Journal of Biochemistry and Molecular Biology, Vol. 33, No. 6, November 2000, pp. 525-530

JOURNAL OF
Biochemistry
and
Molecular Biology

Involvement of Cytosolic Phospholipase A_2 in Nerve Growth Factor-Mediated Neurite Outgrowth of PC12 Cells

Soonwook Choi[1], Eunah Yu[1], Young Seek Lee[2] and Young Sook Yoo[1]*

[1]Bioanalysis and Biotransformation Research Center, Korea Institute of Science and Technology, P.O. Box 131, Cheongryang, Seoul 130-650,

[2]Department of Biochemistry, College of Science, Hanyang University, Kyonggi 425-170, South Korea

Received 10 October 2000, Accepted 14 November 2000

The nerve growth factor (NGF) induces neuronal differentiation and neurite outgrowth of PC12 cells, whereas epidermal growth factors (EGF) stimulate growth and proliferation of the cells. In spite of this difference, NGF-or EGF-treated PC12 cells share various properties in cellular-signaling pathways. These include the activation of the phosphoinositide (PI)-3 kinase, 70 kDa S6 kinase, and in the mitogen-activated protein (MAP) kinase pathway, following the binding of these growth factors to intrinsic receptor tyrosine kinases (RTKs). Therefore, many studies have been attempted to access the critical signaling events in determining the differentiation and proliferation of PC12 cells. In this study, we investigated the cytosolic phospholipase A_2 ($cPLA_2$) in neurite behavior in order to identify the differences of signaling pathways between the NGF-induced differentiation and the EGF-induced proliferation of PC12 cells. We have showed here that the $cPLA_2$ was translocated from cytosol to membrane only in NGF-treated cells. We also demonstrated that this translocation is associated with NGF-induced activation of phospholipase C-γ (PLC-γ), which elevates intracellular Ca^{2+} concentration. These results reveal that the translocation of $cPLA_2$ may be a requisite event in the neuronal differentiation of PC12 cells. Various phospholipase inhibitors were used to confirm the importance of these enzymes in the differentiation of PC12 cells. Neomycin B, a PLC inhibitor, dramatically inhibited the neurite outgrowth, and two distinct PLA_2 inhibitors, 4-bromophenacyl bromide (BPB) and arachidonyltrifluoro-methyl ketone ($AACOCF_3$) also suppressed the neurite outgrowth of the cells, as well. Taken together, these data indicated that $cPLA_2$ is involved in NGF-induced neuronal differentiation and neurite outgrowth of PC12 cells.

*To whom correspondence should be addressed.
Tel: 82-2-958-5076; Fax: 82-2-958-5170
E-mail: ysyoo@kist.re.kr

Keywords: Cytosolic phospholipase A_2, Differentiation, Nerve Growth Factor, PC12.

Introduction

Growth factors are known to induce a great variety of cellular regulations such as cell growth, differentiation, and metabolic homeostasis of individual cells. During the last decade, many growth factors, such as the nerve growth factor (NGF) and the epidermal growth factor (EGF), have been discovered, and the cell surface receptors that mediate their biological functions have been characterized (Eppstein *et al.*, 1985; Godfrey and Shooter, 1986; Levi-Montalcini, 1987; Marchetti and Perez-Polo, 1987; Buxer *et al.*, 1990).

NGF is a neurotropic agent thought to be provided by peripheral tissues for the guidance and substance of outgrowing embryonic, sympathetic and sensory neurons (Server and Shooter, 1977). NGF induces the formation of neurite-like filaments from chick embryo dorsal root ganglia and from rat pheochromocytoma (PC12) cells (Greene and Tischler, 1976). *In vivo*, NGF may be involved in fetal development (Ayer-Lelievre *et al.*, 1983; Taniuchi *et al.*, 1986) and nerve regeneration (Thornburn, 1985). NGF may also play a physiological role within the central nervous system (Ayer-Lelievre *et al.*, 1983; Dreyfus, 1989; Ebendal, 1989). The epidermal growth factor (EGF) is a 6 kD polypeptide discovered by Cohen and Levi-Montalcini. The biological role of EGF includes the inhibition of gastric acid secretion (Gregory, 1975), the support of growth and differentiation during fetal development (Thornburn, 1985), and neuromodulation in the central nervous system (Brown *et al.*, 1989). Stimulation of PC12 cells with EGF, which activates the EGF receptor tyrosine kinases, also leads to the induction of cell growth and proliferation (Chao, 1992).

