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REGULATION OF PROTEIN KINASE C IN NG108-15 CELL DIFFERENTIATION

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Summary: The involvement of PKC in NG108-15 cell differentiation was investigated. Differentiation with dBcAMP was associated with a decrease in total cellular phorbol ester binding. The histone-directed PKC activity was decreased in the soluble fraction. Northern and Western blotting revealed the presence of only PKC α but not PKC β and PKCγ among the calcium-dependent isoforms. Differentiation induced a decrease of cytosolic PKC α immunoreactivity, with no changes of mRNA content or appearance of PKC β and PKC γ isoforms. The low levels of PKCα in the soluble fraction suggest that the mRNA for this species is less efficiently translated in differentiated NG108-15 cells. The data suggest that down-regulation of PKC α protein and kinase activity are associated with induction of neuronal morphology in NG108-15 cells.

Protein kinase C (PKC) is a family of serine/threonine kinases composed of at least 10 distinct isoforms, 4 of which, the α, βI, βII and γ species are dependent on calcium ions for full activation (1). Protein kinases C are directly involved in cell proliferation, differentiation and specific phenotype expression (2-5). For example SK-N-SH cells differentiation to a neuronal or fibroblast phenotype is associated with a decrease or an increase of PKC activity, respectively (6).

Neuronal differentiation has been associated with changes in the activity of PKC and in the expression of specific isoenzymes (6-9). Different results however have been reported depending upon the cell line and the type of differentiating agent used. In LAN-5 neuroblastoma cells, the differentiation with interferon γ induces increased PKC activity (9) while in SH-SY5Y and Neuro 2a neuroblastoma cells, differentiation with either PKC activators (used in conditions

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Abbreviations: PKC, protein kinase C; dBcAMP, dibutyryl cyclic AMP; PdBu, phorbol 12,13-dibutyrate; FCS, fetal calf serum; BSA, bovine serum albumine; PBS, phosphate-buffered saline.

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leading to down-regulation of PKC) or inhibitors is associated with a decrease in PKC activity and selected alteration in isoenzyme expression (8,10-12). In this case the changes in enzyme activity and levels, in addition to the association with cell differentiation, may be a consequence of a direct interaction of the differentiating agent with the cellular PKC system.

We have used NG 108-15 cells as a model to study neuronal differentiation. This clone, upon treatment with agents increasing intracellular cAMP levels, such as.dBcAMP, differentiates to a neuronal phenotype by extending neurites and developing the potential of forming functional synapses with myotubes (13,14). The differentiated phenotype is associated with an increase in voltage-dependent calcium channels of L and N type and with an increase in calcium fluxes (14-16). In NG108-15 cells PKC has been previously characterized in non-differentiated conditions where it regulates phosphatidylcholine biosynthesis (17) and voltage-dependent calcium channel function (18). In addition, NG108-15 cells have been used to investigate neurite extension induced by the combined action of dBcAMP and phorbol esters; in this case PKC activity was inversely correlated with the extension of neurite processes (19).

The aim of this study was to characterize the PKC system in NG108-15 cells and to examine the effect of treatments leading to differentiation with agents not directly affecting PKC. For this purpose we have used dBcAMP alone as differentiating agent. The PKC system has been investigated at multiple levels, including phorbol ester binding, histone-directed activity and calcium-dependent isoenzyme expression at both the mRNA and protein levels.

MATERIALS AND METHODS

Cell culture and chemicals The neuroblastoma x glioma hybrid, NG 108-15 cells, were cultured in DMEM supplemented with 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 0.1 mM hypoxantine, 10 µM aminopterin and 17 µM thymidine. Differentiation was induced by treatment for 5 days with 1 mM dBcAMP and by reducing the FCS concentration to 2% (13,15). Non differentiated cells were used in the logarithmically phase of growth. Differentiated cells stopped dividing and had rounded cell bodies with extensive neurite outgrowth. From a biochemical point of view such cells expressed higher levels of voltage-dependent calcium channels of L and N type, as previously observed (15). The cells had been passed 26-30 times when they were used in the experiments. Cell culture reagents, peptides PKC_{19-36} and [Ser^{25}] PKC_{19-31} were from GIBCO BRL (Mascia Brunelli, Milano). Electrophoresis reagents were from Bio-Rad (Segrate, Milano). All other reagents were of biochemistry or molecular biology grade unless specified and were obtained from Sigma (Sigma Chimica , Milano).

