FEBS Letters 508 (2001) 126–130 FEBS 25435

A PKCβ isoform mediates phorbol ester-induced activation of Erk1/2 and expression of neuronal differentiation genes in neuroblastoma cells

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Received 5 September 2001; revised 15 October 2001; accepted 16 October 2001

First published online 25 October 2001

Edited by Guido Tettamanti

Abstract Protein kinase C (PKC) activation induces neuronal differentiation of SH-SY5Y neuroblastoma cells. This study examines the role of PKCβ isoforms in this process. The PKCβ-specific inhibitor LY379196 had no effect on 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced neurite outgrowth from SH-SY5Y neuroblastoma cells. On the other hand, PKCβ inhibition suppressed the TPA-stimulated increase in neuropeptide Y mRNA, activation of neuropeptide Y gene promoter elements, and phosphorylation of Erk1/2. The TPA-induced increase in neuropeptide Y expression was also inhibited by the MEK inhibitor PD98059. These data indicate that activation of a PKCβ isoform, through a pathway involving Erk1/2, leads to increased expression of neuronal differentiation genes in neuroblastoma cells.

Key words: Growth-associated protein 43; Mitogen-activated protein kinase; Neuroblastoma cell; Neuronal differentiation; Neuropeptide Y; Protein kinase C

1. Introduction

Neuroblastoma cells are derived from the sympathetic nervous system and phenotypically resemble immature sympathetic neuroblasts [1]. It has been suggested that neuroblastoma cells have been arrested at an immature differentiation state and one way to halt the malignancy of these cells would be to induce them to differentiate towards mature sympathetic neurons.

Several neuroblastoma differentiation protocols have been characterized in vitro, for instance treatment with retinoic acid [2]. In the SH-SY5Y neuroblastoma cell line treatment with phorbol esters, at concentrations that activate but do not down-regulate protein kinase C (PKC), leads to sympathetic neuronal differentiation with neurite outgrowth and increased synthesis of noradrenaline and expression of neuropeptide Y (NPY) and growth-associated protein 43 (GAP-43). These effects are mediated by and dependent on PKC [3–6].

PKC constitutes a family of related serine/threonine kinases. Based on structural and regulatory properties this family can be divided into classical (PKCα, βI, βII, and γ), novel (PKCδ, ε, η, and θ), and atypical (PKCι/λ and ζ) isoforms. We have previously demonstrated that a classical isoform, most likely PKCβI, has a stimulatory effect on neuroblastoma cell proliferation [7]. We have also shown that the novel isoform PKCε can elicit morphological changes, such as neurite outgrowth, associated with neuronal differentiation [8]. However, during 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced differentiation of neuroblastoma cells it is likely that a classical isoform is involved in the induction of differentiation marker genes [9]. The aim of this study was to elucidate whether the PKCβ isoforms are implicated in the TPA-induced neuronal differentiation of SH-SY5Y neuroblastoma cells.

2. Materials and methods

2.1. Cell culture

SH-SY5Y cells, kindly provided by Dr. J. Biedler, Memorial Sloan-Kettering Cancer Center, New York, NY, USA, were maintained in Eagle's minimal essential medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco).

2.2. Morphology studies

Cells were seeded in regular growth medium at a density of 150 000 cells per 60 mm dish. When indicated, medium was supplemented with 16 nM TPA (Sigma) or LY379196 (kindly provided by Eli Lilly Research Laboratories). This compound was shown to have in vitro IC_{50} values of 50 nM for PKCβI, 30 nM for PKCβII, and 600 nM or above for other PKC isoforms present in neuroblastoma cells (as communicated by Eli Lilly Research Laboratories). After 4 days phase contrast images were captured with a Sony DKC 5000 camera system.

2.3. Northern blot analysis

4×10^6 SH-SY5Y cells were seeded in 100 mm dishes and 24 h later TPA and kinase inhibitors were added. After an additional incubation for 24 h total RNA was extracted using Trizol (Gibco) essentially according to the manufacturer's instructions. 15 μg RNA was separated on a denaturing agarose gel and thereafter transferred to Hybond N membrane (Amersham) by Northern blotting as described [10]. Hybridization was done with [^{32}P]dCTP-labeled NPY, GAP-43 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes.

