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Nerve growth factor increases the cyclic GMP level and activates the cyclic GMP phosphodiesterase in PC12 cells

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Nerve growth factor (NGF) rapidly increases the cyclic GMP (cGMP) level about 2–3-fold and enhances the cGMP phosphodiesterase (PDE) activity about 2-fold in rat pheochromocytoma PC12 cells. No changes in the level of cyclic AMP (cAMP) and in the activity of cAMP PDE were found. GTP and a nonhydrolysable analog of GTP, GMP-PCP, at 100 μM, were able to mimic the effect of NGF on the cGMP PDE activity. These results suggest that the cGMP system may be one of the second messengers of NGF action in PC12 cells.

Nerve growth factor; cyclic GMP; cyclic AMP; Cyclic-GMP phosphodiesterase; Cyclic-AMP phosphodiesterase; (PC12 cell)

1. INTRODUCTION

Nerve growth factor (NGF) is a target-derived trophic protein regulating the survival, development, and maintenance of sympathetic and some sensory neurons [1]. In PC12 cells, NGF induces outgrowth of neurites, increases electric excitability and the amount of acetylcholine receptors [2]. NGF also induces some rapid changes in PC12 cells, such as the phosphorylation of several proteins, activation of c-*fos* and β-actin genes, redistribution of F-actin, etc. [2–5]. Although the mode of action of NGF in various cell types has been well studied, the question about the second messengers involved in the action of NGF is unclear. The evidence concerning the participation of classical second messengers – cyclic nucleotides, and especially cAMP, in the mechanism of NGF action is conflicting [3,6–17]. Moreover, very little is known about the behaviour of the cGMP system in NGF-treated PC12 cells. For this reason we decided to reinvestigate the possible role of cyclic nucleotides in the action of NGF on PC12 cells.

Here we provide experimental evidence that the cGMP system may be one of the second messengers of NGF action in PC12 cells.

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Abbreviations: NGF, neural growth factor; PDE, phosphodiesterase; GMP-PCP, guanylyl (β,γ-methylene)diphosphonate; IBMX, 3-isobutyl-1-methylxanthine

2. MATERIALS AND METHODS

PC12 cells (kindly provided by Dr J. Patrick, Salk Institute) were grown in DMEM medium (Sigma, USA) containing 10% horse serum. For cyclic nucleotide assay, suspended cells were sedimented by centrifugation and resuspended in Tyrode physiological saline (pH 7.4) or RPMI medium (Sigma) and preincubated at 37°C for 10 min. A sample for the determination of the basal levels of cyclic nucleotides was taken just before the treatment of PC12 cells with mouse 7 S NGF (provided by Dr V. Kalyunov, Minsk). During 10 min after the treatment of PC12 cells with NGF, 60-μl samples of cell suspension were collected at 1-min intervals and transferred to 100 μl ice-cold 10% trichloroacetic acid. IBMX (Serva, FRG), an inhibitor of phosphodiesterase (PDE), was added 5 min before NGF. The content of cGMP and cAMP in collected samples was determined by the standard procedure with cAMP and cGMP ^{125}I RIA Kits (Chemapol, Czechoslovakia).

For measurements of the cGMP PDE and cAMP PDE activity the suspension of PC12 cells was incubated with 7 S NGF

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(100 ng/ml) from 1 to 10 min at 37°C. The cells were sedimented by centrifugation and homogenized in ice-cold 40 mM Tris-HCl buffer, pH 7.8, 5 mM $MgCl_2$. The PDE activity was measured in 40 μl homogenization buffer containing 20 μM [8-^{3}H]cGMP or 20 μM [8-^{3}H]cAMP, respectively (Isotop, USSR). In some experiments the incubation buffer also included 100 μM GTP or 100 μM GMP-PCP (Boehringer Mannheim, FRG). The samples were incubated for 15 min at 37°C, the reaction being stopped by adding 40 μl ice-cold 10% trichloroacetic acid. The reaction products were separated on Silufol 254 TLC plates (Kavalier, Czechoslovakia) [18]. The spots were visualized under UV light and the radioactivity counted in 5 ml dioxane-based scintillation fluid using a Rackbeta (LKB, Sweden) liquid scintillation counter. Protein was determined by the method of Lowry et al. [19].

For studying the NGF-triggered F-actin redistribution of PC12 cells, cells were grown on glass coverslips coated with poly-L-lysine (Serva) and stained with rhodamine-phalloidin [20].

3. RESULTS AND DISCUSSION

3.1. *cAMP and cGMP levels after treatment with NGF*

If cyclic nucleotides serve as second messengers in the action of NGF, then their intracellular level in target cells must change in response to NGF within a few minutes. In the first series of experiments, PC12 cells were preincubated with 0.1 mM IBMX. IBMX is an inhibitor of PDE widely used in studying changes in levels of cyclic nucleotides in cells. In the presence of IBMX the level of cAMP and cGMP in NGF-treated PC12 cells remains unchanged.

