Volume 264, number 1, 75–77 FEBS 08398 May 1990

Nerve growth factor stimulates protein kinase C translocation in PC12 cells

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Received 28 February 1990

The effect of nerve growth factor (NGF) on the activity and intracellular localization of protein kinase C (PKC) in pheochromocytoma PC12 cells was studied. By using immunoblotting, immunofluorescence method and phorbol ester binding, NGF was found to induce PKC translocation from the cytoplasm into the cell membrane. This process was accompanied by an increase in the protein kinase activity in the membrane fraction. Translocation was completely blocked by H-7, a protein kinase inhibitor potentiating the neurite-stimulating activity of NGF.

Nerve growth factor; Pheochromocytoma PC12; Protein kinase C; H-7

1. INTRODUCTION

Nerve growth factor (NGF) is a polypeptide that is required for the development and maintenance of sympathetic, sensory, and certain central cholinergic neurons. It was shown that the effects of NGF on target cells are the result of its binding with the cell surface receptors. It is becoming clear that these effects are mediated by a number of second messengers, but the exact nature of these second messengers is unclear [1,2].

It is obvious at present that the NGF signal is mediated by a number of protein kinases (EC 2.7.1.37), and the evidence obtained by several research teams indicates that different protein kinases modulate different effects of NGF. It was shown that NGF changes the activities of several protein kinases including cAMP-dependent protein kinase [3], Ca^{2+}-calmodulin-dependent protein kinase III [4], protein kinase C (PKC) [3,5], and NGF-sensitive S6 kinase [6].

A suitable approach to study the role of protein kinases in the regulation of different functions of NGF is the use of selective inhibitors. A PKC inhibitor, sphingosine, is known to inhibit the NGF-dependent neurite outgrowth in PC12 cells [7,8]. A potent protein kinase inhibitor, K-252a, completely blocks many of the NGF effects [9,10]. Its derivative, staurosporine, has the same effect at low concentrations and shows a neurite-stimulating activity in PC12 cells at high concentrations [11]. H-7, an inhibitor of cyclic nucleotide-dependent protein kinases and PKC, stimulates the neurotrophic action of EGF, FGF in neurons derived from the CNS of neonatal rats [12], and of NGF and FGF in PC12 cells [8]. Besides, H-7 induces neurite outgrowth in neuro 2a, a neuroblastoma cell line [13]. However, the available literature data are insufficient for identification of a protein kinase responsible for the induction of neurite outgrowth.

Some publications contain evidence indicating that PKC is not a key enzyme mediating the NGF signal [9,14]. At the same time, PKC activates tyrosine hydroxylase in PC12 cells [3], it participates in the regulation of neurotransmitter secretion [15], and its activity in the cytoplasm increases after incubation of the cells with NGF [5]. In turn, NGF stimulates the phosphoinositide turnover generating a PKC activator – diacylglycerol [16].

These data suggest the involvement of PKC at certain stages in the NGF action on PC12 cells. The present study deals with the effect of NGF and H-7 (a protein kinase inhibitor increasing the neurite-stimulating activity of NGF) on the activity and localization of this enzyme in PC12 cells. It has been shown that NGF induces PKC translocation into PC12 cell membranes and H-7 blocks this process.

2. MATERIALS AND METHODS

Pheochromocytoma PC12 cells were cultured in DMEM (Gibco) supplemented with 10% heat-inactivated horse serum, 50 μg/ml streptomycin and 50 U/ml penicillin. The cells were incubated in a humidified atmosphere containing 5% of CO_2 and 95% of air.

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Published by Elsevier Science Publishers B.V. (Biomedical Division)

