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DISCRETE SUBCELLULAR LOCALIZATION OF PHOSPHOINOSITIDASE C β, γ AND δ IN PC12 RAT PHEOCHROMOCYTOMA CELLS

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Phosphoinositidase C activity was revealed in nuclei isolated from PC12 rat pheochromocytoma cells incubated with tritiated phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate. Phosphoinositide breakdown was found to be optimal at neutral pH and Ca^{++} concentrations ranging from endogenous levels to millimolar values. To characterize the enzymes involved, three monoclonal antibodies directed against the β, γ and δ phosphoinositidase C isoforms were employed. A combination of Western blot immunochemical analysis on cytoplasmic and nuclear fractions and of *in situ* immunocytochemistry on intact cells and isolated nuclei indicated that phosphoinositidase C γ, though predominantly cytoplasmic, was present in both cell compartments. On the contrary, phosphoinositidase C β was exclusively localized in the nucleus, whereas phosphoinositidase C δ was restricted to the cytoplasm. These data suggest that inositol lipid breakdown is controlled by different phosphoinositidase C isozymes in the various cell compartments, and support the notion that a separate phosphoinositide signalling system is located in the nucleus.

Receptor occupancy by many types of cell agonists activates a lipid dependent intracellular signalling involving the hydrolysis of PIP_2 by PIC in the plasma membrane, with the consequent production of DG and IP_3, which activate PKC and mobilize Ca^{++} from intracellular stores, respectively (1). Some of these events take also place in the cell nucleus, where the existence of inositol lipids and related enzymes (2-5) and their agonist dependent modulation has led to the contention that a nuclear inositide signalling is operative and responds to extracellular stimuli (6-12). This notion is also supported by the evidence that PKC is located in the nucleus as well (13,14), and is translocated to the nuclear compartment and activated after treatment of various cell types with mitogens and differentiating agents (10,15-19).

Considerable progress has been made in the studies of PICs, and it is rather well established that there are at least five distinct forms of the enzyme (20). However, despite the number of reports

Abbreviations. PIC, phosphoinositidase C ; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; DG, 1,2-diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; PKC, protein kinase C; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HS, horse serum.

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concerning phosphoinositide breakdown in nuclei (3,5,6,10,21), the phosphodiesterases involved have not been identified. The work reported here has approached the subcellular distribution of PIC β, γ and δ in PC12 rat pheochromocytoma cells, which are one of the few cell lines expressing these three isoforms of the enzyme (22). The PICs of nuclear and cytoplasmic fractions have been identified by Western blot and immunocytochemical analysis employing specific monoclonal antibodies (23), and characterized for pH and Ca^{++} dependency of hydrolytic activity.

MATERIALS AND METHODS

Cell culture and fractionation. All culture media were from Biochrom KG (Berlin, Germany). PC12 cells (obtained from dr. G. Ferrari, Fidia, Abano Terme, Italy) were grown as monolayers in DMEM medium supplemented with 5% FCS, 10% HS, 50 μg/ml streptomycin, 50 U/ml penicillin at 37° C in a humidified atmosphere containing 7.5% CO_2. Twice a week cells were subcultured and the medium was changed. After synchronization with 5 μg/ml aphidicolin (Sigma Chemical Co, St. Louis, Mo) for 24 hrs, followed by several washes with fresh medium, the cells were either employed for *in situ* immunocytochemistry or fractionation studies. Nuclei were isolated as described earlier for Friend leukemia cells (6), except that 0.5% NP-40 substituted for Triton X-100 and 50 μg/ml leupeptin, 50 μg/ml soybean trypsin inhibitor, 1 μg/ml E 64, 2 μg/ml aprotinin (all from Sigma) were present in the cell fractionation buffers. When indicated, 50 μg/ml of calpain inhibitor I and II (Boehringer Mannhein, Germany) were also included. Cytoplasm was obtained by the same procedure, and consisted of the postnuclear supernatant after cell homogenization in the absence of detergent. Purity of nuclei was assessed by morphological and biochemical criteria (5).

Western blot analysis. Proteins from whole cells, cytoplasmic and nuclear fractions (35 μg), were separated on 7.5% polyacrylamide denaturing gels (24) and blotted to Trans-Blot nitrocellulose membrane (Bio Rad Laboratories, Richmond, Calif.). Monoclonal antibodies against the three isoforms β, γ and δ of PIC, originally developed by Rhee and coworkers (23), were purchased from UBI (Lake Placid, NY), and employed according to ref. 23. Alkaline phosphatase-coniugated anti-mouse Ig G (Sigma) was used as secondary antibody (19).