PC12 cells are established from a transplantable rat adrenal pheochromocytoma (Greene and Tischler, 1976). These cell lines serve as a useful model system for examining

mechanisms underlying neuronal- differentiation and signal transduction. Exposure of PC12 cells to NGF results in differentiation of the cells, i.e. the conversion from a chromaffin-like phenotype into a sympathetic-neuron-like phenotype (Green and Tischler, 1982). Yet, stimulation of PC12 cells with EGF, which activates the EGF receptor tyrosine kinases, leads to the induction of cell growth and proliferation (Edelman *et al.*, 1987; Czech *et al.*, 1988). NGF- and EGF-treated PC12 cells share several common properties including rapid membrane ruffling, flatting of cells, and increases in cell adhesion (Connolly *et al.*, 1984). However, NGF but not EGF stimulates neurite outgrowth. EGF functions as a typical mitogen for PC12 cells, including the same early responses with NGF, but none of the late differentiation-associated responses (Chao, 1992). Much experimental attention has been paid to examine the differences of the signaling pathway in the NGF-induced differentiation and EGF-induced proliferation of PC12 cells (Traynor, 1984; Kim *et al.*, 1991; Vetter *et al.*, 1991; Hashimoto *et al.*, 1994; Mark *et al.*, 1995; Marshall, 1995; York *et al.*, 1998).

In the previous study about the relationship between phospholipid metabolism and neurite extention, the PLA_2 is thought to be involved in NGF-induced neuronal differentiation of PC12 cells (Traynor, 1984). However, its signaling pathway is not clarified yet. Therefore, we attempted to investigate the roles of $cPLA_2$ in neurite outgrowth. $cPLA_2$ has now been implicated to function in various cellular responses such as mitogenesis (Sa and Das, 1999; Van *et al.*, 2000), differentiation (Burke *et al.*, 1999; Sjursen *et al.*, 2000), inflammation (Adam *et al.*, 1998; Fukuda *et al.*, 1999), and cytotoxicity (Sapirstein *et al.*, 1996; Huh *et al.*, 1998; Pirianov *et al.*, 1999). Depending on the tissue or cell type, $cPLA_2$ can play a functional role through its ability to trigger arachidonic acid release and eicosanoid production (Cristina, 1997).

In this study, we demonstrated the translocation of $cPLA_2$ from cytosol to membrane in the NGF-stimulated PC12 cells, unlike to the EGF-stimulated PC12 cells. We also demonstrated that this translocation is accomplished by the activity of PLC-γ. In addition, we confirm the involvement of PLC-γ and $cPLA_2$ in the differentiation of a PC12 cell using the neurite assay method, showing that various phospholipase-inhibitors, such as neomycin B sulfate, 4-bromophenacyl bromide (BPB), and arachidonyltrifluoromethyl ketone ($AACOCF_3$), dramatically suppressed the NGF-induced neurite outgrowth of the PC12 cells.

Materials and Methods

Materials PC12 cells were obtained from the American Type Culture Collection (ATCC). NGF, EGF, neomycin B sulfate, BPB, anti-rabbit IgG polyclonal antibody(pAb), and rat collagen were from Sigma (St. Louis, USA). $AACOCF_3$ was obtained from BIOMOL Research Laboratories (Plymouth Meeting, USA). Anti-PLC-γ monoclonal antibody (mAb) mixture, anti-phosphotyrosine mAb, and anti-mouse IgG pAb were purchased from Upstate Biotechnology (Lake Placid, USA), and anti- $cPLA_2$ mAb was obtained from the Santa Cruz Biotechnology (Delaware, USA). Protein G Sepharose, Protein A Sepharose, and Western blotting-detection reagent ECL™ were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). The RPMI1640 medium, fetal bovine serum (FBS), and horse serum were purchased from GIBCO BRL (Gaithersburg, USA).

Cell Cultures PC 12 cells were cultured in a RPMI 1640 medium supplemented with 10% fetal bovine serum and 5% horse serum. Cells were grown in a 37°C humidified incubator supplied with 5% CO_2 and passaged every third day.