In the second series, the content of cGMP and cAMP was measured in PC12 cells in the absence of IBMX. Under these conditions, NGF increases the intracellular cGMP level about 2–3-fold within a few minutes. After 6 min of stimulation with NGF the cGMP level reaches a plateau (fig.1). Statistically significant changes in cAMP level were not observed (fig.1). A possible explanation for the increase in cGMP level only in the absence of IBMX is that NGF regulates the activity of cGMP PDE rather than that of guanylate cyclase. Therefore, we assumed that NGF may inhibit the activity of cGMP PDE and the latter may lead to the accumulation of cGMP within the cells.

3.2. *cGMP PDE activity changes in PC12 cells treated with NGF*

Surprisingly, direct measurement of cGMP PDE activity shows that NGF does not inhibit but

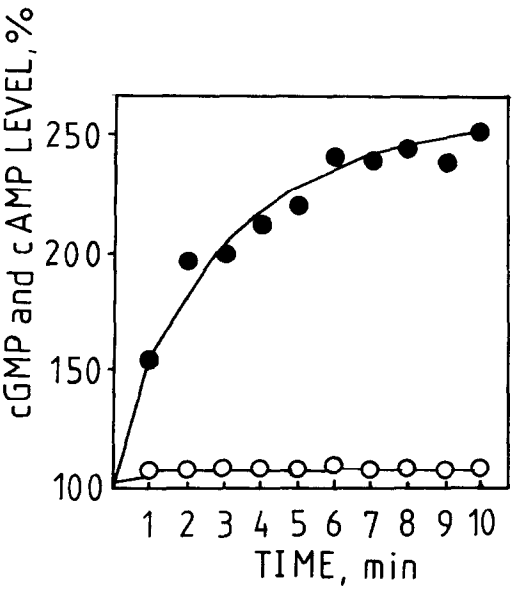

Fig.1. Effect of NGF on the levels of cGMP (●) and cAMP (○) in PC12 cells. 100% – the levels of cGMP and cAMP in control cells (not treated with NGF).

stimulates the cGMP PDE activity about 2-fold (fig.2). This stimulation occurs very rapidly; the increase in activity was completed within 1 min of the treatment with NGF. At the same time NGF had no effect on the activity of cAMP PDE (fig.2).

The data on receptor-mediated PDE activity regulation in other systems indicate that in this

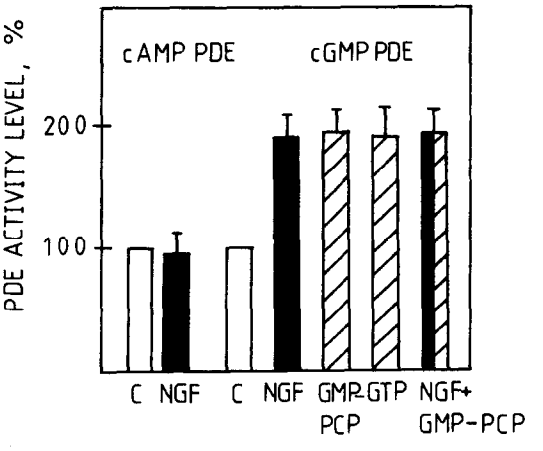

Fig.2. Effects of NGF, GTP and GMP-PCP on cAMP PDE and cAMP PDE activities in PC12 cells. 100% – the cAMP PDE and cGMP PDE activities in control cells (not treated with NGF). NGF, 100 ng/ml; GTP and GMP-PCP, 100 μM.

process a guanine nucleotide regulatory protein (G-protein) is involved [21–23]. For example, a well-characterized G-protein, transducin, couples the light-activated receptor (rhodopsin) of the retinal rod outer segment to activation of a cGMP PDE [24]. It has been proposed that the NGF receptor may also be coupled with G-like protein. This assumption is supported by the facts that microinjection of G-like ras protein p21 into PC12 cells causes the outgrowth of neurites similar to that induced by NGF [25,26] and that neurite outgrowth induced by NGF in PC12 cells is inhibited by microinjection of monoclonal antibodies into Harvey v-ras p21 [27]. Examination of the NGF receptor amino acid sequence has shown that the portion of the NGF receptor extending into the cytoplasm is large enough to possess an enzymatic activity or to interact with other proteins that may mediate signal transduction [28].

If G-protein is involved in the regulation of cGMP PDE activity in PC12 cells, then GTP or its nonhydrolysable analogs ought to mimic the action of NGF. We investigated this possibility and showed that GTP and GMP-PCP (a nonhydrolysable analog of GTP) are indeed able to stimulate the increase in cGMP PDE activity in homogenates of PC12 cells (fig.2). The stimulatory effects of NGF and GMP-PCP were not additive (fig.2). This indicates that the regulation of PDE activity by NGF and GMP-PCP may occur through the same effector system. In fact, these experiments support the idea that G-like protein is involved in the regulation of cGMP PDE activity in PC12 cells. Furthermore, our preliminary experiments indicate that G-protein which is involved in the action of NGF can be blocked by pertussis toxin (PT). On incubation of the cells with PT, no stimulation of cGMP PDE activity by NGF was elicited (not shown).