NGF was isolated from bovine seminal plasma as described previously [17]. H-7 was purchased from Seikagaki Kogyo Co. To determine the activity of PKC, PC12 cells were plated on Petri dishes (20000 cells/cm^2). The effectors were added in the following concentrations: NGF 100 ng/ml, H-7 50 μM, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) 0.1 μM. After 15 min of incubation, the medium was removed, the cells were washed twice in Hank's balanced salt solution and placed in an ice-cold lysing buffer (20 mM Tris-HCl, pH 7.5, 250 mM sucrose, 5 mM EGTA, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.01% leupeptine, 0.4 mM phenylmethyl sulphonylfluoride). The cells were mechanically removed from a dish and homogenized in a Teflon-glass homogenizer (B. Braun). The homogenate was centrifuged at 1000 × *g* for 5 min to remove the nuclei; then the membranes and cytoplasm were separated by ultracentrifugation at 100000 × *g* for 70 min. The pellet was resuspended in a lysing buffer containing 1% detergent NP-40. The cytosol and membrane fractions were applied onto columns with DEAE cellulose DE-52 (1 ml vol.) balanced with 20 mM Tris-HCl buffer, pH 7.5, containing 0.1 mM EGTA, 0.1 mM EDTA, 1 mM dithiothreitol. The columns were washed with 2 vols of the same buffer, and protein kinase C was eluted using 3 ml of the same buffer supplemented with 0.2 M NaCl. Protein kinase C activity was determined according to Kikkawa et al. [18].

Protein concentration was measured by the method of Bradford [19].

Binding of phorbol 12,13-dibutyrate ([^{3}H]PDBu) to PC12 cells was performed as described previously [20]. PDBu concentration in the medium was 3 nM.

Immunoblotting was performed as described earlier [21]. After homogenization of the cells treated with the effectors and separation of the membrane and cytosol fractions (see above) the samples were subjected to SDS-electrophoresis according to Laemmli [22] in gradient (4–20%) polyacrylamide gel with subsequent electrophoretic transfer on nitrocellulose sheets.

Monoclonal antibodies to PKC (Amersham) were conjugated with FITC [23]. PC12 cells were treated with the effectors (see above), carefully washed in phosphate-buffered saline (Flow) containing 0.02% sodium azide and 0.1% bovine serum albumin, and placed into a solution of FITC-labelled antibodies to PKC (1:10). The cells were incubated for 1 h at 4°C, carefully washed, mechanically removed, suspended in 1 ml of the same buffer and examined on a flow sorter EPICS C (Coulter). Cell viability was controlled by ethidium bromide staining [24].

3. RESULTS AND DISCUSSION

The influence of NGF on the activity and localization of PKC in PC12 cells was studied. PKC activity was determined in the cytoplasmic and rough membrane fractions (see section 2) of pheochromocytoma PC12 cells after incubation with NGF, TPA, and NGF + H-7 (Fig. 1A,B). The data presented in Fig. 1A indicate that NGF and TPA caused a slight increase in the cytoplasmic PKC activity. In the membrane fraction a sharp increase in the PKC activity was observed after treatment with these effectors (Fig. 1B). The data obtained demonstrate that NGF, as well as TPA, induces PKC translocation from the cytoplasm into the membrane in PC12 cells, which is a characteristic feature of activation of this enzyme [25]. Since we showed earlier that the neurite-stimulating activity of NGF is sharply enhanced in the presence of H-7 [8], we studied the effect of the latter on NGF-induced changes in the PKC activity. It appeared that NGF-induced enhancement of the activity was practically completely blocked in both cytosols and membrane fractions (Fig. 1A,B).

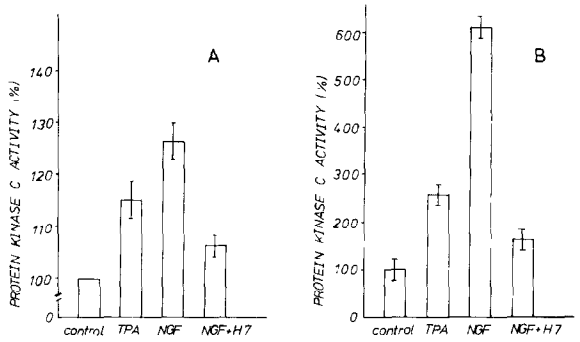

Fig. 1. Effect of TPA, NGF and NGF + H-7 on the activity of PKC in the cytosol (A) and membrane (B) fractions of PC12 cells. PKC activity in the control was 14500 ± 270 cpm/min per mg of protein in the cytosol, and 9789 ± 120 cpm/min per mg of protein in the membrane fraction.