[^{3}H]-PdBu binding After removing growth medium, cells were washed with PBS, detached with a rubber policeman and washed twice with PBS by low speed centrifugation. Cells were homogenized with a Polytron in PBS containing calcium to facilitate transfer of soluble PKC to the membrane fraction (20, 21) and centrifuged at 48,000 x g for 15 min. The binding assay was carried out in a final volume of 1 ml PBS containing 50 µg of cell membrane extract protein, 1 mg/ml BSA and [^{3}H]-PdBu (S.A. 13.2 Ci/mmol, Dupont NEN) for 60 min at 25°C. Non-specific binding was assessed in the presence of 10 µM unlabeled PdBu. The incubation was stopped by filtration through Whatman GF/C filters pre-soaked for 15 min in 0.5% polyethyleneimine and washed with cold PBS. Bound radioactivity was determined with Formula 989 (Dupont NEN) scintillation fluid.

Protein kinase C activity assay The cell pellets were resuspended in homogenization buffer containing 20 mM Tris (pH 7.4), 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, 1 mM PMSF, 20 µg/ml leupeptin (HB). The samples were homogenized with a glass-Teflon pestle and the

soluble and particulate fractions prepared as described (22), with the exception that the latter fractions were obtained by solubilizing the pellet with 3-[(3-cholamidopropoyl)-dimethylammonio]-1-propane-sulfonate (CHAPS) (10 mg/ml) under constant stirring for 45 min at 4°C. The fractions were partially purified through DEAE 52 (Whatman) columns (500 µl bed volume, every 10^7 cells). The column was washed and partially purified PKC was eluted with buffer containing 120 mM NaCl. PKC activity was measured using histone Type H1 III S or peptide [Ser^{25}]PKC $_{19-31}$ as substrates, in the presence of 2 mM calcium chloride, 0.2 µg diolein and 2.0 µg phosphatidylserine. Basal activity was measured by replacing the calcium, diolein and phosphatidylserine with 2mM EGTA. Incubations were performed for 10 min with 4-8 µg of eluted protein at 30 °C in a final volume of 250 µl. The reaction buffer consisted of 60 mM Tris-HCl (pH 7.5), 15 mM magnesium acetate, 20 µM[γ-^{32}P]-ATP (10^5 cpm/nmol) (S.A. >5000 Ci/mmol, Amersham). Proteins were measured using the micromethod of Bradford (23) using BSA as a standard.

RNA extraction and Northern blotting Cell pellets were resuspended in 4M guanidinium isothiocyanate and centrifuged at 12,000xg for 30 min at 4°C. The supernatant was ultracentrifuged through 5.7 M cesium chloride. Total RNA was separated on 1% agarose/2.2M formaldehyde gel and transferred to nitrocellulose paper. Filters were prehybridized overnight in 50% formamide, 4xSSC (1xSSC is 0.15 M NaCl/0.015M sodium citrate pH 7.4), 0.5 mg/ml salmon sperm DNA, 1x Denhardt's solution, 0.25 mg/ml tRNA and 10% dextran sulfate. Hybridization was performed at 37°C for 18 hrs with labeled probes ($2x10^6$ cpm/ml). Filters were then washed at 55°C four times with 1xSSC/0.1% SDS and dried (24). Dehybridization was performed with hot 0.05xSSC, 10 mM EDTA (pH 8.0), 0.1% SDS for 15 min followed by wash with 0.01xSSC at room temperature. Synthetic oligonucleotides for PKCα, γ and for β-actin were purchased from Dupont NEN; for PKCβ the oligonucleotide complementary to bases 2171-2218 (25) was used. The probes were 3' end-labeled using terminal deoxynucleotidyl transferase (Promega, Firenze) and [α ^{32}P] dATP (>3000 Ci/mmol, Dupont NEN) as described (24). These probes have been reported to react with murine tissues (26). β-actin mRNA was used as an index non influenced by differentiation (27) for controlling RNA loading. Densitometric analysis was performed using the program Image 1.44 for digital image processing (Wayne Rasband, NIMH, Bethesda, USA).

SDS-PAGE and Western blotting Cell pellets were sonicated in HB then centrifuged at 100,000 x g for 60 min. The supernatant represented the soluble fraction, while the particulate fraction was solubilized utilizing HB containing 0.2% Triton X-100 with constant stirring for 45 min at 4°C, sonicated and centrifuged as described above. Aliquots of the fractions were taken for protein assay while the remainder was diluted with sample buffer and boiled for 5 min (28). Proteins were loaded onto a 10% SDS-PAGE gel, electrophoresed, and transferred by electroblot to nitrocellulose. After incubation for 1 hr in TBS (TBS is 50 mM NaCl/2 mM Tris pH 7.5) containing 0.02 % Tween 20 (TBST), the nitrocellulose sheets were incubated for an additional hr in TBST containing 5% non-fat dry milk. After incubation overnight at 4°C with specific antisera (PKCα 1: 2000, PKCβI, βII and γ 1: 500) (29), filters were washed twice in TBST and then in TBS each for 10 minutes. Samples were incubated with secondary antiserum (goat antirabbit IgG conjugated with alkaline phosphatase at 1:3000 dilution) for 1 hr. The nitrocellulose membrane was finally washed and color developed with nitroblue tetrazolium chloride and 5-bromo-chloro-3-indolylphosphate-p-toluidine salt. Rat brain tissue was run in parallel as a positive control (29). Densitometric analysis was performed as described in the previous section. For specificity of immunoblotting cellular extracts were run in presence of the appropriate antigenic peptide (2 µg/ml) added to the respective primary PKC antisera just before addition to the blot.