Preparation and Treatment of Growth Factors To prepare a stock solution, reconstitute the growth factors such, as NGF and EGF, in a solution of tissue culture media containing 0.1% bovine serum albumin (BSA) to a concentration of 10 μg/ml. This may be diluted immediately before use to the final working concentration.

Detection for Tyrosine Phosphorylation of PLC-γ Cells were grown to about 90% confluence on a 10 cm-culture dish and starved for 24 h in a serum-free defined RPMI 1640 medium containing 10 μg/ml insulin, 6.7 mg/ml sodium selenite, 5.5 μg/ml sodium transferrin, 110 μg/ml pyruvate, 0.5% (w/v) glucose, 1 mM glutamine, 20 nM progesterone, and 0.1 mM putrescine. Cultures were incubated for an additional 10 min in the presence or absence of NGF (100 ng/ml) or EGF (100 ng/ml). Incubation was terminated by aspirating the medium, and the cells were washed with 2 ml portions of ice-cold phosphate-buffered saline. Each plate was treated with 1 ml of an ice-cold lysis buffer (20 mM Hepes, pH 7.2, 1% Triton X-100, 10% glycerol, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 10 μg/ml aprotinin), and incubated for 10 min at 4°C. After centrifugation (12,000 rpm, 10 min), the cell extracts were then treated with 5 μg anti-PLC-γ monoclonal antibodies for 6 h, and then with 30 μl of the protein G-sepharose beads for 2 h. The immunoprecipitates were recovered by centrifugation in a microcentrifuge and washed four times with a washing buffer containing 1% Triton X-100, 1% deoxycholate, 1% SDS, 150 mM NaCl, and 50 mM Tris, pH 8.5. The immunoprecipitated proteins were dissolved in a 10 ml 6X Laemmli buffer and heated for 5 min at 95°C. The precipitations were centrifuged for 10 min, and the proteins present in the supernatant fraction were analyzed by 7.5% SDS-polyacrylamide gel electrophoresis. For Western blotting analysis, the gels were transferred to a nitrocellulose membrane (MSI, Westborough, USA) in a Western blot apparatus (Hoefer Scientific, San Francisco, USA). The membranes were blotted with anti-phosphotyrosine mAb for 3 h and horseradish peroxidase linked with anti-mouse IgG pAb for 30 min. The phosphorylation of the protein was detected using ECL™.

Detection for Translocation of $cPLA_2$ The cells were grown in the same conditions as in the mobility-shift assay. Each plate was treated with 1 ml of an ice-cold, detergent-free lysis buffer (20 mM Hepes, pH 7.2, 10% glycerol, 50 mM NaF, 1 mM

phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 10 μg/ml aprotinin), incubated for 10 min at 4°C, and sonicated for 30. The nuclei, organelles, and unbroken cells were pelleted by centrifugation at 1,000 × g for 10 min. The postnuclear supernatant was centrifuged at 100,000 × g for 60 min to separate cytosolic and membrane fractions, and both fractions were adjusted to the same volume. Both fractions were then treated with 5 μg anti-$cPLA_2$ mAb for 6 h, and then with 30 μl of the protein G-sepharose beads 1 : 1 slurry for 2 h. The immunoprecipitates were recovered and analyzed by 7.5% SDS-polyacrylamide gel electrophoresis as described above. After transferring the gels to nitrocellulose membranes, the membranes were blotted with anti-$cPLA_2$ monoclonal antibodies (Santa Cruz Biotechnology, USA) for 3 h and anti-mouse Ig, horseradish peroxidase linked with whole antibodies for 30 min. The $cPLA_2$ in each fractions were detected using ECL™.

Neurite Assay For the evaluation of the neurite outgrowth, following neurite assays were performed. The PC12 cells were cultured with the growth medium described above for 24 h in 60 mm-culture dishes. Before treatment of growth factors to the cells, the medium was changed to a serum-free defined medium and incubated for an additional 12 h. A portion of the cells were pretreated various phospholipase inhibitors for the indicated times before and during 60 min of treatment with 100 ng/ml NGF. Controls which received 0.1% dimethyl sulphoxide (DMSO) were also perforemed. DMSO by itself, however, had no effect. After incubation in various conditions, the cells were washed with a phosphate-buffered saline (PBS) and then fixed with 4% formaldehyde in PBS for the neurite outgrowth measurement. Cells in two culture dishes, with 5 counting areas on each dish, were examined for each experimental groups to calculate the ratio of neurite-bearing cells observed per area. Neurite was defined as such if its length was longer than a diameter of the cell body. All the data are reported as mean±standard error of the mean (S.E.M).

