Volume 322, number 2, 120–124 FEBS 12413 May 1993

Protein kinase C isoenzymes in human neuroblasts

Involvement of PKCε in cell differentiation

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Received 1 March 1993; revised version received 24 March 1993

Although neuronal cells are a major target of phorbol ester action, the activity of the various protein kinase C (PKC) isoenzymes have not been studied in detail in human neuroblasts. Differentiation of the LAN-5 human neuroblastoma cell line by interferon-γ (IFN-γ) is accompanied by a twofold increase in PKC activity. Since PKC is a multigene family, we investigated which isoforms were expressed in control and differentiated cells, and which of these isoenzymes is involved in neuronal differentiation. We found that: (1) PKC activity is higher in differentiated than in undifferentiated cells; (2) RT-PCR analysis showed the expression of mRNA for PKCα, -γ, -δ, -ε and -ζ and the absence of mRNA for β in untreated LAN-5 cells; (3) Western blot evaluation with PKC isoform-specific antibodies showed the same pattern of PKC expression in non-differentiated cells; (4) Expression of PKCε mRNA was significantly enhanced by IFN-γ-induced differentiation, while the other isoforms were not affected; (5) Differentiation of LAN-5 cells with IFN-γ or retinoic acid induced overexpression of the PKCε protein, while inhibition of cell proliferation by fetal calf serum starvation was without effect. These findings suggest that expression of PKCε isoform is tightly coupled with neuronal differentiation and may play a role in the maintenance of the differentiated state.

Neuroblastoma; Cell differentiation; Protein kinase C; Isoform

1. INTRODUCTION

Protein kinase C (PKC) is a phospholipid-dependent protein kinase which phosphorylates proteins on serine and threonine residues and binds phorbol ester [1–3]. PKC is now known to be a multigene family with at least seven members: α, β, γ, δ, ε, ζ and n/L [1–4]. In addition, the β gene undergoes differential splicing, resulting in two distinct RNA products, β_1 and β_2 [5]. The PKC family can be divided into two groups. PKCα, β and γ have four constant domains, C1–4, at which a high degree of sequence homology exists between the different isoenzymes [3]. It has been suggested that the aspartic acid- and the glutamic acid-rich sequences in the C2 domain are involved in Ca^{2+} interaction [6]. Although PKCδ, ε, ζ and n/L have C1, C3, and C4, they lack the C2 domain and consistently show a Ca^{2+} independent activation [7–9]

PKC isoenzymes are important regulatory enzymes. They are involved in a large number of cellular processes, including cellular proliferation, gene regulation, ion channel and receptor activation, and neurotransmitter release [3,10]. PKCs are highly expressed in brain tissue [11] and they play a role in several brain-specific functions, including long-term potentiation, neurite outgrowth, and neuronal differentiation [12,13]. Several neuroblastoma (NB) cell lines can be induced to differentiate to cells with neuronal characteristics and have been used as models for neuronal differentiation [14]. Human LAN-5 NB cells can be induced to mature after treatment with retinoic acid (RA) and interferon-γ (IFN-γ) giving rise to non-proliferative terminally differentiated ganglion-like cells [15,16]. Studies from a number of laboratories have demonstrated that changes in the activity of PKC may be involved in the differentiation pathway of several cell types indicating both increased expression and down-regulation of different PKC isoenzymes [17–20]. A recent study demonstrates that PKC isoforms are capable of selective isotype switching and their expression may differ with respect to the inducer used to stimulate differentiation [21]. Moreover, several attempts to correlate PKC activity to NB differentiation did not afford any consistent result [22–25]. The discovery of multiple PKC isoforms led to the suggestion that different isoenzymes serve distinct functions through differences in activator and/or substrate specificity [2].

The aim of the present study was therefore to investigate in more detail the PKC activity and the mRNA and protein levels of the various isoenzymes during neural cell differentiation using the human NB cell line LAN-5 as a model.