2.4. Luciferase assay

A plasmid, kindly provided by Dr. Carolyn Minth-Worby [11], containing the NPY gene promoter element −87 to −47 fused to the minimal herpes simplex virus thymidine kinase promoter, was used as template for a PCR reaction. In this reaction primers containing *Hin*dIII and *Sal*I sites were used to amplify a fragment encompassing the NPY promoter elements and the minimal thymidine kinase promoter. The fragment was initially inserted into the pEGFP-1 vector (Clontech) and thereafter a *Sma*I/*Sac*I fragment was transferred to the pGL3Basic vector (Promega) which contains cDNA for luciferase as reporter thereby generating the NPY(−87/−47)TKLuc

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GAP-43, growth-associated protein 43; NPY, neuropeptide Y; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate

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PII: S0014-5793(01)03043-5

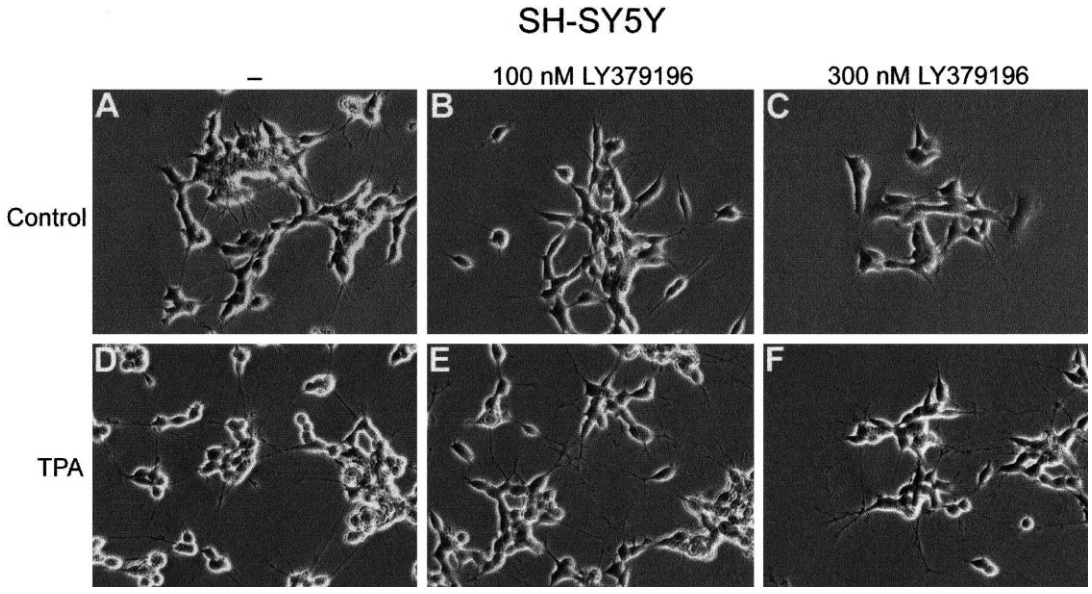

Fig. 1. Inhibition of PKCβ isoforms does not lead to suppression of TPA-induced neurite outgrowth. Phase contrast images of SH-SY5Y cells grown for 4 days in serum-containing medium in the absence (A–C) or presence (D–F) of 16 nM TPA together with vehicle only (A and D), 100 nM (B and E) or 300 nM (C and F) of the PKCβ inhibitor LY379196.

vector. The resulting PCR product was sequenced to exclude the possibility of base changes in the PCR reactions.

For promoter activity analysis, SH-SY5Y cells were seeded at a density of 1×10^6 per 60 mm dish. Cells were transfected 24 h later with the NPY(−87/−47)TKLuc vector using Lipofectamine (Gibco) according to the supplier's protocol. After transfection cells were incubated for 16 h in serum-free medium with TPA, and GF109203X, Gö6976, Gö6983, PD98059 (all Calbiochem) or LY379196. Luciferase activity was measured with a Luciferase Assay System (Promega) according to the supplier's protocol. The coefficient of analytical variation for this assay was determined to be 0.042.

2.5. Analysis of Erk1/2 phosphorylation

Following stimulation cells were put on ice and washed with ice-cold phosphate-buffered saline and harvested in lysis buffer (25 mM Tris–HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl, 1 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid and Complete Protease Inhibitor cocktail [Roche]). Lysates were centrifuged for 10 min at 15 000 rpm and 25 μg proteins from the supernatants were separated on a 10% polyacrylamide gel and transferred to Hybond-C extra nitrocellulose filter (Amersham Pharmacia Biotech). Filters were analyzed with primary antibodies towards phosphorylated (New England Biolabs) and total (Transduction Laboratories) Erk1/2 followed by secondary horseradish peroxidase-labeled antibodies. Immunoreactivity was visualized with Super Signal (Pierce) and chemiluminescence was captured with a CCD camera (Fujifilm).