Results

NGF Stimulates Phosphorylation of Phospholipase C-γ in PC12 Cells To investigate whether or not the NGF or EGF treatment of PC12 cells induced phosphorylation of PLC-γ, we used a mixture of anti-PLC-γ mAbs to immunoprecipitate PLC-γ from lysates of untreated, NGF-treated, and EGF-treated PC12 cells. After immunoprecipitation was performed, immunoprecipitates were probed by Western blotting analysis with antibodies directed against phosphotyrosine. Although serine or threonine residues are also present at the phosphorylation sites of the protein in addition to tyrosine, it is well known that tyrosine is the major phosphorylation residue of PLC-γ (Vetter *et al.*, 1991). The tyrosine phosphorylation of PLC-γ was increased significantly by the NGF treatment. On the other hand, the increase in phosphorylation of PLC-γ was not observed when cells were treated with EGF or left untreated (Figure 1).

NGF Induces Translocation of $cPLA_2$ from Cytosol to Membrane in PC12 Cells Activation of $cPLA_2$ requires not only phosphorylation, but also Ca^{2+}-dependent translocation of the enzyme from cytoplasm to membranes (Kast *et al.*, 1993; Lin *et al.*, 1993; Qiu *et al.*, 1993; Marshall, 1995; Cristina, 1997). To specifically demonstrate $cPLA_2$ translocation, membrane and cytosolic fractions from unstimulated, NGF-treated, and EGF-treated PC12 cells were isolated and subjected to immunoprecipitation and Western blotting with anti- $cPLA_2$ mAb. The result in Figure 2A shows that the quantity of $cPLA_2$ in the membrane fraction was increased in response to the NGF treatment. Increases of $cPLA_2$ quantity in

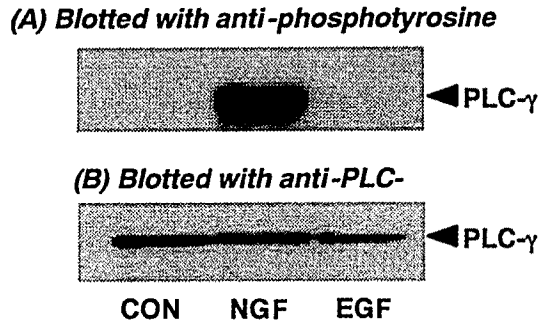

Fig. 1. Effect of NGF and EGF on the tyrosine phosphorylation of PLC-γ in PC12 cells. Lysates from unstimulated cells (CON) or from cells stimulated with either NGF (100 ng/ml) or EGF (100 ng/ml) were immunoprecipitated with anti-PLC-γ Ab and immunoblotted with anti-phosphotyrosine Ab (A). After exposure, the nitrocellulose membrane was stripped and then blotted with anti-PLCγ Ab (B).

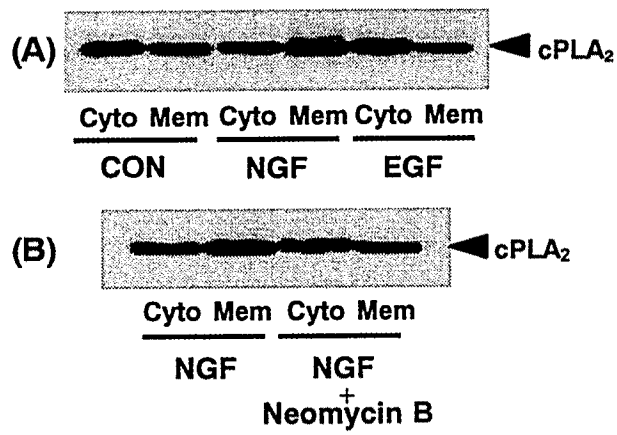

Fig. 2. NGF-stimulated translocation of $cPLA_2$ from cytosolic to membrane fractions (A). PC12 cells were incubated with 100 ng/ml of NGF at 37°C for 30 min. Lysates were prepared and the cytosolic (Cyto.) and membrane (Mem.) fractions were isolated by ultracentrifugation. The fractions were subjected to immunoprecipitation and immunoblotted with anti-$cPLA_2$ mAb. Inhibition of $cPLA_2$ translocation by the PLC inhibitor, neomycin B (B). To investigate the relationship between PLC activity and the translocation of $cPLA_2$, PC12 cells were incubated with 100 ng/ml of NGF and 0.5 μM neomycin B, inhibitor of PLC.

