Phorbol 12-myristate 13-acetate (PMA) increases the expression of the nerve growth factor (NGF) gene in mouse L-929 fibroblasts

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The rise of the NGF mRNA pool which takes place following exposure of L-929 fibroblasts to serum was prevented in the presence of 5 μM K-252a, a compound which inhibits several species of protein kinase activities. To characterize further this phenomenon, L-929 cells growing in a serum-free medium were exposed to cyclic nucleotide analogs, to a divalent cation ionophore or to the phorbol ester PMA. Only this latter compound induced an enhancement of the NGF mRNA pool, suggesting an involvement of protein kinase C in the upregulation of the NGF transcripts. The effects of PMA or serum also require a synthesis of protein since the level of NGF transcripts remained stable in the presence of cycloheximide.

Nerve growth factor; Phorbol ester; Serum; mRNA; Protein kinase; Protein synthesis

1. INTRODUCTION

Nerve growth factor (NGF) is a neurotrophic protein which supports the survival and promotes the terminal differentiation of several types of peripheral and central neurons [1]. In vivo, levels of expression of the NGF gene are tissue-specific and developmentally regulated [2–6]. However, the molecular mechanisms involved in the control of the synthesis of NGF remain largely unknown. Studies performed in vitro with NGF-producing L-929 fibroblasts have provided evidence for a regulatory role of some effector molecules, such as glucocorticoids or retinoic acid, which decrease and increase, respectively, the cellular levels of NGF mRNA [7–9].

L-929 fibroblasts have also been shown to respond to serum, which enhances the steady state level of NGF mRNA by a factor of about 5-fold [10,11]. This effect of serum on the transcription or stability of NGF mRNA is not restricted to L-929 cells, as it has also been observed in primary fibroblasts or iris transplants [12].

Polypeptide hormones or growth factors are known to mediate their effect through secondary messengers. Cyclic nucleotide synthesis, calcium mobilization or diacylglycerol production may cause an activation of specific protein kinases which play a critical role in cell responsiveness [13]. Therefore, we have investigated whether the effect of serum on the cellular level of NGF mRNA could be impaired by a protein kinase inhibitor. Alternatively, cells grown in the absence of serum were exposed to various compounds known to activate different types of protein kinases, in order to determine if they could increase the NGF mRNA pool.

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2. MATERIALS AND METHODS

L-929 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% horse serum. Cells were plated in 10 cm diameter dishes at an initial density of 10^3 cells/cm^2. After 16 h, the medium was replaced by a serum-free mixture made of DMEM and F12 media (3:1 v/v) containing insulin (5 μg/ml), transferrin (5 μg/ml) and selenium ions (2.5×10^{-8} M). After 3 days, the serum-free medium was renewed, and cells were exposed to the additives listed in table 1.

Cells were collected after 8 h of treatment and total RNA was extracted by the LiCl/urea procedure [14]. Amounts of poly(A)$^+$ RNA were estimated by hybridization with [^{3}H]poly(U), followed by RNase digestion and TCA precipitation [10]. Glyoxylated RNA samples containing identical amounts of poly(A)$^+$ RNA were fractionated on agarose gels, transferred to a nylon membrane and hybridized with a NGF cDNA probe [15] previously labelled by nick translation. After several washes of the filters, radioactivity associated with the NGF mRNA was revealed by radioautography, and relative levels calculated by densitometric analysis.

Cyclic nucleotide analogs, PMA, ionophore A23187, forskolin, insulin and transferrin were purchased from Sigma Chemicals (France); K-252a was the kind gift of Dr Y. Matsuda (Kyowa Hakko Kogyo Co., Japan) while [^{3}H]poly(U), [^{32}P]dCTP and the nick translation kit were obtained from Amersham (UK). Culture media were from Gibco (France) and tissue culture dishes from Nunc (Denmark).

3. RESULTS

3.1. *The protein kinase inhibitor K-252a counteracts the effect of serum*

K-252a is an inhibitor of protein kinase C, as well as of cyclic AMP- or cyclic GMP-dependent protein kinases [16], thus providing a tool to investigate

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whether the stimulation of NGF synthesis depends on either of these enzymatic activities. L-929 cells maintained in serum-free medium for 3 days were exposed to K-252a during 1 h, and serum was added to the growth medium 1 h later. After 8 h of incubation, mRNAs were extracted and subjected to Northern blot analysis. Data presented in fig.1A and table 1 show that the increase of NGF mRNA pool elicited by serum is abolished in the presence of the kinase inhibitor.

3.2. *PMA mimicks the effect of serum*

In order to characterize which of the different kinase activities inhibited by K-252a mediates the effect of serum, NGF mRNA levels were measured in L-929 cells following exposure for 8 h to cyclic nucleotide analogs, to the divalent cation ionophore A23187 or to PMA. The effect of forskolin, an activator of adenylate cyclase, was also investigated. A typical Northern blot is presented in fig.1B, while the results of the densitometric analyses are summarized in table 1. They demonstrate a 4-fold stimulatory effect of 10^{-6} M PMA. In contrast, forskolin, 8-bromo-cyclic AMP or ionophore A23187 did not have any effect on the steady state level of NGF mRNA, at least at the concentrations tested in these experiments. 8-Bromo-cyclic GMP had a weak stimulatory action. A23187, forskolin or 8-bromo-cyclic GMP, present at the concentrations listed in table 1, did not have any synergistic effect when added together with 1 μM PMA (not shown). Unlike PMA, the unesterified compound 4-α phorbol, which has no kinase C activating activity, was without effect on the NGF mRNA level.

