared to control cells. In contrast, for the detection of PI-PLCβ1b we used the monoclonal antibody to the N-terminus epitope. As shown in Figure 5B, immunoblotting analysis revealed in three clones a similar overexpression of

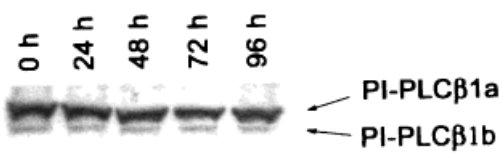

Fig. 3. PI-PLCβ1a and b expression during NGF-induced differentiation of PC12 cells. A 80 μg of protein from whole cell homogenates (prepared at the indicated times of NGF treatment) was separated by SDS–PAGE and blotted to nitrocellulose sheets, that were then probed with a monoclonal antibody recognizing both the forms of PI-PLCβ1. Bands were visualized by ECL.

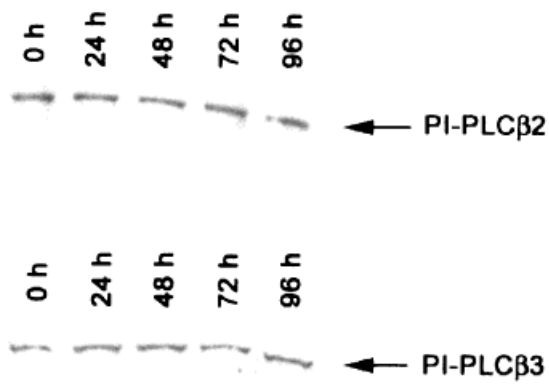

Fig. 4. Expression of PI-PLCβ2 or PI-PLCβ3 during NGF-induced differentiation of PC12 cells. A 80 μg of protein from whole cell homogenates (prepared at the indicated times of NGF treatment) was separated by SDS–PAGE and blotted to nitrocellulose sheets, that were then probed with polyclonal antibodies recognizing either PI-PLCβ2 or PI-PLCβ3. Bands were visualized by ECL.

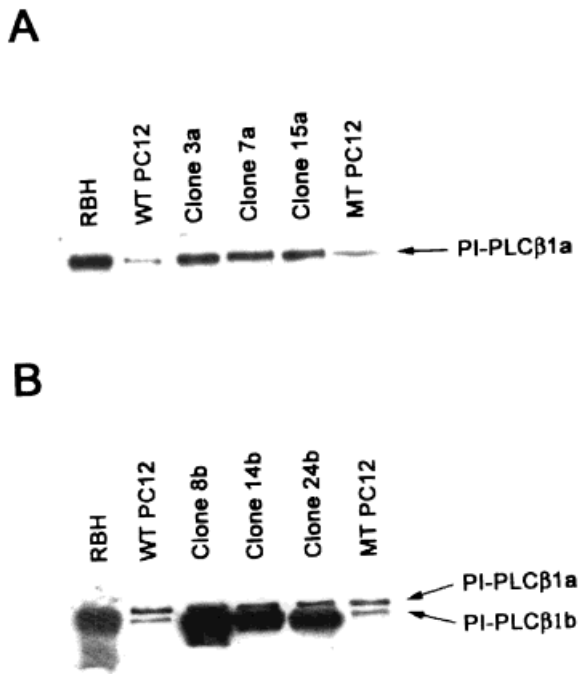

Fig. 5. Immunoblotting analysis of PI-PLCβ1 forms in wild type (WT) PC12 cells, stably transfected clones, and mock-transfected (MT) PC12 cells. A 80 μg of protein from whole cell homogenates was separated by SDS–PAGE and blotted to nitrocellulose sheets, that were then probed with either a polyclonal antibody to PI-PLCβ1a (**A**) or a monoclonal antibody recognizing both PI-PLCβ1a and b (**B**). Bands were visualized by ECL. RBH: rat brain homogenate.

PI-PLCβ1b but not of PLCβ1a. Once again, mock-transfected cells did not display any increase in either of the two proteins.

For immunofluorescence analysis we employed the monoclonal antibody to the N-terminus. It should be emphasized that for this set of experiments we deliberately chose a primary antibody concentration (1:100) which barely stained wild type cells whereas overexpressing clones appeared very brilliant. As presented in Figure 6, immunostaining revealed that in two representative stably-transfected clones (3a, overexpressing PI-PLCβ1a, and 14b, overexpressing PI-PLCβ1b) both the forms of the phospholipase were located in the cytoplasm. Similar results were obtained with the other clones (data not presented).