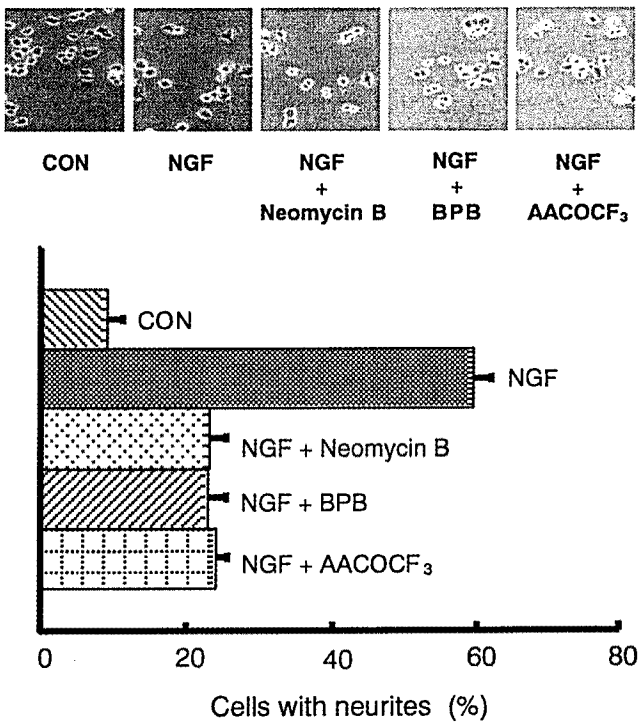

Fig. 3. Effects of various phospholipase inhibitors on the NGF-induced neurite outgrowth. The various phospholipase inhibitors pretreated for the indicated times before and during NGF treatment with concentration of 100 ng/ml (60 min); Neomycin B (60 min), BPB (30 min), $AACOCF_3$ (30 min). PC12 cells were cultured for more than 3 days in a serum-free medium with NGF. A control group was cultured without NGF. A total of over 300 cells were counted by using 2 dishes per treatment group. Each bar shows the percent of cells bearing neurite.

the membrane fraction were not detected when the cells were treated with EGF or untreated. These data clearly show that $cPLA_2$ is translocated to membrane after stimulation of cells with NGF, but not with EGF.

Requirement for Phospholipase C-γ for Translocation of $cPLA_2$ by NGF To determine whether an increase of intracellular Ca^{2+} concentration upon activation of PLC-γ by NGF treatment was also associated with the translocation of $cPLA_2$, we investigated the translocation of $cPLA_2$ from cytosol to the membrane after treatment of neomycin B sulfate, which is a PLC inhibitor that suppresses the enzyme activity via binding to inositol phospholids (Smalheiser *et al.*, 1966). In Figure 2B, the translocation of $cPLA_2$, which was clearly shown in NGF-stimulated cells, was inhibited by Neomycin B treatment.

Effect of Phospholipase Inhibitors on Neurite Outgrowth Important effects of PLC-γ and $cPLA_2$ on NGF-induced neuronal differentiation and neurite outgrowth was examined by neurite assay. Treatment of PC12 cells with 0.5 mM of PLC inhibitor, neomycin B, for both 1 h before and during NGF treatment significantly inhibited NGF-induced neurite outgrowth for the differentiated PC12 cell cultures (Figure 3).

We also treated two distinct PLA_2 inhibitors to investigate the relationship between $cPLA_2$ activity and neurite behavior. Treatment with 0.1 μM BPB, one of the generally used as a PLA_2 inhibitors, inhibited the NGF-induced neurite outgrowth dramatically. A similar inhibitory effect was also observed on the NGF-induced neurite outgrowth with the treatment of 50 μM $AACOCF_3$ which is a cell-permeable trifluoromethyl ketone analog of arachidonic acid and specific inhibitors for $cPLA_2$ on its activity (Figure 3).