3. Results

To explore whether PKCβ isoforms are involved in TPA-induced morphological differentiation of SH-SY5Y cells, the cells were treated with 16 nM TPA for 4 days in the absence or presence of the specific PKCβ inhibitor LY379196 (Fig. 1). This TPA treatment leads to neuronal differentiation of SH-SY5Y which can be blocked by PKC inhibitors [3,6]. Inclusion of TPA in the medium elicited a morphological differentiation of the cells with neurites bearing varicosities. Concomitant incubation with 100 or 300 nM LY379196 did not influence this morphological effect and thus implies that the activity of PKCβ isoforms is not crucial for the induction of morphological features of neuronal differentiation.

Another feature of TPA-differentiated SH-SY5Y cells is the increased expression of sympathetic neuronal marker genes such as NPY and GAP-43. This TPA effect is completely blocked by PKC inhibitors [9]. To investigate whether a PKCβ isoform mediates this effect, we analyzed the expression of NPY and GAP-43 upon treatment with 16 nM TPA in the absence or presence of 100 nM LY379196 (Fig. 2). This experiment revealed that 100 nM LY379196, a concentration that is two to three times the reported in vitro IC_{50} value for PKCβ but less than one fifth of the corresponding value for other isoforms present in neuroblastoma cells, abolished TPA-induced NPY expression in the absence of serum and markedly suppressed it in the presence of serum. Since PKC activation frequently leads to activation of the mitogen-activated protein kinases Erk1/2 and since activation of these kinases is associated with neuronal differentiation, we also investigated the effect of the MEK inhibitor PD98059, which will block the activation of Erk1/2 (Fig. 2). This compound also suppressed the TPA-stimulated increase in NPY mRNA indicating that Erk1/2 is involved in this effect. The pattern was similar for the TPA-stimulated increase in GAP-43 mRNA levels. In the absence of serum, LY379196 blocked the TPA effect and in the presence of serum it was suppressed. PD98059 inhibited the increase in GAP-43 mRNA both in the absence and in the presence of serum.

A part of the sequence upstream of the transcription start in the NPY gene has been identified to be responsive to stimuli that lead to increased NPY expression [11]. Using reporter genes the sequence has been shown to drive transcription upon stimulation with several factors, including TPA, that elicit neuronal differentiation in SH-SY5Y cells [12,13]. We subcloned this promoter sequence in the pGL3Basic vector which contains luciferase cDNA as the reporter gene. SH-SY5Y cells were transfected with the vector and thereafter treated with TPA and increasing concentrations of two PKC inhibitors, Gö6976 which only inhibits classical PKC isoforms and the PKCβ-specific inhibitor LY379196 (Fig. 3A). Both inhibitors attenuated the TPA-stimulated luciferase expression at concentrations which are compatible with a mechanism

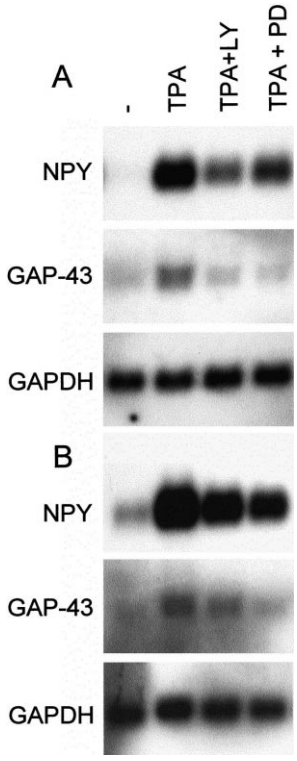

Fig. 2. TPA-stimulated increase in NPY and GAP-43 expression is suppressed by inhibitors of PKCβ and MEK. SH-SY5Y cells were grown for 24 h in the absence (A) or presence (B) of serum and treated with 16 nM TPA in the absence or presence of 100 nM LY379196 (LY) or PD98059 (PD). Total RNA was thereafter extracted and the expression of NPY, GAP-43 and GAPDH was analyzed by Northern blot. The blots are representative of three separate experiments.

involving a PKCβ isoform. This TPA effect was also blocked by the pan-PKC inhibitors Gö6983 and GF109203X and by suppression of the Erk1/2 pathway by PD98059 (Fig. 3B).

