Phosphatidylcholine-phospholipase C mediates the induction of nerve growth factor in cultured glial cells

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Abstract Addition of phosphatidylcholine-hydrolyzing phospholipase C (PC-PLC) to cultured glial cells increased the levels of nerve growth factor (NGF) mRNA and the amount of cell-secreted NGF. The effect of PC-PLC was 2.5 times higher than that elicited by 4β-phorbol 12β-myristate 13α-acetate. In cells in which protein kinase C (PKC) was fully inhibited or down-regulated, the effect of PC-PLC was reduced – though still evident – and similar to that exerted by sphingosine. Results thus indicate that PC-PLC induces the synthesis of NGF by glial cells by a PKC-dependent and PKC-independent mechanisms.

Key words: Nerve growth factor; phosphatidylcholine; Protein kinase C; Sphingosine; Glial cell; Astrocyte

1. Introduction

Nerve growth factor (NGF) is a neurotrophic protein required for the development and survival of several populations of neurons [1]. In the central nervous system, NGF is synthesized by certain neurons, but during brain development and under different pathological alterations of the nervous system, there is a local increase in the production of NGF by astrocytes [2–5]. Thus, substantial present effort is directed toward an understanding of the influences that control NGF synthesis, because of the possibility that increasing the endogenous synthesis of NGF might provide clinical advantage in degenerative diseases of central nervous system [6]. However, little is known about the effectors that induce the synthesis of NGF by those cells and about how this synthetic process is up-regulated. It seems that several NGF-inducing agents act through different pathways. One of them is the signalling network involving the activation of protein kinase C (PKC). Studies performed with cultured astrocytes and fibroblasts have shown that 4β-phorbol 12β-myristate 13α-acetate (PMA), a well-documented PKC activator, induces the synthesis and secretion of NGF, and this effect is counteracted by PKC inhibitors like H-7 or H-9 [7,9]. PMA induces the expression of c-*fos*, the product of which enhances NGF synthesis by the same route as PMA [5,9,10].

PKC is physiologically activated by diacylglycerol (DAG) resulting from agonist-induced hydrolysis of inositol phospholipids [11]. However, this DAG rapidly disappears and seems to be responsible for a transient activation of PKC [12]. In contrast, hydrolysis of other membrane phospholipids, particularly phosphatidylcholine (PC), produces DAG at a relatively later phase. This DAG may be the responsible for the sustained activation of PKC that leads to long-term cellular responses [13]. Mechanisms of cellular response to PC hydrolysis have been demonstrated to occur in many cell types (reviewed in [12,14]). Despite extensive studies, the biochemical mechanisms underlying signal-induced activation of PC breakdown are still poorly defined. Phospholipase C (PLC)-catalyzed hydrolysis of PC has been shown to be activated in response to a number of agonists [15], in transformed cells [16] and in signalling cascades triggered by growth factors [17]. PC-PLC provides a positive signal for PKC by inducing its translocation to membrane though not its down-regulation [16]. It has also been shown that PC hydrolysis by a PC-PLC mimics the ability of carbachol to inhibit adenylyl cyclase [18]. Furthermore, a role of PC-specific phospholipase D (PLD) in signal transduction has been described [13,19].

In the present study we investigated the possible relationship between PC hydrolysis and PKC activation in the control of NGF synthesis by astrocytes. Previous experimental evidence showed that activation of PKC with PMA stimulated NGF synthesis by glial cells [7] while exogenously added PC-PLC was able to activate PKC in fibroblasts [18]. This provinded us a tool to investigate whether NGF synthesis by glial cells depends on PC breakdown.