Discussion

Many previous studies (Traynor, 1984; Kim *et al.*, 1991; Vetter *et al.*, 1991; Tsukada *et al.*, 1994; Blumberg *et al.*, 1995; Marshall, 1995; Smalheiser *et al.*, 1966;York *et al.*, 1998) were performed to clarify what differences lead to deciding the fate of cells, such as the differentiation or proliferation upon NGF- or EGF- stimulated PC12 cells respectively, and to identify many differences in the growth factor-stimulated PC12 cell. Traynor reported that the difference of phospholipid metabolism was related to neurite behavior in PC12 cells (Traynor, 1984). In 1991, the difference of phosphoinositide(PI)-hydrolysis in NGF- or EGF-treated PC12 cells was also observed (Kim *et al.*, 1991). Therefore, we first attempted to investigate PLC as a candidate enzyme associated with these differences, since PLC hydrolyzes various phospholipid in the cells. PC12 cells have three immunologically distinct PLC isozymes, PLC-β, PLC-γ, and PLC-δ, and it is well known that only PLC-γ isozyme is phosphorylated when NGF was treated to PC12 cells (Kim *et al.*, 1991). Thus, we also attempted to observe the phosphorylation of PLC-γ with NGF- or EGF-treatment to the cells. As shown in Figure 1, only NGF treatment showed stimulation of the tyrosine phosphorylation of PLC-γ. This result provides insight into the critical signaling event to identify the differences between NGF-induced differentiation and EGF-induced proliferation of PC12 cells. To confirm the relationship between the activity of this enzyme and NGF-induced differentiation of PC12 cells, the effect of neomycin B, a PLC inhibitor on NGF-induced neurite outgrowth, was investigated by morphological analysis. And the inhibitory effect of neomycin B on the NGF-induced neurite outgrowth was shown in Figure 2B. Together, these results represent convincing evidence that the phosphorylation and activation of PLC-γ play an important role in the NGF-induced differentiation of PC12 cells.

NGF has been also shown to stimulate arachidonic acid (AA) release, a product of $cPLA_2$ (Tsukada *et al.*, 1994) in PC12 cells, however, the functional outcome of $cPLA_2$ activation has not been defined. Therefore, we attempted to investigate whether $cPLA_2$ would related to NGF-induced neuronal differentiation of PC12 cells. Accordingly, we

investigated the extent of translocation of $cPLA_2$ from cytosol to membrane upon NGF stimulation. Since, the process of translocation of $cPLA_2$ and the phosphorylation of $cPLA_2$ are thought to be equally important and necessary for the activation of the enzyme (Kast *et al.*, 1993; Lin *et al.*, 1993; Qiu *et al.*, 1993; Marshall, 1995; Cristina, 1997).

As shown in Figure 2A, $cPLA_2$ was translocated only with NGF treatment. In EGF-stimulated PC12 cells, the translocation of PLA_2 from cytosol to the membrane was not detected as in the group of unstimulated PC12 cells. Therefore, it is clear that the translocation of $cPLA_2$ is critical signaling event in NGF-induced differentiation of PC12 cells.

Translocation of $cPLA_2$ is achieved by the binding of Ca^{2+} to the calcium-binding (CaLB) domain of the enzyme (Kast *et al.*, 1993; Qiu *et al.*, 1993). Thus, increasing the level of intracellular calcium ion is very important for $cPLA_2$ activation. The increase in intracellular Ca^{2+} level is closely related to the activation of PLC that produces diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). IP3 controls the Ca^{2+} mobilization from the endoplasmic reticulum (ER) to cytosol, which leads to the elevation of the concentration of calcium ion in the cytosol (Fay *et al.*, 1994; Fernando and Barritt, 1994; Vandeheijning *et al.*, 1994). Therefore, the relationship between the activity of PLC and the translocation of $cPLA_2$ came into our interest, and this relationship was examined in this study as well. When PC12 cells were pretreated with neomycin B, which is a specific inhibitor of PLC, treatment with NGF to the cells did not stimulate the translocation of $cPLA_2$, as shown in Figure 2B. This result confirms that the activity of PLC-γ is closely coupled to the translocation of $cPLA_2$.