***In situ* immunocytochemical analysis.** PC12 cells were grown on glass coverslips coated in a row with Ca^{++} and Mg^{++}, poly-L-lysine and collagen. After synchronization, cells were fixed in freshly made 4% paraformaldehyde/67 mM PBS pH 7.3 for 30 min at room temperature, and permeabilized at -20° C in pure methanol or 0.2 % Triton X-100 for 8 min with similar results. Isolated nuclei were placed on poly-L-lysine coated glass microscopy slides and fixed with the same solution containing 5mM $MgCl_2$, 0.5 mM PMSF. Immunocytochemical labelling was then performed as previously described (19).

Assay of phosphoinositide-specific phospholipase C. Nuclei and cytoplasm (50 μg protein) from PC12 cells were incubated in a final volume of 100 μl for 30 min at 37° C in the presence of 3 nmoles of either [^{3}H]PI, [^{3}H]PIP or [^{3}H]PIP_2 (15,000 dpm/nmol, Amersham, UK). The incubation included 0.06 % Na taurodeoxycholate, 0.6 % NaCl and, for the different pH values, either 100 mM Mes (pH range 5-6.5) or 100 mM Tris-HCl (pH range 7-8). Free Ca^{++} concentration was stabilized using Ca^{++}-EGTA buffers (25). Where indicated, endogenous Ca^{++} was chelated with 2 mM EGTA.

The reaction was stopped by addition of chloroform:methanol:conc. HCl (200:100:0.75, v/v), and organic and aqueous phases were separated (26). Water-soluble radioactivity was determined by direct counting of the dryed aqueous phases.

RESULTS

In Western blots, the three antibodies specifically localized the β, γ and δ PIC isoforms in PC12 cell fractions (Fig. 1). The β and δ antibodies recognized a single band present only in nuclei and

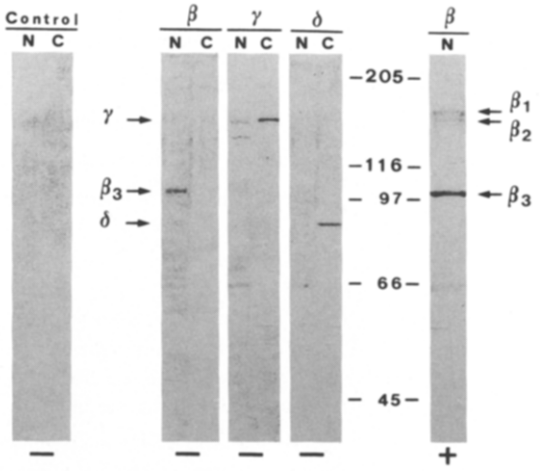

Figure 1. Western blot analysis of the localization of PIC β, γ and δ in PC12 cell fractions, and comparison of PIC β pattern in nuclei extracted in the absence (-) or presence (+) of calpain inhibitors. Antibody dilution was 1:250 for anti β and δ, and 1:500 for anti γ.
N, nuclei; C, cytoplasm; Control, secondary antibody only.
Molecular weight markers are indicated in Kd.

cytoplasm respectively, whereas the reactivity to the γ antibody, though mainly cytoplasmic, was present in both cell compartments. While the γ and δ isoforms displayed the native size of 145 and 85 Kd, the β isozyme showed a molecular weight of 100 Kd, corresponding to the calpain cleavage product β3 (20). When calpain inhibitors were used, PIC β was partly protected from proteolytic degradation, and the doublet of β1 (150 Kd) and β2 (145 Kd) bands appeared, in agreement with the molecular mass reported for the β form immunoprecipitated from PC12 cell lysates with the same antibody (22). In the nuclear fraction, all antibodies recognized additional bands in the 65-70 Kd region, and the anti-PIC γ also reacted with a protein of approximately 130 Kd.

Additional proof of the subcellular distribution of the three PIC isoforms was obtained by *in situ* immunocytochemical analysis (Fig. 2). The cytoplasm of cells reacted with the anti- PIC γ and δ, while no staining occurred with the anti-PIC β. In the same intact cells, the nuclei resulted stained with the antibodies against PIC β and, even though to a lower extent, PIC γ, but not with the anti-PIC δ. Further confirmation of this intracellular pattern was obtained by immunocytochemical analysis on isolated nuclei, wich reacted only with the anti PIC β and γ.