Increased Expression of PI-PLCβ1 Forms is Associated With Increased Rates of Phosphoinositide Hydrolysis In Vivo

To determine whether or not the increased expression of either PL-PLCβ1a or b was accompanied by an increased rate of inositol lipid hydrolysis in vivo, [^{3}H] inositol phosphate production was determined. In preliminary experiments, we ascertained that in all the different samples there was an equivalent incorporation of [^{3}H] inositol into lipid respect to protein. Moreover, radioactivity was recovered primarily in phosphatidylinositol (~94%), with the remainder present in phosphatidylinositol 4-phosphate and PtdIns(4,5)P_2 (data not presented). As shown in Table 1, direct stimulation of PI-PLC with the Ca^{2+} ionophore ionomycin produced an increase in inositol phosphate generation which was higher (about four-/six-fold) in clones overexpressing PI-PLCβ1 forms than in either wild-type or mock-transfected PC12 (about two-fold). Clones overexpressing PI-PLCβ1b produced a higher amount of inositol phosphates when compared to clones transfected with the PI-PLCβ1a

TABLE 1. Inositol Phosphate Production by Wild Type, Mock-Transfected, and Stably Transfected PC12 Cells

Sample	Inositol phosphates in unstimulated cells (% total dpm)	Inositol phosphates in stimulated cells (% total dpm)
Wild type cells	0.32 ± 0.04	0.63 ± 0.08
Mock-transfected cells	0.29 ± 0.04	0.57 ± 0.07
Clone 3a	0.61 ± 0.07	2.56 ± 0.29
Clone 7a	0.76 ± 0.07	3.02 ± 0.34
Clone 15a	0.70 ± 0.09	2.97 ± 0.39
Clone 8b	0.86 ± 0.09	4.93 ± 0.55
Clone 14b	0.89 ± 0.09	5.32 ± 0.61
Clone 24b	0.92 ± 0.08	5.93 ± 0.77

Cells were pre-labeled with 20 μCi/ml [^{3}H] inositol for 72 h. Intact cells were incubated in the presence or absence of 1 μM ionomycin at 37°C for 15 min. Inositol phosphates were quantified as described in Materials and Methods and expressed as the difference between the means of experimental and control replicates. Data shown are the mean from three different experiments ± SD.