RESULTS

PKC was first evaluated by analyzing [^{3}H]-PdBu binding to NG108-15 cell membrane extracts. Upon differentiation, the binding at two different concentrations of [^{3}H]-PdBu was decreased (-45 and -44% at 21 and 46 nM free ligand respectively) (Table I). PKC activity was also examined using different PKC phosphorylation substrates and the highly specific inhibitor

Table I

Binding of [^{3}H]-PdBu to NG 108-15 cells: effect of differentiation

	[^{3}H]-PdBu binding	
	Cell membrane extracts (fmol/mg prot)	
Free [^{3}H]-PdBu concentration	21 nM	46 nM
Non-differentiated	3083	3500
dBcAMP differentiated	1687 (-45)	1944 (-44)

Values are the means of two independent experiments utilizing duplicate samples for specific and non specific binding. The percentage variation with respect to non-differentiated cells is enclosed in parentheses.

PKC$_{19-36}$ (30). Partially purified proteins from the soluble fraction of non-differentiated NG108-15 cells phosphorylated both histone and the specific PKC peptide substrate [Ser^{25}] PKC$_{19-31}$. At a substrate concentration of 10 μM, peptide-directed phosphorylation was three times higher when compared with histone phosphorylation (1604 versus 528 pmol/min/mg prot). The peptide-directed phosphorylation was inhibited by PKC$_{19-36}$ (30) with an IC$_{50}$ of 18 μM; similar results were observed with particulate partially purified proteins (data not shown). As routine assay for the subsequent experiments histone was used as substrate.

Differentiation of NG108-15 cells with dBcAMP treatment was accompanied by a decrease in histone-directed PKC activity in the soluble fraction with no modification in the membrane fraction (Figure 1). To analyze the PKC isoforms involved in NG 108-15 cell differentiation, we determined which of the various calcium-dependent PKC isoforms were expressed in these cells.

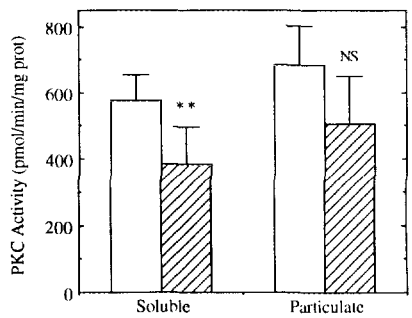

Figure 1.
PKC activity in soluble and particulate fractions in non-differentiated (open columns) and dBcAMP-differentiated (hatched columns) NG 108-15 cells.
The values reported are the means ± S.E.M. of 7 different experiments.
** $p < 0.02$, NS not significantly different, paired Student's t test.

Table II

PKCα mRNA and protein expression in NG 108-15 cells

	PKCα		
		Protein	
	mRNA (%)	Soluble (%)	Particulate (%)
Non-differentiated	0.662 (100)	1085 (100)	735 (100)
dBcAMP differentiated	0.644 (97)	836 (77)	699 (95)

mRNA values are expressed as ratio of the optical density peaks for mRNA for PKCα and β–actin, respectively. Protein values are expressed as areas (in pixels) of the optical density peaks. All values are from a representative experiment out of two with similar results.

Northern blot analyses indicated that the PKCα gene was expressed as a major and an occasional minor transcript at 4 and 9 kb, respectively. Similar sized PKCα mRNA species were also detected in rat brain (Figure 2A). Cell differentiation was not associated with any changes in the expression of the 4 kb PKCα transcript (Table II). The mRNAs for PKCβ and γ were not detected in NG108-15 cells under either differentiated or non-differentiated states. The presence of PKCα was confirmed by immunoblot with antiserum specific for PKCα (Figure 2B). NG108-15 cells contained a 80 kDa PKCα immunoreactive material which was the same size as that found in rat brain. This PKCα immunoreactive band in the cells was successfully competed by preabsorbtion with the immunizing peptide (Figure 2B). In accordance with the PKC activity data cellular differentiation was associated with a decrease in PKCα immunoreactivity in soluble but not in particulate fractions (Table II). In contrast to the Western blot PKCα results, there was no evidence that PKC βI, βII or γ were present in differentiated or non differentiated NG108-15 cells (Figure 2B).