2. Experimental

2.1. Culture conditions

Primary cultures of rat brain glial cells were prepared from cerebral hemispheres of 1–2-day-old rat pups. Cerebral hemispheres were dissected in phosphate buffered saline (PBS) supplemented with 0.33% glucose and treated with trypsin (5 mg/ml, 30 min at 37°C). Trypsin was subsequently inhibited by the addition of 10% foetal calf serum (FCS), before treatment with DNase I (10 μg/ml, 5 min at 37°C). Brain cells were dissociated by fluxation with a Pasteur pipette and next sedimented by low speed centrifugation (1000 × *g*, 5 min). The pellet was gently resuspended, and cells were seeded at a density of 3×10^4 cells/cm^2. All plastic supports were previously coated with 5 μg/ml poly-L-ornithine in water. Cells were cultured for 3 weeks in basal medium containing a mixture of Dulbecco's modified Eagle medium and Ham's F12 (1/1, v/v), supplemented with 0.66% glucose, 5 μg/ml streptomycin, 5 U/ml penicillin and 10% FCS. Three days before the experiment, FCS was removed and cells were transferred to a chemically defined medium consisting of basal medium supplemented with 25 μg/ml insulin, 100 μg/ml human transferrin, 20 nM progesterone, 50 μM putrescine and 30 nM sodium selenite. Immunocytochemical studies showed that 90% of cultured cells were positive for glial fibrillary acidic protein, indicating that cultures were largely enriched in astrocytes.

2.2. Assay of NGF and NGF mRNA

Cell supernatants were collected 24 h after the different treatments described in the text and diluted in 1 volume of PBS containing 0.1% Tween-20 and 0.5% gelatin. NGF released by the cells was assayed in triplicate, with a double-site-ELISA, using a monoclonal anti-NGF antibody, coupled or not to β-galactosidase (Boehringer Manheim), according to [20]. Northern blot analysis was performed by standard procedures [7]. After 6 h of treatment, total RNA was extracted from

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cells by the LiCl/urea method. Glyoxal-treated RNAs were fractionated in agarose gel, transferred to a nylon membrane by capillary blotting, and hybridized with a ^{32}P-labelled NGF cDNA and a amyloid precursor protein (APP) cDNA, used to verify the loading of the gels.

2.3. Identification of PKC by immunoblotting

Glial cells (treated as described in the legend to Fig. 3) were scraped into 0.5 ml of ice-cold 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 10 mM 2-mercaptoethanol containing 1 μg/ml leupeptin, 1 μg/ml aprotinin, 10 μg/ml benzamidin, 5 μg/ml soybean tripsin inhibitor and 0.1 mM PMSF, and rapidly centrifuged at 5,000 × *g* for 5 min to eliminate debries. Following denaturation in SDS sample buffer, proteins were resolved in a 8% SDS-PAGE and transferred electrophoretically onto nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% of fat-free dried milk and incubated with 2 μg/ml of specific anti-PKC antiserum (clone MC5, Amersham International). Following incubation with horseradish conjugated anti-mouse antibody, the blots were developed with an enhanced chemiluminescence detection kit (Amersham International).

2.4. Other chemicals

Tissue culture plastic wares were purchased from Nunc (Denmark), culture media from Gibco (France) and FCS from Eurobio (France). PC-PLC and bisindolylmaleimide (GF 109203X) were from Calbiochem (USA). Sphingosine and most other reagents were obtained from Sigma Chemicals (USA).

3. Results

3.1. PC-PLC enhances the synthesis of NGF

Cultured glial cells were treated for 24 h with different doses of exogenously added PC-PLC from *Bacillus cereus* and NGF levels were determined in the supernatant media 24 h later. Results presented in Fig. 1 show a dose-dependent and saturable increase in the extracellular levels of NGF, which become maximal at 0.5 U/ml of PC-PLC. Doses over 1 U/ml were toxic to cells. Stimulation of cells with 100 nM of PMA was quantitatively lower than that induced by PC-PLC (Fig. 1).