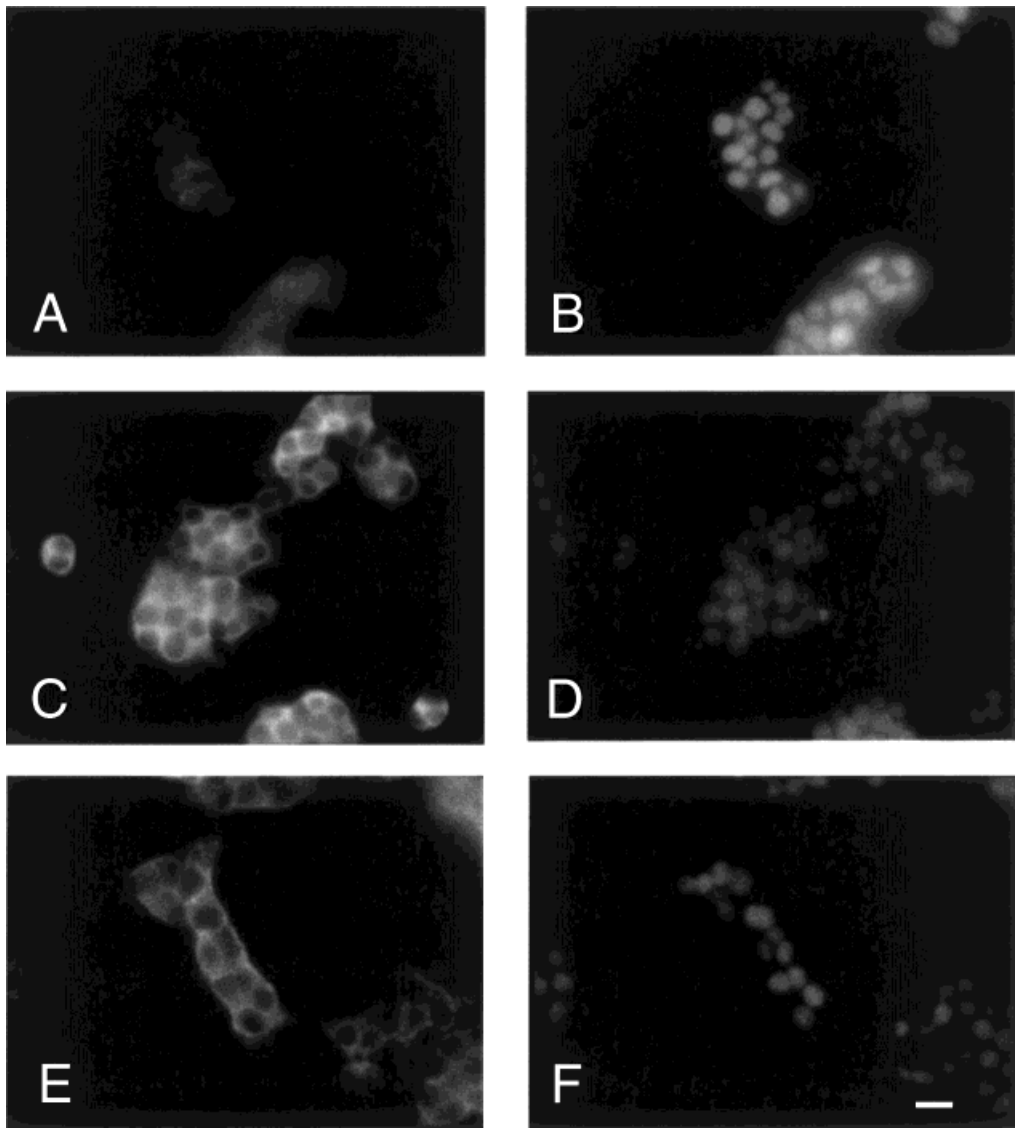

Fig. 6. Immunofluorescence staining of PI-PLCβ1 in wild type PC12 cells (**A**), clone 3a (**C**), and clone 14b (**E**). **B**, **D**, and **F** are the corresponding pictures showing DAPI staining. A monoclonal antibody recognizing both the forms of PI-PLCβ1 was employed for this set of experiments. Please note that for FITC fluorescence (A, C, E) the same exposure time was employed. Scale bar: 10 μm.

cDNA. Conceivably, this depends on the fact that the former clones synthesize a higher quantity of the enzyme.

Effect of PI-PLCβ1a or b Stable Overexpression on NGF-Induced Neuronal Differentiation

Next, we analyzed the effect of overexpressing PI-PLCβ1 forms on the NGF-induced differentiation of PC12 cells. However, as shown in Figure 7 and Table 2, no significant changes were seen in any of the clones along the differentiative pathway.

Effect of PI-PLCβ1a or b Stable Overexpression on Serum Deprivation-Induced Apoptosis of PC12 Cells

In a successive series of experiments, we analyzed whether or not the clones stably overexpressing PI-PLCβ1 forms were more resistant to cell death induced by serum starvation. Cell viability was measured by direct counting of viable cells using trypan blue dye exclusion [Mesner et al., 1992]. However, as shown in Figure 8, no significant differences in