The question arises as to whether there is any evidence on the physiological role of regulation of cGMP PDE activity by NGF. According to our previous data, NGF causes rapid redistribution of F-actin in PC12 cells [3]. Preincubation of PC12 cells for 30–60 s with 0.1 mM IBMX prior to NGF treatment blocks the redistribution of F-actin (fig.3). Unfortunately, IBMX is not a specific inhibitor for the cGMP PDE, also inhibiting the activity of cAMP PDE. Therefore, we do not know

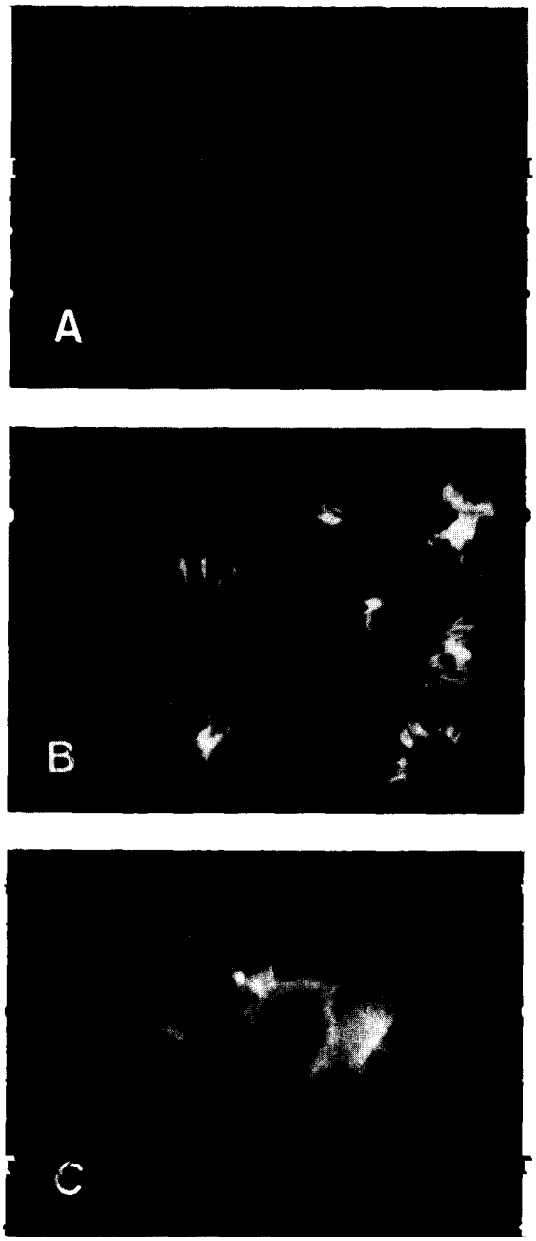

Fig.3. Effect of IBMX on NGF-induced redistribution of F-actin in PC12 cells: (A) untreated, (B) treated with NGF (50 ng/ml) for 10 min, (C) treated simultaneously with NGF and IBMX (0.1 mM) for 10 min. Cells were stained with rhodamine-phalloidin; magnification, 600 ×.

whether the effect of IBMX is due to the increased basal level of these nucleotides or, indeed, to the blockage of the NGF-induced increase in cGMP level (see above). During long-term incubation,

IBMX, analogously to theophylline [29], does not prevent the outgrowth of neurites in PC12 cells. Thus, the rapid and long-term responses caused by NGF may be independent of each other.

The regulation of cGMP PDE activity by NGF in PC12 cells is similar to that by light-activated receptors of the retinal rod outer segment. Hence, one can speculate that in PC12 cells, as in retinal rods, the main role is played by cGMP, regulating the movement of Na^+ and K^+ through the membrane. In this case, cGMP PDE has the function of regulating the cGMP level. This hypothesis is supported by data showing that NGF regulates the permeability of membranes of chick embryo ganglionic neurons and PC12 cells for Na^+ and K^+ [30–33] and that Na^+ responses to NGF are independent of cAMP [34].

Another question to arise concerns the reason why, in spite of the NGF-induced increase in cGMP PDE activity, the intracellular level of cGMP also increases. We suppose that the increase in cGMP level after NGF treatment may be explained by the enhanced guanylate cyclase activity or the compartmentalization of cGMP and cGMP PDE in PC12 cells. In the first case, the activation of guanylate cyclase activity in PC12 cells must be related to the influence of NGF on the PDE, since IBMX, a PDE inhibitor, blocked the NGF-mediated increase in cGMP level. However, further experimental evidence is needed. Taken together, our results with PC12 cells indicate that the cGMP system may be one of the second messenger systems in the action of NGF.

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