Since inhibition of either Erk1/2 or PKCβ isoforms suppresses TPA-stimulated NPY expression and promoter activity we investigated whether TPA treatment would induce phosphorylation of Erk1/2 through a pathway that is dependent on PKCβ activity. SH-SY5Y cells were treated with TPA and the phosphorylation of Erk1/2 was analyzed (Fig. 4). Initially a time course study for the TPA effect was performed and this demonstrated that there is a rapid phosphorylation of Erk, detectable already after 3 and peaking after 10 min of TPA treatment (Fig. 4A). We thereafter examined the effect of PKC inhibitors and the MEK inhibitor PD98059 on TPA-stimulated phosphorylation of Erk (Fig. 4B,C). These experiments showed that blocking MEK activity with PD98059 or inhibiting all PKC isoforms using GF109203X and Gö6983 abolished TPA-stimulated Erk1/2 phosphorylation both in the absence and in the presence of serum. The PKCβ inhibitor LY379196 at 100 nM markedly suppressed the same effect indicating that a PKCβ isoform at least partially mediates the TPA-induced activation of Erk1/2.

4. Discussion

There are several reports demonstrating a role for different PKC isoforms in the induction of neuronal differentiation. Primarily the novel isoforms PKCδ and ε have been suggested to be involved in this process [6,14–18]. In neuroblastoma cells, PKCε has been shown to induce neurite outgrowth without an accompanying increase in the expression of neuronal differentiation marker genes [8]. This study demonstrates that a PKCβ isoform can mediate the induction of the neuronal marker genes NPY and GAP-43. Hence different PKC isoforms seem to mediate different aspects of the neuronal differentiation process which indicates that it is necessary to activate several isoforms to obtain a complete differentiation.

The PKCβ inhibitor did not completely abolish the TPA-stimulated increase in NPY and GAP-43 mRNA. This could be due to the concentration of the inhibitor, 100 nM, which is only two to three times the reported IC_{50} for inhibition of PKCβI and βII in vitro. We refrained from using higher concentrations in most experiments since under such conditions other PKC isoforms may also be inhibited. Thus the lack of complete suppression may be caused by a fraction of the PKCβ activity that is not inhibited by 100 nM LY379196. In the reporter assay, where higher concentrations were used, the TPA-stimulated NPY promoter activity was indeed abolished by LY379196 which indicates that a complete suppression of PKCβ activity abolishes the TPA effect on NPY expression. However, it cannot be excluded that at the higher LY379196 concentrations other PKC isoforms are also affected which could potentially explain the complete suppression by the higher concentrations. This would imply that there

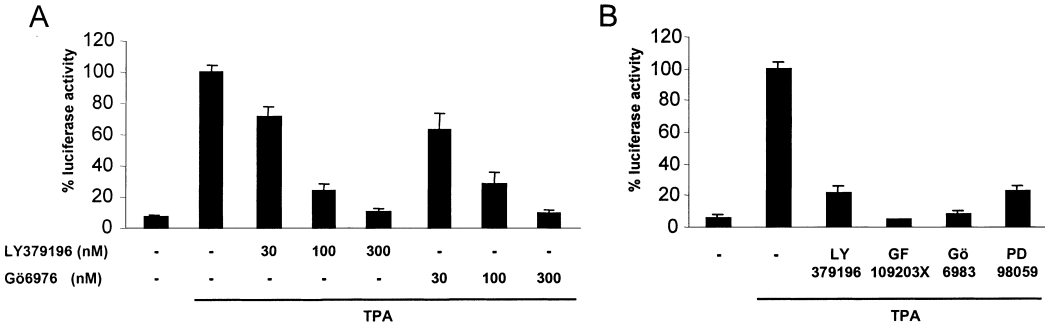

Fig. 3. TPA-stimulated activation of the NPY promoter is attenuated by PKC and MEK inhibitors. SH-SY5Y cells were transfected with the vector encoding the luciferase reporter gene under the control of the NPY promoter element −87 to −47 and the minimal thymidine kinase promoter. Cells were grown for 16 h in serum-free medium with or without 16 nM TPA and treated with (A) increasing concentrations of the PKCβ inhibitor LY379196 or the cPKC inhibitor Gö6976, or (B) either the PKCβ inhibitor LY379196 (100 nM), two general PKC inhibitors GF109203X (2 μM) and Gö6983 (2 μM), or the MEK inhibitor PD98059 (50 μM). Luciferase activity was thereafter assayed and the data are shown as relative values where the activity for TPA-stimulated cells is 100%.