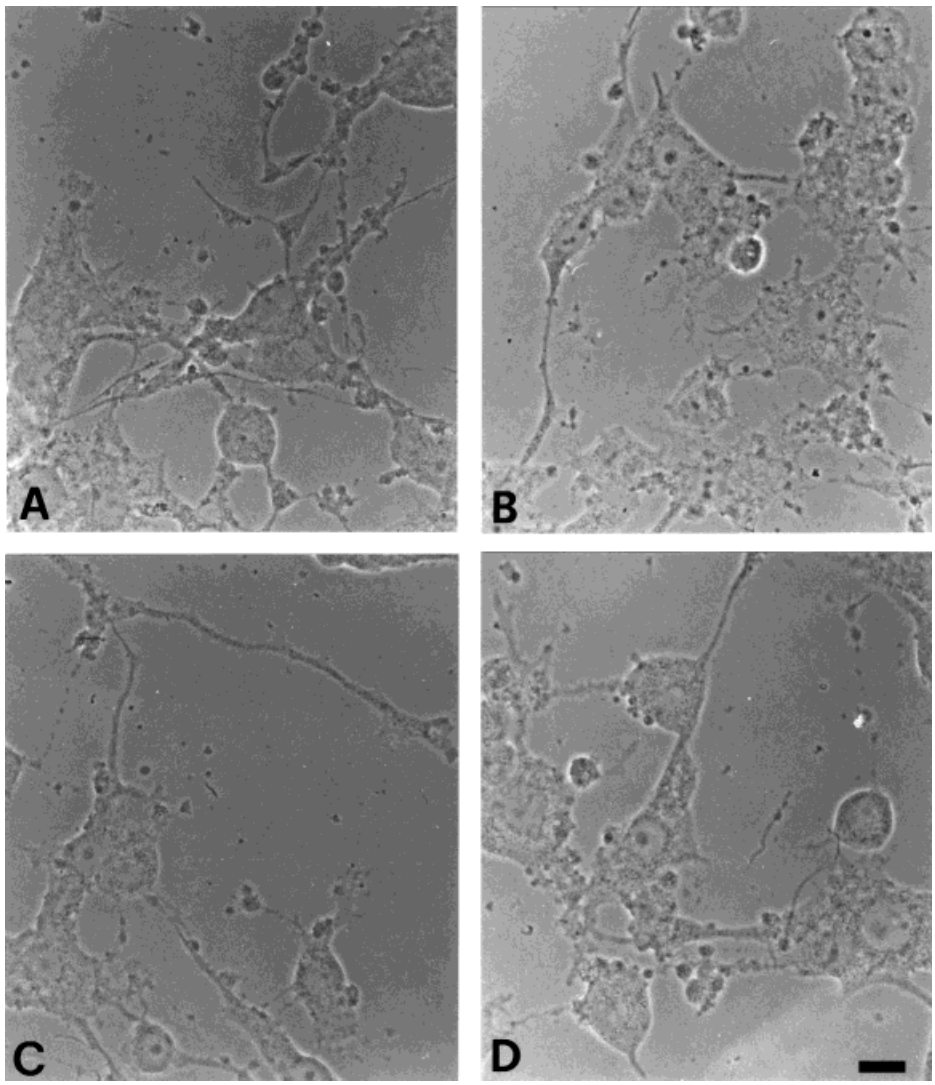

Fig. 7. Morphological assay of stably transfected cell clone differentiation in response to NGF (50 ng/ml for 4 days). **A:** clone 3a; **B:** Clone 7a; **C:** clone 8b; **D:** clone 14b. Also the two other clones gave similar results (not shown). Scale bar: 10 μm.

TABLE 2. Percentage of Cells With Neurites in Wild Type, Mock-Transfected, and Stably Transfected PC12 Cells Treated With 50 ng/ml NGF for Various Times

Sample	0 h	48 h	72 h	96 h
Wild type cells	2.4 ± 1.2	11.7 ± 2.3	48.9 ± 5.9	62.3 ± 7.4
Mock-transfected cells	1.8 ± 1.0	14.4 ± 3.9	54.4 ± 5.7	67.2 ± 8.9
Clone 3a	1.5 ± 1.3	12.8 ± 3.5	45.4 ± 5.0	61.1 ± 6.9
Cone 7a	2.7 ± 1.1	10.1 ± 3.3	53.8 ± 5.2	65.5 ± 8.8
Clone 15a	3.3 ± 1.6	9.8 ± 2.5	46.4 ± 4.9	64.8 ± 7.9
Clone 8b	1.7 ± 1.0	13.9 ± 3.8	49.1 ± 5.6	66.6 ± 8.1
Clone 14b	3.6 ± 1.9	13.2 ± 3.4	55.2 ± 5.4	65.7 ± 6.3
Clone 24b	3.1 ± 2.0	14.4 ± 4.0	46.7 ± 4.8	68.3 ± 7.1

Data are the mean from three different experiments ± SD.