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2. MATERIALS and METHODS

2.1. *Chemicals*

Human recombinant IFN-γ and human recombinant Tumour Necrosis Factor-α (TNF) were obtained from Genzyme Corporation (Boston, MA, USA) and kept in aliquots (1000 IU/μl) in phosphate-buffered saline (PBS) (Flow Laboratories, Milan, Italy) plus 0.1% bovine serum albumine (BSA) at −80°C. RA (Sigma, St. Louis, MO, USA) was prepared at 10^{-3} M in DMSO every week and kept at −20°C. All other reagents of biochemistry or molecular biology grade were obtained from Sigma.

2.2. *Cell cultures and treatments*

LAN-5 NB cell line was a gift of R. Seeger [26]. Cells were maintained in the logarithmic phase of growth in 75 cm^2 plastic culture flasks (Falcon plastic, Oxnard, CA, USA) in RPMI 1640 medium (Seromed, Biochrom KG, Berlin, Germany), supplemented with 15% heat-inactivated fetal calf serum (FCS) (Seromed), sodium penicillin G (50 IU/ml), and streptomycin sulfate (50 μg/ml) (complete medium) at 37°C in a 5% CO_2–95% air humidified incubator. Cells were split following treatment with 1 mM EDTA in Hank's salts solution (Seromed), washed, counted, and replated in fresh complete medium. Cells were treated with 1000 IU/ml IFN-γ or 200 IU/ml TNF or 10^{-6} M RA or starved in 2% FCS-complete medium for five days as previously described [27,28]. U937(ATCC-CRL 1593) and PC-12 cells (ATCC-CRL1721) were cultured as above.

2.3. *Protein kinase C activity assay*

After the end of the culture period, the cells were lysed on ice with 20 mM Tris, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 1% Triton X-100, 0.5 mM phenylmethylsulfonil fluoride (PMSF), and 10 μg/ml aprotinin. The homogenates were mixed for 1 h at 4°C and centrifuged at 100,000 × g for 1 h at 4°C. The supernatant was loaded onto a DEAE-cellulose column and PKC activity was semipurified as described [29]. Total cellular PKC content was determined using histone type III-S as substrate as described [30]. Specific PKC activity is expressed as pmol ^{32}P transferred per mg substrate protein per min. in the presence of phosphatidylserine, diolein, and $CaCl_2$ minus that in the presence of Ca^{2+} only.

2.4. *RNA amplification and reverse transcriptase-polymerase chain reaction (RT-PCR)*

Total cellular RNA was prepared according to Chomczynski and Sacchi [31]. First strand synthesis and polymerase chain reaction were performed by means of a commercial kit (Perkin-Elmer, Norwalk, CT, USA). For each reaction 1 μg of total RNA was reverse transcribed by priming with random primers and then amplified with the isoform specific oligonucleotide primers synthesized with a Applied Biosystem 391 DNA synthetizer and described in Table I. The same RNAs were primed with the commercial primers for the housekeeping gene G3PDH (Clontech, Palo Alto, CA, USA). Amplification conditions were: 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C for 30 cycles. The amplified products were separated by electrophoresis on a 10% acrylamide gel run in TBE buffer (TBE is 90 mM Tris-HCl, pH 8.3, 90 mM Boric acid, 2 mM EDTA) at 200 V for 2 h. Restriction enzyme digestions were performed according to standard procedures. The same RT-PCR technique was used to quantify the RNA for the noradrenaline transporter of LAN-5 cells [32].