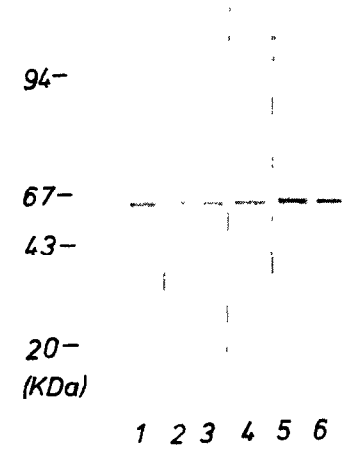

Fig. 2. Electroblotting of PKC from the cytosol (1–3) and membrane (4–6) fractions of PC12 cells. (1, 4) Control; (2, 5) 15 min incubation with TPA; (3, 6) 15 min incubation with NGF.

NGF-induced translocation of PKC in PC12 cells was corroborated by us through detection of phorbol ester binding, by immunoblotting and immunofluorescence method.

PKC in association with phospholipids and calcium

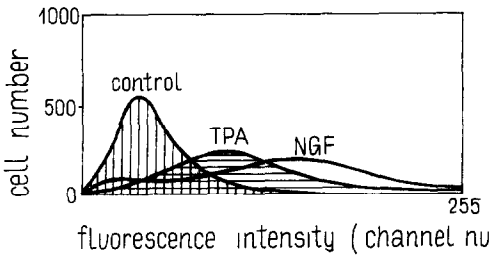

Fig. 3. Fluorescence intensity of PC12 cells labelled with monoclonal antibodies to PKC after 15 min treatment with TPA and NGF.

ions is known to be a receptor of phorbol esters [25]. Therefore [^{3}H]PDBu is often used for estimating PKC concentrations in different tissues [26]. In our studies the specific binding made up 2.25 ± 0.5 pmol/mg of protein upon incubation of control PC12 cells with PDBu. After 15 min of incubation of cells with NGF, this value increased up to 7.1 ± 1.5 pmol/mg of protein. H-7 added to the incubation medium completely eliminated the stimulatory effect of NGF.

PKC translocation into the membrane is most visually detected by the electrophoretic transfer of the cytosol and membrane fractions onto nitrocellulose sheets followed by staining with monoclonal antibodies to the enzyme. As seen from Fig. 2, in control PC12 cells PKC was detected in both cytosol and membrane fractions. 15 min incubation of cells with TPA caused a translocation of the enzyme onto the membrane and a corresponding decrease in its concentration in the cytosol fraction. NGF also produced the same effect. The amount of PKC in the membrane increased 3.4-fold in the presence of TPA and 2.4-fold in the presence of NGF.

The use of a flow sorter permits the detection of the antigen on the outer membrane surface. After 15 min incubation of cells with NGF, an increase was observed in the binding of FITC-labelled monoclonal antibodies to PC12 cells. A similar increase occurred also in the case of TPA (Fig. 3). H-7 alone had no effect, but in combination with NGF it completely blocked the action of the latter. In the course of the experiment, however, a great number of cells (up to 80%) broke down. In this connection, the intensification of fluorescence of cells after their treatment with TPA and NGF can be explained not only by the translocation of PKC and/or the exposure of the antigen onto the outer membrane surface but also by an increase under the action of the effectors in the affinity of FITC-labelled antibodies to PKC penetrating into a broken cell. However, taking into account the results of immunoblotting demonstrating quantitative changes in the PKC content in the cytosol and membrane fractions, as well as the data on PDBu binding and enzyme activity, it can be assumed that the effect observed on EPICS C is associated with an increase in the content of PKC in PC12 cell membranes after treatment with TPA and NGF. Cytoplasmic PKC is most likely to be removed in the course of cell washing. The possibility of PKC exposure on the outer surface of PC12 cell membranes under the action of NGF and TPA is being explored.

The data obtained indicate that NGF induces PKC translocation from the cytoplasm into the membrane resulting in the enhancement of the activity of the enzyme. Besides, a certain increase in the activity of cytoplasmic PKC was observed, which agrees with the data of Hama et al. [5]. The translocation effect was completely blocked by H-7, an inhibitor of cyclic nucleotide-dependent protein kinases and PKC. As shown earlier [8], H-7 is a strong promoter of the neurite-stimulating activity of NGF in PC12 cells. The results obtained suggest that the stimulatory effect of H-7 is associated with blocking of PKC translocation and that PKC itself is involved in the regulation of NGF-induced neurite outgrowth.

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