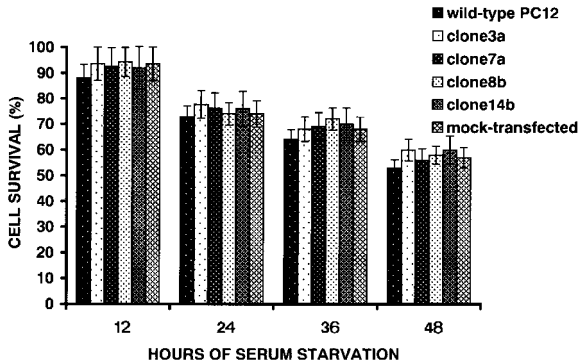

Fig. 8. Quantitative assay of PC12 cell survival to serum deprivation. Cells were serum-starved for the indicated times, then cell viability was assessed by the Trypan blue exclusion test. Results are the mean of three different experiments ± SD.

survival up to 2 days of serum starvation was detected between wild-type, mock-transfected cells, and four of the clones. After 48 h of serum starvation about 60% of cell were still viable, in agreement with the results reported by Mesner et al. [1992]. Also, the other clones gave similar results (not shown).

Effects of PI-PLCβ1a or b Stable Overexpression on Cell Cycle

Finally, we sought to determine whether overexpression of either PI-PLCβ1a or b had any effect on the cell cycle. As shown in Table 3, we did not detect any significant differences in the cell cycle, as analyzed by flow cytometry, between wild type PC12 cells and the over-expressing clones.

DISCUSSION

In response to NGF, PC12 cells cease division and differentiate into sympathetic neuron-like cells with extensive neurites [Greene et al., 1987]. The signaling pathway activated by NGF begins with the sequential activation of a Src-Ras cassette, leading to the activation of mitogen-activated protein (MAP) kinase [e.g. Lloyd and Wooten, 1992; Cowley et al., 1994]. Furthermore, NGF leads to the activation of both p38 kinase [Morooka and Nishida, 1998] and c-Jun N-terminal kinase [e.g., Minden et al., 1994]. In addition, phosphatidylinositol 3-kinase is activated and required for NGF-mediated differentiation [e.g., Jackson et al., 1996; Kobayashi et al., 1997; Neri et al., 1999b]. A wealth of evidence also suggests that atypical protein kinase C isoforms play an important role in the NGF-elicited neuronal differentiation of PC12 cells [e.g., Coleman and Wooten, 1994; Wooten et al., 2000]. NGF plays an important role in PC12 cell survival [e.g., Wert and Palfret, 2000; Shimoke and Chiba, 2001], but in this case it seems that MAP kinase is not required [e.g., Creedon et al., 1996; Klesse et al., 1999], even though other investigators have shown its requirements [Liu et al., 1999]. Following two recent reports dealing with increased expression of PI-PLCβ1 in relationship with neuronal differentiation [Hannan et al., 1998; Novak et al., 2000], we sought to determine whether or not an increased production of this PI-PLC isozyme could be linked to NGF-dependent differentiation of PC12 cells. It needs to be emphasized that in the two aforementioned investigations [Hannan et al., 1998; Novak et al., 2000], it was employed the polyclonal antibody which recognizes PI-PLCβ1a. However, we did not detect changes in the expression of both the forms of PI-PLCβ1 along the differentiation pathway induced by NGF up to 4 days. Moreover, stable overexpression of either PI-PLCβ1a or b did not influence

TABLE 3. Percent Distribution in the Various Phases of the Cell Cycle of Wild-Type, Mock-Transfected, and Stably Transfected PC12 Cells

Sample	G_1	S	G_2/M
Wild type cells	66.0 ± 8.2	21.6 ± 3.6	12.4 ± 2.3
Mock-transfected cells	63.9 ± 9.4	23.5 ± 4.0	12.6 ± 1.9
Clone 3a	67.8 ± 9.9	22.8 ± 3.8	9.4 ± 2.1
Clone 7a	70.1 ± 10.3	19.9 ± 3.5	10.0 ± 1.7
Clone 15a	65.8 ± 8.3	24.1 ± 4.2	10.1 ± 2.0
Clone 8b	74.2 ± 10.9	18.5 ± 3.7	7.3 ± 1.8
Clone 14b	68.8 ± 10.2	22.7 ± 4.4	8.5 ± 2.2
Clone 24b	71.7 ± 9.8	20.4 ± 3.9	7.9 ± 2.5

Cells were analyzed the day after seeding in fresh medium. Data were obtained by flow cytometric analysis and are the mean from three different experiments ± SD.

the number of PC12 cells that differentiated upon exposure to NGF, even though, by measuring in vivo phosphoinositide hydrolysis, we determined that the overexpressed PI-PLCβ1 forms were functionally active.