To test the importance of $cPLA_2$ in NGF-induced neuronal differentiation, neurite assay was also performed using PLA_2 specific inhibitors such as BPB and $AACOCF_3$. $AACOCF_3$ is especially known to be a specific inhibitor for the cytosolic form of the PLA_2 enzyme. Treatment with 0.1 μM BPB, which inhibits the activity of PLA_2, suppressed the NGF-induced neurite induction. Futhermore, treatment of 50 μM $AACOCF_3$, which specifically inhibits the $cPLA_2$ also suppressed the neurite outgrowth of PC12 cells to the same extent as BPB (Figure 3). Based on these data, it is suggested that the activity of cytosolic forms of PLA_2 ($cPLA_2$) is involved in the neurite behavior of PC12 cells.

On the basis of these findings, we suggest that not only the phosphorylation process, but also translocation of the $cPLA_2$ are the critical signaling events. Also, the activation of $cPLA_2$ is involved in NGF-induced differentiation of PC12 cells. Perhaps, AA, which was produced by $cPLA_2$, has an intimate relationship with the neurite behavior of PC12 cells. This suggestion was also supported by other investigation regarding the role of AA in neurite sprouting and the effect of exogenous or endogenous AA in neurite behavior (Smalheiser *et al.*, 1966).

Acknowledgment This work was supported in part by grants (2U02330, 2N19790, 2N19770, 2M10160).

References

Adam-Klages, S., Schwandner, R., Luschen, S., Ussat, S., Kreder, D. and Kronke, M. (1998) Caspase-mediated inhibition of human cytosolic phospholipase A_2 during apoptosis. *J. Immunol.* **161**, 5687-5694.

Ayer-Lelievre, C. S., Ebendal, T., Olson, L. and Seiger, A. (1983) Localization of nerve growth factor-like immunoreactivity in rat nervous tissue. *Med. Biol.* **61**, 296-304.

Blumberg, D., Radeke, M. J. and Feinstein, S. C. (1995) Specificity of Nerve Growth Factor Signalling: Differential Patterns of Early Tyrosine Phosphorylation Events Induced by NGF, EGF, and bFGF. *J. Neurosci. Research* **41**, 628-639.

Brown, G., Nanney, L., Griffen, J., Cramer, A., Yancey, J., Curtsinger, L., Holtzin, L., Schultz, G., Jukiewiez, M. and Lyunch, J. (1989) Enhancement of wound healing by topical treatment with EGF. *New Engl. J. Med* **321**, 76-79.

Burke J. R., Davern L. B., Gregor K. R. and Owczarczak L. M. (1999) Differentiation of U937 cells enables a phospholipase D-dependent pathway of cytosolic phospholipase A_2 activation. *Biochem Biophys Res Commun.* **260**, 232-239.

Buxer, S., Decker, D. and Ruppel, P. (1990) Relationship among types of nerve growth factor receptors on PC12 cells. *J. Biol. Chem.* **265**, 12701-12710.

Chao, M. V. (1992) Growth factor signaling: where is the specificity? *Cell 68*, 995-997.

Connolly, J. L., Green, S. A. and Greene, L. A. (1984) Comparison of rapid changes in surface morphology and coated pit formation of PC12 cells in response to NGF, EGF, and dibutyryl cAMP. *J. Cell. Biol.* **98**, 457-475.

Cristina, C. L. (1997) Properties and Regulation of Cytosolic Phospholipase A_2. *J. Biol.Chem.* **272**, 16709-16712.

Czech, M. P., Klarlund. J. K., Yagaloff, K. A., Bradford, A. P. and Lewis, R. E. (1988) Insulin receptor signaling. Activation of multiple serine kinase. *J. Biol. Chem.* **263**, 11017-11020.

Dreyfus, C. F. (1989) Effects of nerve growth factor on cholinergic brain neurons. *Trends Pharmacol. Sci.* **10**, 145-149.

Ebendal, T. (1989) NGF in CNS: experimental data and clinical implications. *Prog.Growth Factor Res.* **1**, 143-159.

Edelman, A. M., Blumenthal, D. K. and Krebs, E. G. (1987) Protein serine/threonine kinases. *Annu. Rev. Biochem.* **56**, 567-613.

Eppstein, D. A., Marsh Y. V., Scheiber A. B., Newman S. R., Todaro G. J. and Nestor J. J. Jr. (1985) Epidermal growth factor occupancy inhibits vaccinia virus infection. *Nature* **318**, 663-665.