3.3. *Requirement for a protein synthesis*

In order to assess whether PMA and serum may modify the level of NGF mRNA in the absence of protein synthesis, cells grown in serum-free medium were exposed to 10^{-6} M PMA or 5% horse and fetal calf serum, in the absence or presence of 35 μM cycloheximide. The protein synthesis inhibitor prevented the rise of NGF mRNA pool (fig.1C).

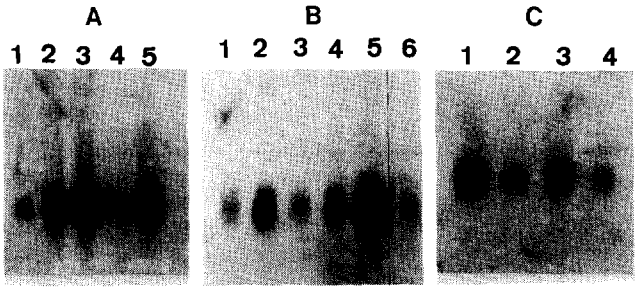

Fig.1. Northern blot analysis of the NGF mRNA of L-929 cells. (A) The protein kinase inhibitor K-252a counteracts the effect of serum. RNAs were extracted from cells growing in serum-free medium (1), or exposed to 5% horse and 5% fetal calf serum plus 0.5 μM K-252a (2), to serum alone (3 and 5), or to serum and 5 μM K-252a (4). (B) PMA mimicks the effect of serum. RNAs were from cells growing in serum-free medium (1), or exposed to 1 μM PMA (2), 10 mM 8-bromo-cyclic AMP (3), 2.8 mM 8-bromo-cyclic GMP (4), 5% horse and 5% fetal calf serum (5) or 0.1 μM A23187 (6). (C) Cycloheximide prevents the effect of serum and PMA. RNAs were extracted from cells exposed for 8 h to 5% horse and 5% fetal calf serum (1), the same and 35 μM cycloheximide (2), 1 μM PMA (3), or 1 μM PMA and 35 μM cycloheximide (4).

Table 1

Levels of NGF transcripts in L-929 fibroblasts

Culture conditions	Relative levels of NGF mRNA ± SD (*n*)
Serum-free medium, no additive	1 (11)[a]
PMA (1 μM)	4.10 ± 0.87 (11)
8-Bromo-cyclic AMP (10 mM)	1.08 ± 0.48 (2)
Forskolin (1 μM)	0.90 ± 0.00 (2)
8-Bromo-cyclic GMP (2.8 mM)	1.86 ± 0.75 (3)
A23187 (0.1 μM)	1.21 ± 0.50 (3)
Horse serum (5%) + fetal calf serum (5%)	8.13 ± 6.70 (9)
Horse serum (5%) + fetal calf serum (5%) + K-252a (5 μM)	1.2 ± 0.56 (2)
Horse serum 5% + fetal calf serum (5%) + cycloheximide (35 μM)	1.46 ± 0.35 (3)
PMA (1 μM) + cycloheximide (35 μM)	1.53 ± 0.35 (3)
4-α-Phorbol (1 μM)	0.99 ± 0.15 (3)

[a] A value of 1 was given to the relative level found in cells cultured in serum-free medium

4. DISCUSSION

Addition of serum to L-929 cells or to primary fibroblasts induces a specific increase of the level of NGF-mRNA [10–12]. We have proposed that the serum effect observed in vitro reflects a wound mechanism, which could take place in vivo in response to vasculature disruption [12]. It was interesting, in this context, to investigate the chemical nature of the intracellular signal which transduces the stimulatory action of serum elements. The response of L-929 cells to serum is prevented in the presence of K-252a, a compound which impairs the activity of several types of protein kinases [16]. Conversely, a 4-fold increase of the pool of NGF mRNA was observed upon addition of PMA to L-929 cells growing in serum-free medium. PMA is known to activate the calcium and phospholipid-dependent protein kinase C [17]. This, and the fact that the unesterified compound 4-α phorbol did not influence the NGF mRNA pool suggest an involvement of protein kinase C in the up regulation of NGF transcripts. These data raise also the possibility that serum factors promoting NGF synthesis may act through this enzyme family.

In contrast, our results suggest that cyclic nucleotide or calcium and calmodulin-dependent protein kinases do not influence the pool of NGF transcripts in L-929 fibroblasts. In this respect, L-929 cells appear different from C6 glioma cells, in which the NGF mRNA levels are regulated by a cyclic AMP-dependent mechanism

[18]. It should be stressed, however, that this cAMP-dependent process was observed in a serum-containing medium, and that serum was reported to enhance the amount of NGF protein released by C6 glioma cells [19] as well as by astrocytes [20]. Thus, it is possible that the serum-mediated regulation of the NGF mRNA, via kinase C, may operate in these cells too.

Use of cycloheximide indicated that transduction of the signal triggered by the presence of serum or PMA requires protein synthesis. It seems possible, therefore, that together with a protein kinase C-dependent process, activation of hitherto silent genes, such as proto-oncogenes, is also involved in the cascade of events leading to a modification of the NGF mRNA pool.

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