PIC activity in cytoplasm and nuclei showed a pH optimum near 7 for the three substrates PI, PIP and PIP_2. Compared to cytoplasm, the nuclei displayed a higher specific activity for PIP and PIP_2 hydrolysis in the pH range 7-8 and with all Ca^{++} concentrations. Both nuclear and cytoplasmic enzymes were near maximally active with Ca^{++} concentrations ranging from endogenous levels to 10^{-3} M for the preferred substrates PIP and PIP_2. PI phosphoinositidase activity increased with calcium for cytoplasm, while for nuclei an optimum was found at 10^{-4} M. Since the predominant nuclear PIC form was the cleaved β3 fragment, it can be concluded that proteolytic digestion of the native β enzyme results in no loss of catalytic activity. The overall substrate specificity was in the order PI<PIP< PIP_2, with an increase from PI to PIP larger than from PIP to PIP_2, regardless of the enzyme source and the assay conditions (Fig. 3).

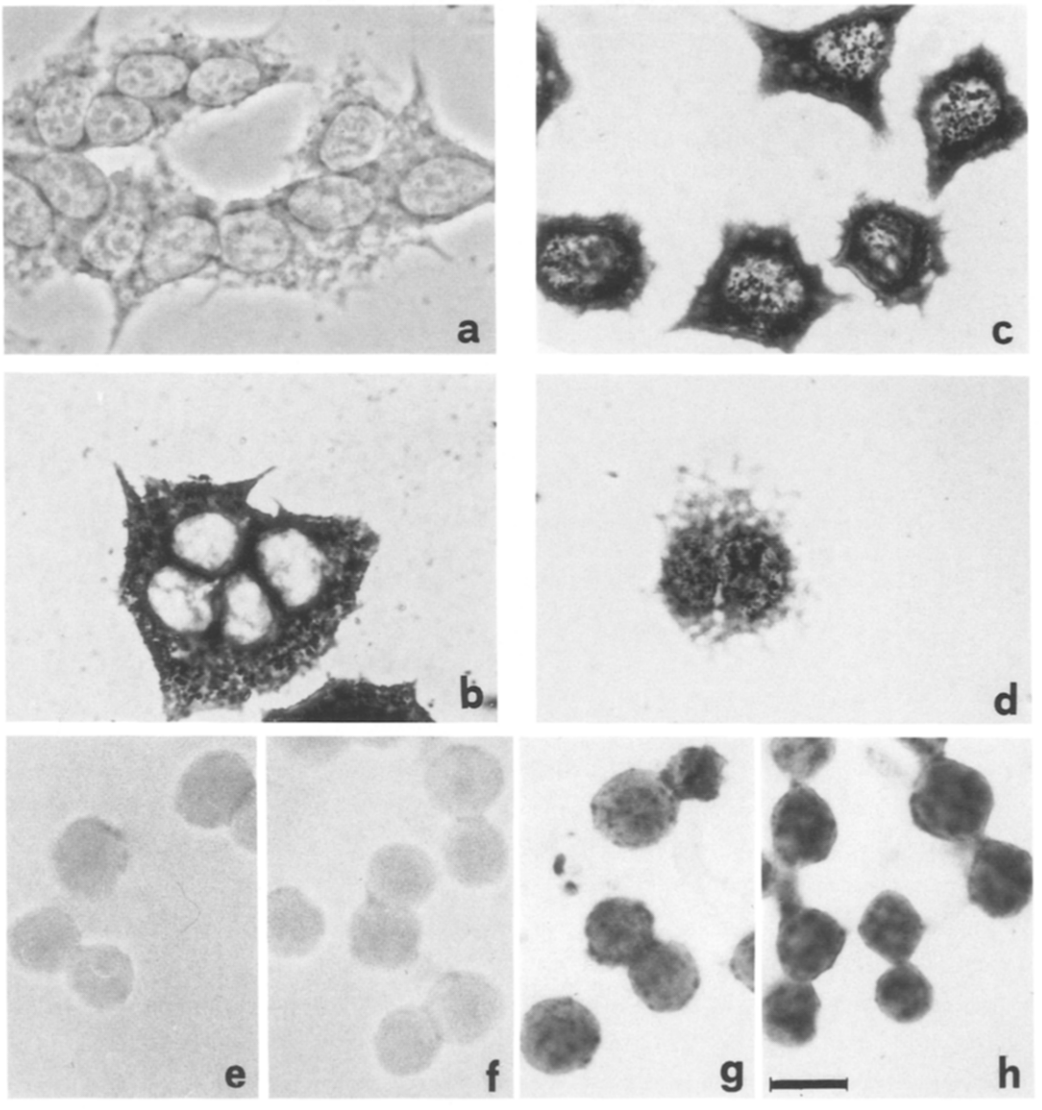

Figure 2. Immunocytochemical localization of phosphoinsitidases C in whole PC12 cells (a-d) and in isolated nuclei (e-h). a,e: Negative controls (not incubated with first antibody). b,f: Anti-PIC δ (dil. 1:25). c,g: Anti-PIC γ (dil. 1:50). d,h: Anti-PIC β (dil. 1:12). Note that, even though the γ isoform stains all cell compartments, the intensity of the labelling is several fold higher in the cytoplasm, whereas the β and δ isoforms are only nuclear and cytoplasmic, respectively.
Bar: 5 μm.

DISCUSSION

In current signal transduction models, PIC catalyzes PIP_2 breakdown at the plasma membrane in response to agonist-receptor coupling. The evidence that several PIC isozymes exist in most cell types, and some of them are cytosolic (see ref.20 for rewiew), has addressed the question of cellular localization and specific function of the different enzyme isoforms. In recent years, an additional signalling mechanism located in the nucleus has been proposed, on the basis of evidence indicating that an inositide pathway, distinct from that found in the plasma membrane, operates at the nuclear

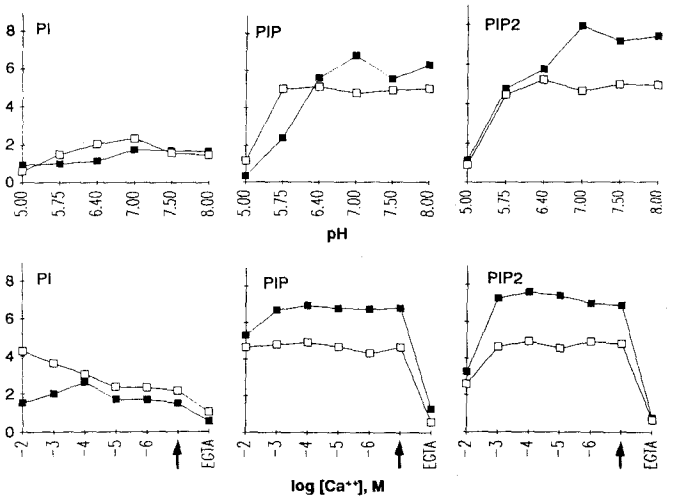