In order to determine whether PC-PLC had a corresponding on the pool of NGF transcripts, total RNA was extracted from cells pretreated with or without the enzyme. Northern blot analysis showed that PC-PLC produced a marked increase in

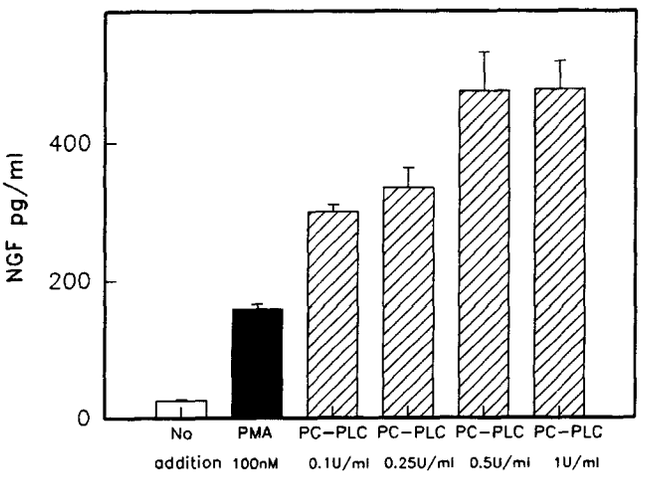

Fig. 1. Effect of increasing concentrations of PC-PLC on the production of NGF. Glial cells were incubated with no additions, PMA (100 nM), or PC-PLC (0.1 U/ml, 0.25 U/ml, 0.5 U/ml and 1 U/ml). The medium was collected after 24 h and NGF was quantified by double-site ELISA. Values are means ± S.D. of 3 independent experiments assayed in triplicate.

Fig. 2. Accumulation of NGF mRNA in astrocytes. Constant amounts of total RNAs were fractionated on an agarose gel, blotted and hybridized with a radiolabelled NGF cDNA probe. Lane 1: control; lane 2: PMA 100 nM; lane 3: PC-PLC 0.5 U/ml; lane 4: cells treated with PMA 500 nM for 48 h and the last 24 h with PMA 100 nM; and lane 5: cells treated with PMA 500 nM for 48 h and the last 24 h with PC-PLC 0.5 U/ml. Essentially identical results were obtained in two independent experiments.

NGF mRNA, as compared to controls, indicating that PC-PLC is able to promote NGF synthesis (Fig. 2, lanes 1 and 3). This effect is more potent than that elicited by PMA (Fig. 2, lane 2).

3.2. The induction of NGF synthesis by PC-PLC is partially dependent on PKC

To investigate whether PKC activation is required for the PC-PLC-triggered induction of NGF synthesis, PKC was down-regulated in glial cells by pretreating the cell cultures with a supramaximal dose of PMA (500 nM) for 48 h. This treatment completely removed PKC from astrocytes as determined by Western blot analysis with a specific monoclonal anti-PKC antibody (Fig. 3). Interestingly, induction of NGF synthesis by PC-PLC emerged reduced but not abolished, in cells in which PKC was down-regulated (Table 1), whereas NGF induction

Table 1
Effect of PKC inhibition and down-regulation and effect of sphingosine on the levels of cell-secreted NGF measured by ELISA

Additions	NGF pg/ml
None	34.68 ± 10
PMA 100 nM	196.51 ± 90
PC-PLC 0.5 U/ml	490.54 ± 119
PMA 100 nM + BIM 2 μM	37.41 ± 3
PC-PLC 0.5 U/ml + BIM 2 μM	130.30 ± 23
PMA 500 nM (48 h) + PMA 100 nM	32.87 ± 2
PMA 500 nM (48 h) + PC-PLC 0.5 U/ml	171.50 ± 27
Sphingosine 10 μM	164.80 ± 12

The values represent the means ± S.E.M. of 2 independent experiments assayed in triplicate.