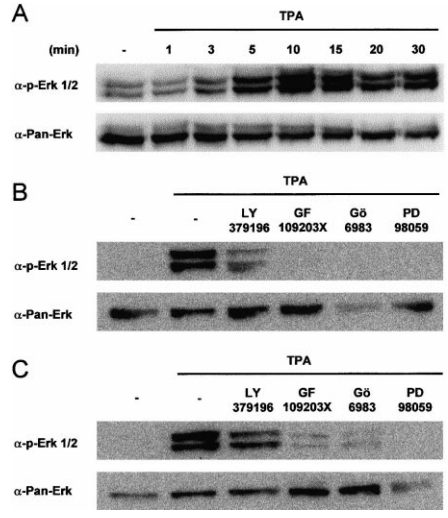

Fig. 4. TPA-stimulated Erk1/2 phosphorylation is suppressed by PKC and MEK inhibitors. SH-SY5Y cells were grown in serum-containing medium (A and C), or serum-free medium (B) prior to stimulation with 16 nM TPA (A) for increasing periods of time or (B and C) for 10 min in the presence of 100 nM LY379196, 2 μM GF109203X, 2 μM Gö6983, or 50 μM PD98059. The inhibitors were added 5 min prior to the addition of TPA. The activation of Erk1/2 was then determined by Western blotting using anti-phospho-Erk1/2 antibody and total Erk1/2 was determined with anti-pan-Erk antibody. The blots are representative of at least three experiments. Although the levels of total Erk1/2 are low in TPA+Gö6983 (B) and in TPA+PD98059 (C) the same suppression of Erk1/2 phosphorylation by these compounds was observed in experiments where total Erk1/2 immunoreactivity was the same as control conditions.

is a redundancy in the system, particularly evident in the presence of serum, with several PKC isoforms mediating the effects on gene expression. We have previously seen that TPA-stimulated NPY expression can be completely abrogated by the inhibitor of classical PKC isoforms Gö6976 [9]. It is thus likely that this alternative PKC isoform is PKCα, since PKCγ is not expressed in these cells.

Activation of Erk1/2 has frequently been connected to induction of a differentiation process and it is activated during neuronal differentiation of neuroblastoma cells [19]. Accordingly, inhibition of Erk1/2 activation suppressed TPA-stimulated NPY and GAP-43 expression. Furthermore, pan-PKC inhibitors abolished TPA-stimulated phosphorylation of Erk1/2 clearly highlighting Erk1/2 as a mediator of PKC-induced increase in the expression of neuronal marker genes. A crucial role for Erk1/2 in the TPA-mediated increase in NPY and GAP-43 expression has also recently been demonstrated [20]. In that study it was also shown that suppression of Erk1/2 did not influence the morphological differentiation induced by TPA. Together with the data in this study this clearly illustrates that a PKCβ-Erk1/2 pathway is involved in the up-regulation of differentiation genes but not in the morphological changes during differentiation of neuroblastoma cells.

In several cell systems activation of PKC leads to stimulation of the Erk1/2 pathway. PKCα [21], β [22], δ [18], ε [21], as well as ζ [23] have been shown to activate the Erk pathway in different cell types. Furthermore, it seems likely that most, if not all, PKC isoforms have the capacity to induce activation of Erk1/2 when overexpressed in the same cell [24,25]. These findings may indicate that there is either a redundancy so that several isoforms can mediate the same effect or that for each cell there are conditions which determine which isoform can transduce the signal. This could be caused by the expression pattern of PKC isoforms in the particular cell or by cell-specific expression of interacting proteins which direct particular isoforms to the site of action in the transduction cascade. It illustrates the necessity to identify the PKC isoform involved in each individual case of PKC-mediated Erk1/2 activation. Our data clearly show that a PKCβ isoform is involved in phorbol ester-stimulated activation of Erk1/2 in the SH-SY5Y neuroblastoma cells. However, the PKCβ inhibitor did not completely abolish the phosphorylation of Erk1/2 as the pan-PKC inhibitors did. This could be due to either a redundancy or incomplete suppression by LY379196 as discussed above for the induction of NPY expression.

In conclusion, this study demonstrates that activation of PKCβ can induce the expression of neuronal differentiation genes in neuroblastoma cells through activation of the Erk1/2 pathway. The PKC-stimulated morphological differentiation, on the other hand, is not dependent on PKCβ illustrating that different PKC isoforms elicit different features of the neuronal differentiation program.

Acknowledgements: Drs. Carolyn Minth-Worby and Eli Lilly Research Laboratories are gratefully acknowledged for supplying the NPY(−87/−47)TKCat plasmid and LY379196 respectively. Financial support was obtained from the Swedish Cancer Society, the Children's Cancer Foundation of Sweden, the Swedish Society for Medical Research, the Crafoord; Magnus Bergvall; Gunnar, Arvid and Elisabeth Nilsson; Ollie and Elof Ericsson; and John and Augusta Persson Foundations, and Malmö University Hospital Research Funds.

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