2.5. *Preparation of cell lysates and Western blot analysis*

After detachment from flasks, cells were washed twice with ice-cold PBS and resuspended at 10^7/ml in lysing buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 0.5% Triton X-100. Brains from 10-week-old Sprague–Dawley rats, from Charles River (Calco, Italy) were homogenized in the same lysing buffer as above. After 60 min on ice, cell membranes were pelleted by centrifugation at 100,000 × g for 60 min at 4°C. 100 μg of cytosolic proteins, determined with the Coomassie protein reagent (Pierce, Rockfort, IL, USA) were loaded onto a 7.5% SDS-polyacrylamide gel, electrophoresed at 35 mA for 5 h and then electroblotted onto Hybond C super membranes (Amersham, Buckingamshire, UK). After blocking in 3% dry milk in TBST (10 mM Tris-HCl, pH 8.0, 154 mM NaCl, 0.05% Tween 20), filters were incubated overnight with the various isoform-specific antibodies. Anti α and β isoforms were monoclonal antibodies purchased from UBI (Lake Placid, NJ, USA), while anti γ, δ, ε, and ζ isoform antibodies were raised in rabbit and obtained from Gibco-BRL (Gaithersburg, MD, USA). Specific PKC antigen peptides were obtained from Gibco-BRL. Filters were sequentially incubated for 2 h with a biotinylated anti mouse or anti rabbit Ig and for 1 h with a streptavidine-alkaline phosphatase conjugate, and then developed with NBT and BCIP as indicated by ICN (Costa Mesa, CA, USA).

3. RESULTS AND DISCUSSION

Previous reports have documented changes in PKC during neuronal maturation [22–24,33]. While these studies have demonstrated modulation of some PKC isoenzymes during treatment of neural cells with PKC inhibitors or they have focused on only few PKC isoforms, they have, however, failed to address whether true differentiation-promoting agents, such as IFN-γ and RA, affect the individual isoforms in a differential manner within the same neuronal model. In this study, changes in total PKC activity in LAN-5 human NB cells treated with IFN-γ over the time course of full differentiation have been documented. Moreover, induction of NB terminal differentiation with IFN-γ or RA is followed by a significant selective enhancement of the mRNA and protein level for the PKCε isoform.

We first measured PKC activity in detergent-solubilized cell homogenates under conditions where enzyme activity varies linearly with enzyme concentration [30]. The total specific activity of PKC in LAN-5 cells rose in a time-dependent manner with the IFN-γ treatment, reaching a maximum (approximately twofold the level of untreated cells) after five days of culture (Fig. 1), concomitant with acquisition of the neuronal morphol-

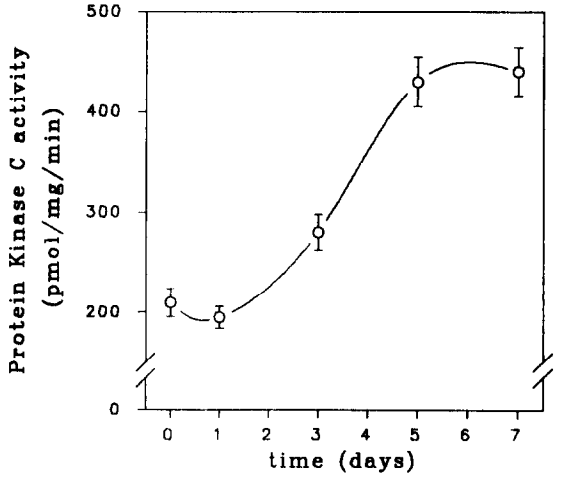

Fig. 1. Time course of IFN-γ treatment on total PKC activity of LAN-5 cells. Cells were treated with 1000 IU/ml of IFN-γ. Values are expressed as means ± S.D. of four independent experiments each done in triplicate.

ogy [16]. The magnitude of the unspecific PKC activity measured in the presence of only $CaCl_2$ was about 10% in all the time courses examined. The multiple effects attributed to PKC [3,10,12,13] indicate that PKC serves as a central mediator involved in the transduction of various signals in a wide variety of cell types, but how this enzyme can fulfill so many divergent functions is not understood. PKC is a growing multigene family and many studies indicate that PKC isoenzymes are expressed in a tissue-specific manner and that individual PKC isoforms might play cell type-specific roles in cellular responses and differentiation [3,10–13,17–21]. Since IFN-γ is able to induce neurogenesis and biochemical maturation of LAN-5 cells [16], as well as documented changes in PKC activity (Fig. 1), experiments were undertaken to address whether changes in the individual isoforms of PKC could account for the observed differences in PKC activity. Initial characterization of the model was obtained by investigating the RNA levels of the different kinase isoenzymes in cultured LAN-5 cells by RT-PCR analysis using specific oligonucleotide primers deduced from the sequences of the PKC cDNA clones (Table I). We found that LAN-5 cells express five PKCs: α, γ, δ, ε, and ζ, the α and ζ being the prominent constitutive isoforms (Fig. 2). In contrast to a previous observation on another NB cell line [23] we did not find any PKC β mRNA. This could be ascribed to differences in method evaluation or to a further cell clone-specificity. The specificity of the messages was verified by the expected sizes, by positive and negative controls (Fig. 2, lanes 1 and 2, respectively), and by the expected restriction digestion patterns (data not shown). Induction of differentiation by treatment of LAN-5 cells with 1000 IU/ml of IFN-γ for five days induced a significant increase in the level of PKCε RNA, while the other isoforms were totally unchanged (Fig. 2).