DISCUSSION

The results show that following differentiation the [^{3}H]-PdBu binding to NG 108-15 cell membrane extract (representing the soluble+membrane PKC pool) is decreased upon differentiation. This observation is in agreement with the PKC activity data in which soluble PKC is decreased. In addition the kinase activity measurements agree with data obtained in other murine and human neuroblastoma differentiated cells (6,8). In NG108-15 cells Weeks and coworkers have proposed a role for decreased protein phosphorylation in neuritogenesis (31); the data here presented are in agreement with that proposal.

The fact that the changes are observed only in soluble but not in particulate PKC activity may indicate that in differentiated cells there is not enzyme redistribution between soluble and

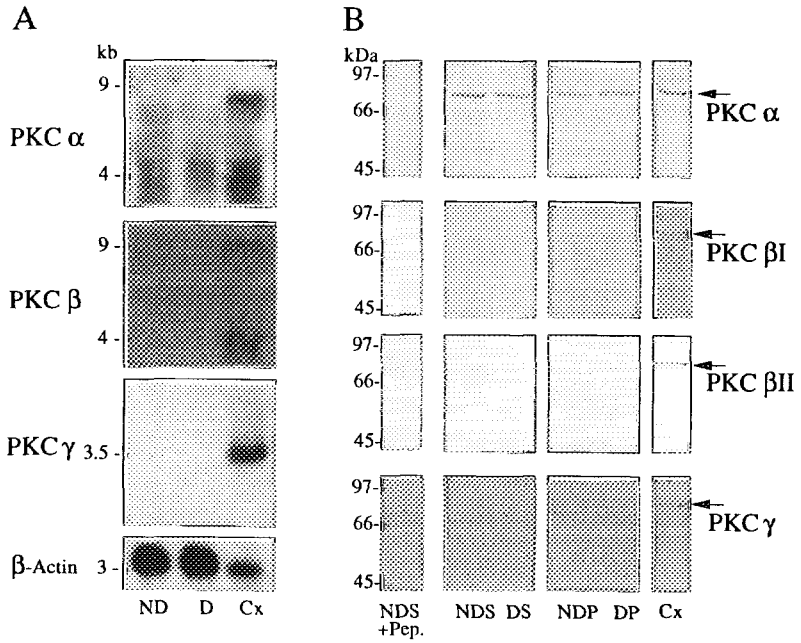

Figure 2.
Analysis of calcium-dependent PKC isoforms in NG108-15 cells. A: Northern blot analysis. Total RNA was extracted and 40 μg of RNA from non-differentiated (ND) and differentiated (D) cells were run on agarose-formaldehyde gels; 10 μg of RNA from rat cortex (Cx) was used as a positive control. The RNA was transferred to nitrocellulose and the blots were hybridized with oligonucleotide probes to PKC isoforms. Filters were stripped and rehybridized with the β–actin probe. The sizes of the hybridizing species are given in kilobases (kb). B: Western blot analysis. Twenty μg of soluble (S) or particulate (P) NG 108-15 cell proteins from non differentiated (ND) and differentiated (D) cells were run in each lane. The rat cortex (Cx) lane was loaded with 5 μg of soluble protein. Molecular weight standards are shown on the left of the figure and are reported in kilodaltons (kDa). The arrows refer to 80 kDa. The respective competing peptide (Pep) was added at a concentration of 2 μg/ml.

particulate fractions and that the cytosolic pool, in spite of being reduced, is still sufficient to sustain membrane PKC levels under resting conditions.

Among the calcium-dependent PKC isoforms only PKCα is detected in NG 108-15 cells while the β and γ isoforms are not expressed as determined by either Northern or Western blot criteria. Differentiation was associated with a decrease in the protein levels of the α isoform, with no modification in its mRNA levels. The absence of parallel changes of PKCα mRNA and protein levels in our cells could be related to a differentiation-dependent modification in protein turnover not coupled with significant changes in mRNA levels (32). The importance of down-regulation of the calcium-dependent PKCα in the process of neuronal differentiation is confirmed by data utilizing the SK-SY5Y neuroblastoma cells and showing that the intracellular delivery of PKCα, but not -β or -γ, antisera induces a differentiated phenotype (33).

It seems rather intriguing that the PKC β and γ isoforms (that are highly expressed and specific for neuronal tissues respectively (1)) were not observed even in the differentiated cells. Their level of expression may be either cell specific and/or below the limits of detection for the assay utilized in our experiments. Similar results, however, have been reported in human neuroblastoma (10,26) and in other murine hybrid cell lines (34).

Our results demonstrate that neuronal differentiation of NG 108-15 cells involves a decrease in PKC activity and PKCα immunoreactivity. The observation that calcium-dependent PKC is down-regulated, irrespective of the agent used to induce differentiation i.e phorbol esters combined with dBcAMP (19) or dBcAMP alone (this study) underlines the importance of PKC in transformation of logarithmically dividing NG108-15 cells to a mature neuronal phenotype.

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