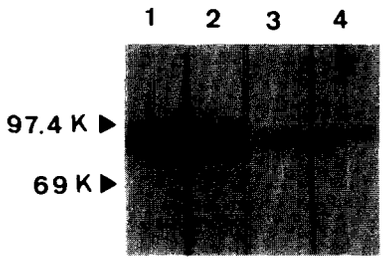

Fig. 3. Western blot analysis of PKC levels in cell lysates. Lane 1: control; lane 2: PMA 100 nM; lane 3: cells treated with PMA 500 nM for 48 h and the last 24 h with PMA 100 nM; and lane 4: cells treated with PMA 500 nM for 48 h and the last 24 h with PC-PLC 0.5 U/ml.

as elicited by PMA was completely abolished in down-regulated cells. This effect was also observed at the mRNA levels, as determined by Northern blot analysis (Fig. 2, lanes 4 and 5).

Further experimental evidence to support these data was obtained by the use of bisindolylmaleimide (BIM), a potent and higly specific inhibitor of PKC [21]. Inhibition of PKC by BIM completely abolished the increase in NGF levels induced by PMA, whereas treatment of cells with BIM only reduced in part the increase in NGF levels produced by PC-PLC (Table 1). Hence, PKC seems to be only partially involved in the NGF inducing response elicited by PC-PLC.

3.3. *Sphingosine increases the production of NGF*

In order to study whether another mechanism of signal transduction could be involved in the synthesis of NGF, we determined the effect of sphyngosine on NGF secretion by glial cells. Results reported in Table 1 show that sphingosine increases the extracellular levels of NGF to the same extent as PC-PLC does in PKC down-regulated cells.

4. Discussion

Despite the recent efforts performed to unravel the mechanisms of NGF induction, the signal-transducing pathways leading to the synthesis of this neurotrophic protein remain largely unknown. Many of the agents which are able to stimulate NGF synthesis are dependent on PKC activation [9]. However, steroids and 1,25-dihydroxyvitamin D_3 have been shown to induce NGF protein and mRNA by a mechanism different to serum and PMA, and independent of PKC and c-*fos* gene activation [22,24]. Although this indicates that the the control of NGF synthesis does not depend on a unique pathway, the different putative mechanisms up-regulating NGF synthesis operate by increasing the steady-state levels of intracellular NGF mRNA.

In this report we demonstrate that a PC-PLC induces the synthesis of NGF by glial cells by a mechanism that is partially dependent on PKC activation. The fact that PC-PLC is able to produce an accumulation of NGF transcripts indicates that at least part of the stimulatory effect takes place at the pre-translational level. One of the particular features of the action of PC-PLC, alone or in combination with phosphatidate phosphohydrolase, is that the increase in intracellular DAG concentration is prolonged, indicating that it may be responsible for long-term cellular responses [13]. Our results are in agreement with this notion since NGF is generally considered to be coded by a late expression gene. Results also indicate that PC-PLC acts by at least two different pathways, one dependent on PKC and another independent of PKC. We and others have previously found that PC-PLC triggers some cellular responses, namely induction of DNA synthesis or activation of nuclear transcription factor systems by a PKC-independent mechanism [17,24]. It has been described that PC-PLC is coupled to a sphingomyelinase that catalyzes the breakdown of sphingomyelin into ceramide [24]. A second messenger function for the sphingomyelin cycle and ceramide in the control of cell growth, differentiation and apoptosis has been recently proposed [25]. Our data show that sphingosine induces NGF synthesis to a similar extent than the PKC-independent mechanism elicited by PC-PLC. The effect exerted by sphyngosine may be mediated by its conversion to ceramide, as it has been suggested for other biological responses mediated by sphingosine [26]. On the other hand, vitamin D_3, which induces NGF by a PKC-independent mechanism, has been shown to elicit an early and reversible hydrolisis of sphingomyelin with a concomitant generation of ceramide [27]. Two mechanisms may be therefore involved in the generation of NGF by astrocytes, one represented by serum and DAG acting through PKC, and another one induced by vitamin D_3 acting through the sphingomyelin cycle. Our results suggest that the breakdown of PC by a PLC may trigger both mechanisms.

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