To investigate the regulation of PKC isoenzymes by IFN-γ induction, we performed Western blot analysis with PKC isoenzyme-specific antibodies. No PKCβ immunoreactivity could be found in untreated and IFN-γ-treated cells, confirming that these NB cells do not express this PKC isoform. Antibodies against PKCα, γ, δ, and ζ recognized single major immunoreactive bands of 85 (PKCα and γ) and 80 kDa (PKCδ and ζ) in untreated LAN-5 cells (Fig. 3, lanes 3). These bands were not modulated by the IFN-γ-treatment, confirming the data of the RT-PCR. Moreover, the antibody to PKCε recognized a band of about 90 kDa in the control cells that was drastically enhanced by the induction with IFN-γ (Fig. 3, lanes 4), indicating a possible involvement of the PKCε isoform in the IFN-γ-induced neuronal differentiation. It could be noted that the pattern of isoenzyme expression seen in RNA is also reflected at the protein level, suggesting that PKC expression in NB cells is regulated mainly at the mRNA level.

To further address the question whether PKCε isoenzyme is generally involved in neuronal maturation and is not simply linked to a selective IFN-γ-dependent modulation, we cultured LAN-5 cells for five days in the presence of RA or TNF or 2% FCS, and then we analysed the level of PKCε protein. RA is able to induce terminal differentiation of LAN-5 cells, while these cells are completely resistant to the TNF action [27], al-

Table I

Sequence of isoform specific oligonucleotide primers used for RT-PCR analysis

Isoform	Sequence 5′→3′		Position[b]	Size[c]	Ref[d]
α	F[a]	GGG ACG AGG AAG GAA ACA TGG AAC	898–921	177	38
	R[a]	AAC TCC CCT TTC CCA ACA CCA TGA	1052–1075		
β I/II	F	CCA GAA GGA AGT GAG GCC AAT GAA G	938–962	226	39
	R	AGC TCA TCT GTG CCT TTT CGT TCT G	1139–1164		
γ	F	TAC CCC CTG GAA TTG TAT GAG	1114–1134	128	39
	R	GGA GAT GTG CAG TCG TCC AG	1223–1242		
δ	F	AGT GAC CCA GAA AGC TTC CCG GA	1231–1254	238	7
	R	TAC CTT TCC TTG CCC TTC AGT TCT GC	1444–1469		
ε	F	AGC CGG CTT CTG GAA ACT CCC	1176–1196	264	7
	R	AGC TGC CTT TGC CTA ACA CCT TGA T	1416–1440		
ζ	F	TGT CAT GCC TTC CCA AGA GCC TC	258–280	231	7
	R	ATA GCT TCC ACG CCC GAT GAC TCT	466–489		

[a]F = Forward, R = Reverse
[b]Position of the primers on the clone sequence
[c]Expected size of the amplified fragment (bp)
[d]Reference for the clone sequence.