There are at least two possible explanations for this discrepancy. First of all, PC12 cells is a cell line originally established from a rat pheochromocytoma. As such, they derive from adrenal medulla and neural crest [Greene et al., 1987]. Hannan et al. [1998] investigated the expression of PI-PLCβ1 in the neurons of rat somatosensory cortex while Novak et al. [2000] studied NT2 cells. NT2 cells are a human embryonal carcinoma cell line which can be differentiated into post-mitotic neuron-like cells upon exposure to retinoic acid. Interestingly, when grafted into mouse brain they differentiate into both neuron- and oligodendrocyte-like cell types [Ferrari et al., 2000]. Both cortical neurons and NT2 cells derive from the neural tube. Therefore, the different embryonal origin could also explain the different results. Moreover, also the different inducer of neuronal differentiation could play an important role, given that PC12 cells respond to NGF, whereas NT2 are sensitive to retinoic acid and cortical neurons presumably respond to a number of physiological factors (collectively referred to as neurotrophins) that are present in vivo during maturation of cerebral hemispheres [e.g., Cavanaugh et al., 2001]. Interestingly, two of these factors are IGF-I [Niblock et al., 2000] and NGF [e.g., McAllister et al., 1995; Zhou et al., 1996]. IGF-I and insulin are capable of activating PI-PLCβ1 through a MAP kinase dependent phosphorylation [Martelli et al., 1999b; Martelli et al., 2000; Xu et al., 2001]. Moreover, disruption of PI-PLCβ1 signaling negatively affects the MAP kinase pathway [Beckman et al., 1998]. Also the NGF signaling cascade leads to the activation of MAP kinase [e.g., Lloyd and Wooten, 1992; Cowley et al., 1994], so that an involvement of a PI-PLCβ1 during PC12 cell differentiation would not have been unprecedented. However, it should be reminded that NGF blocks proliferation of PC12 cells [e.g., Bang et al., 2001], whereas, when IGF-I activates PI-PLCβ1, there is a concomitant increase in cell proliferation [Martelli et al., 1999b; Martelli et al., 2000]. The fact that in stably-transfected PC12 cell clones overexpressing PI-PLCβ1a or b, both of these proteins were located in the cytoplasm and not in the nucleus is consistent with the recent findings of others who have studied the localization of these subtypes in transiently transfected PC12 cells [Caricasole et al., 2000]. In contrast, in rat C6Bu-1 glioma cells PI-PLCβ1b was found by immunoblotting and immunocytochemistry to be predominantly located within the nucleus, while PI-PLCβ1a was located preferentially in the cytosol [Bahk et al., 1998]. In MEL cells, PI-PLCβ1b was exclusively nuclear, whereas PI-PLCβ1a was predominantly located within the nucleus [Faenza et al., 2000]. In these cells, overexpression of PI-PLCβ1a blocked the erythroid differentiation induced by dimethyl sulfoxide (DMSO) [Matteucci et al., 1998], while stable overexpression of either PI-PLCβ1a and PI-PLCβ1b led to enhanced synthesis of both cyclin D3 and its kinase, cdk4 even during serum starvation [Faenza et al., 2000]. Moreover, MEL cells stably overexpressing either of the two forms of PI-PLCβ1 were able to proliferate in the absence of serum. We have been unable to reproduce these results in PC12 cells, because our overexpressing clones did not showed differences in NGF-induced differentiation, survival to serum starvation, and cell cycle. A possible explanation for our different findings, other than the different cell line employed, is that only when present within the nucleus PI-PLCβ1 is capable of influencing cell proliferation and differentiation, as well as cell survival. In this sense, our results resemble those obtained by others [Matteucci et al., 1998; Faenza et al., 2000] who overexpressed in MEL cells a mutated form of PI-PLCβ1a (referred to as M2b) which does not localize to the nucleus [Kim et al., 1996]. Cell clones overexpressing such a mutated form still differentiated along the erythroid pathway in response to DMSO, and neither proliferated in the absence of serum nor overexpressed cyclin D3.

In conclusion, we have demonstrated that expression of PI-PLCβ1 forms could not be related to the NGF-induced neuronal differentiation of PC12 cells, and that such an isozyme is not involved either in cell survival or cell cycle control in this cell line. We have also shown that the nuclear localization of PI-PLCβ1 does not occur universally, but, conceivably, this observation strengthens the contention that, when it is located within the nucleus, this isoform of PI-PLC markedly affects the growth characteristics of cells.

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