Figure 3. pH and Ca^{++} dependency of PIC activity on exogenous tritiated PI, PIP and PIP_2 in isolated nuclei and cytoplasmic fractions from PC12 cells. Ordinate indicates the recovery of labelled acqueous compounds, expressed as dpm/mg protein x 10^{-5}. The results are the average of at least 3 experiments, with standard deviation < 9%. The arrows indicate endogenous Ca^{++} only.
■—■ nuclei; □—□ cytoplasm.

level producing PKC-mediated effects in response to extracellular agonists (4-13,27). A yet unresolved question concerns the nature of the enzymes responsible for polyphosphoinositide breakdown in the nucleus, even though we and others have reported the existence of hydrolytic activities in membrane-deprived nuclei (5,6,21,27,28).

From the present data, obtained with monoclonal antibodies employed in a number of recent studies (20,22,23,29-32), a discrete distribution of three of the different PIC isoforms is evident, and particularly intriguing appears the nuclear localization of PIC β. A cytoplasmic β form was not recognized by the antibody employed, and its existence remains to be determined. Similar findings have been reported by our group in Swiss 3T3 cells (33) and, for the γ isoform, in rat embryo fibroblasts by other investigators (29).

Since the nuclei employed are completely stripped of the nuclear membrane, nuclear PIC β and γ appear membrane unrelated, as reported, on the other hand, for many components of the lipid-dependent nuclear signalling (3,5,6,11,27).

The regulation of nuclear PICs remains an open question. However, the modulatory mechanisms as yet identified could be effective also at the nuclear level, i.e. tyrosine phosphorylation for PIC γ (22,34), PKC-dependent serine phosphorylation of PIC γ and β (20,23), and G-protein coupling for PIC β (35,36). A potential basis for such nuclear modulation stems from the evidence that G-proteins are associated to the nuclear lamina (37) and PKC is also a nuclear enzyme (9,13-15).

Our data add to the widely demonstrated complexity of the PIC family the specificity of cellular localization. This supports the contention that the various agonists may generate diversified cellular responses by selective activation of different PIC isozymes, including the forms involved in nuclear inositol lipid metabolism.

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