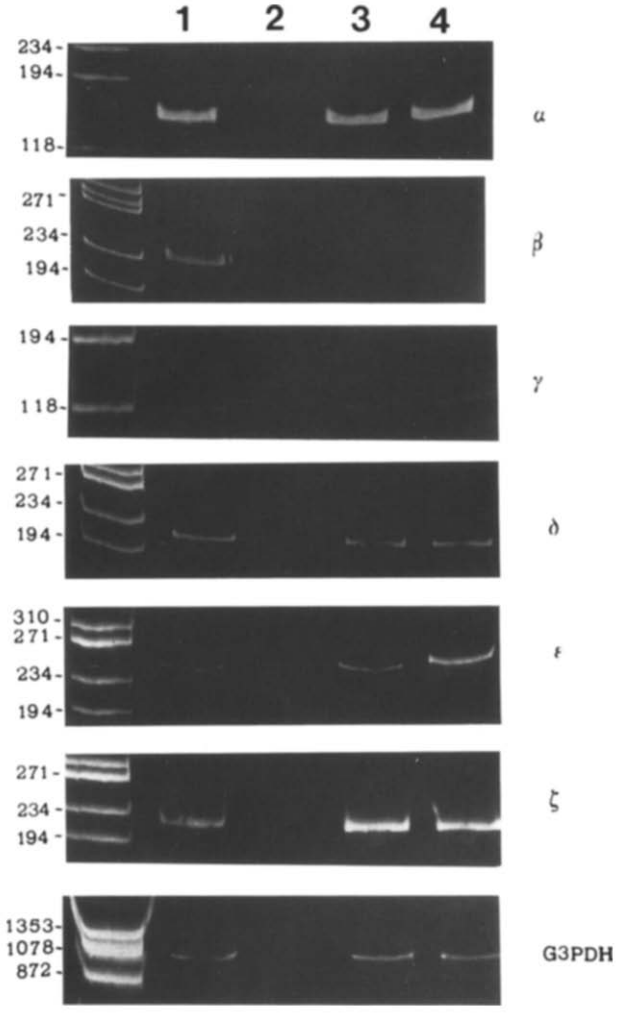

Fig. 2. RT-PCR analysis of PKC isoforms. The expression of the six PKC isoforms tested and of the housekeeping gene G3PDH, were analyzed in: control cell lines (U937, positive for α, β, and γ isoforms; PC-12, positive for δ, ε, and ζ isoforms), lane 1; negative control (absence of template RNA), lane 2; untreated LAN-5 cells, lane 3; IFN-γ-treated (1000 IU/ml) LAN-5 cells for 5 days, lane 4. Molecular weight markers from $\phi\chi$ 174 RF DNA digested with *Hae*III are shown on the left side.

though they bear high-affinity cell surface TNF-receptors [34]. Moreover, starvation of these cells in 2% FCS inhibits cell proliferation without affecting neural maturation [28]. Fig. 4 clearly shows that only IFN-γ (lane 3) and RA (lane 4) treatments were able to enhance PKCε levels, while the other treatments were without effect (lanes 5,6). Immunoreactivity against the PKCε protein was blocked by the presence of a specific antigen peptide (lane 7), confirming the specificity of the isoform induced.

The discrepancy between the relatively modest (two-fold) increase in PKC activity observed after differentiation of LAN-5 cells with respect to the marked enhancement of mRNA and protein levels of PKCε could have several reasons. Histone type III-S is a relatively

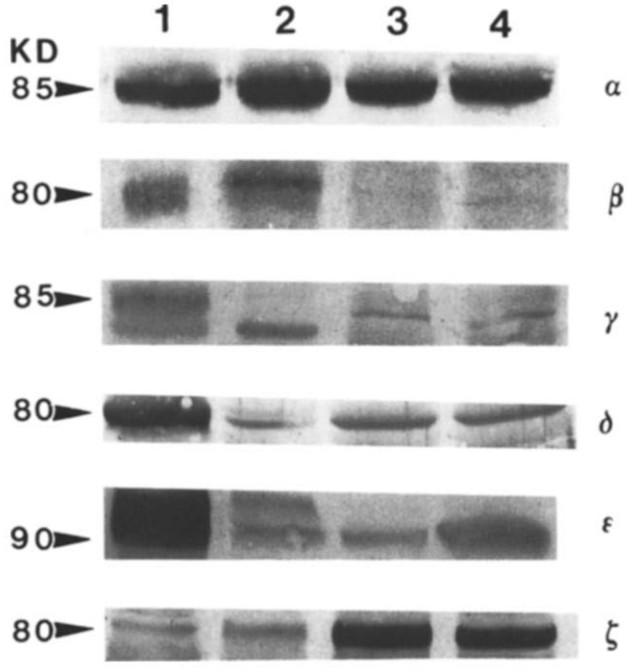

Fig. 3. Western blot analysis of PKC isoforms, 100 μg of cytosolic proteins from: rat brain, lane 1; U937 cells, lane 2; untreated LAN-5 cells, lane 3; LAN-5 cells treated with 1000 IU/ml of IFN-γ for 5 days, lane 4, were electrophoresed and probed with the isoform specific antibodies as described in section 2. Molecular weights are indicated on the left.

poor substrate for PKCε [35] i.e., although PKCε binds phorbol ester (PMA) and displays phospholipid- and PMA-dependent kinase activity on substrate peptides, it does not phosphorylate histone to a significant extent.

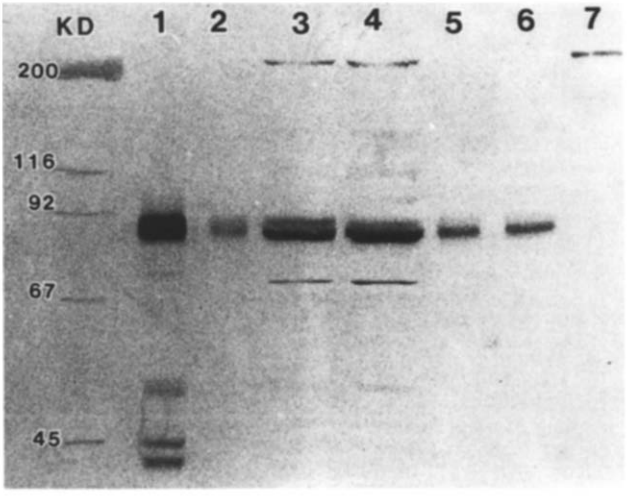

Fig. 4. Modulation of PKCε expression during NB cell differentiation. 100 μg of cytosolic proteins from: rat brain, lane 1; untreated LAN-5 cells, lane 2; LAN-5 cells treated for 5 days with 1000 IU/ml IFN-γ, lane 3, or with 10^{-6} M RA, lane 4, or with 200 IU/ml TNF, lane 5, or cultured in the presence of 2% FCS, lane 6, were electrophoresed and probed with the PKCε specific antibody as described in section 2. The blot in lane 7, containing the same amount of protein as lane 3, was incubated for 2 h with the immunogen PKCε peptide and then probed with the PKCε antibody.

Therefore our measurements of PKC activity in cell homogenates may have underestimated the amount of PKCε present. Moreover, although DEAE chromatography removes endogenous inhibitors that interfere with the PKC assay [36], some inhibitory activity can still be present in the preparation. Finally, since measurements of PKC activity results from the combined effects of PKC, proteases, phosphatases, and inhibitors, the PKC activity assay with total cell homogenates may have underestimated IFN-γ-induced increase in PKC levels.

These results clearly demonstrate modulation of PKCε isoform protein and RNA levels via specific differentiation-inducing agents and support the notion that each of these isotypes plays a discriminate role in the cell. Several proteins which participate in neurofilament reorganization during neural differentiation are themselves substrates for PKC [37]. Moreover, PKCε is nearly exclusively localized in the membranes and processes in NB cells [23]. Thus, it is possible that both the PKCε isoform and particular substrates colocalize during neurite extension and that proximal distribution of the kinase and substrate plays a key role in the acquisition of neuronal phenotype.

Acknowledgements: We thank Mr. F. Guarnaccia for technical assistance and Mr. T. Carlucci for editing. This paper was partially supported by Grant Ricerca Finalizzata No. 91.171.02.F and the Associazione Italiana Ricerca sul Cancro.

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