Fay, M. J., Friedmann. A. S., Yu, X. M. and North, W. G. (1994) Vasopressin and vasopressin receptor immunoreactivity in small-cell lung carcinoma (SCCL) cell lines: Disruption in the activation cascade of Vla-receptors in variant SCCL. *Cancer Lett.* **82**, 167-174.

Fernando, K. C. and Barritt, G. J. (1994) Characterization of the inhibition of the hepatocyte receptor-activated Ca^{2+} inflow system by gadolinium and SK&F96365. *BBA-Mol. Cell Res.* **1222**, 383-389.

Fukuda, T., Kim, D. K., Chin, M. R., Hales, C. A. and

Bonventre, J. V. (1999) Increased group IV cytosolic phospholipase A_2 activity in lungs of sheep after smoke inhalation injury. *Am J Physiol.* **277**, L533-542.

Godfrey, E. and Shooter, E. (1986) Nerve growth factor receptors on chick embryo sympathetic ganglion cells: binding characteristics and development. *J. Neurosci.* **6,** 2543-2550.

Greene, L. A. and Tischler, A. S. (1976) Establishment of a noradrenergic slonal lone of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA.* **73**, 2424-2428.

Green. L. A. and Tischler, A. S. (1982) PC12 pheochromocytoma cultures in neurobiological research. *Adv. Cell. Neurobiol* **3**, 373-414.

Gregory, H. (1975) Isolation and structure of urogastrone and its relationship to epidermal growth factor. *Nature* **257**, 325-327.

Hashimoto, Y., Matuoka, K., Takenawa, T., Muroya, K., Hattori, S. and Nakamura, S. (1994) Different interaction of Grb_2/Ash molecule with the NGF and EGF receptors in rat pheochromocytoma PC12 cells. *Oncogene* **9**, 869-875.

Huh, D. H., Kang, M. S., Sohn, D. H., Na, D. S. and Kim, D. K. (1998) Enhancement of a liver form of cytosolic phospholipase A_2 activity by methylmercury. *J. Biochem. Mol. Biol.* **31**, 189-195.

Kast, R., Furstenberger, G. and Marks, F. (1993) Activation of cytosolic phospholipase A_2 by transforming growth factor-alpha in HEL-30 keratinocytes. *J. Biol. Chem.* **268**, 16795-16802.

Kim, U., Fink, D. Jr., Kim, H. S., Oark, D. J., Contreras, M. L., Guroff, G. and Rhee, S. G. (1991) Nerve growth factor stimulates phosphorylation of phospholipase C-γl in PC12 cells. *J. Biol. Chem.* **266**, 1359-1362.

Levi-Montalcini, R. (1987) The nerve growth factor 35 years later. *Science* **237**, 1154-1162.

Lin, L.-L., Wartmann, M., Lin, A. Y., Knopf, J. L., Seth, A. and Davis, R. J. (1993) $cPLA_2$ is phosphorylated and activated by MAP kinase. *Cell* **72**, 269-278.

Marchetti, D. and Perez-Polo, J. (1987) Nerve growth factor receptors in himan neuroblastoma cells. *J. Neurochem.* **49**, 475-486.

Mark, M. D., Liu, Y., Wong, S. T., Hinds, T. R. and Storm, D. R. (1995) Stimulation of neurite outgrowth in PC12 cells by EGF and KCl depolarization: A Ca^{2+}-independent phenomenon. *J. Cell Biol.* **130**, 701-710.

Marshall, C. J. (1995) Specificity of receptor tyrosine kinase signaling: Transient versus sustained extracellular signal-regulated kinase activation. Cell **80**, 179-185.

Pirianov G., Danielesson C., Carlberg C., James S. Y. and Colston K. W. (1999) Potentiation by vitamin D analogs of TNF alpha and ceramide-induced apoptosis in MCF-7 cells is associated with activation of cytosolic phospholipase A_2. *Cell Death Differ.* **6**, 890-901.

Qiu, Z. -H., De Carvalho, M. S. and Leslie, C. C. (1993) Regulation of phospholipase A_2 activation by phosphorylation in mouse peritoneal macrophages. *J. Biol. Chem.* **268**, 24506-24513.

Sa, G. and Das, T. (1999) Basic fibroblast growth factor stimulates cytosolic phospholipase A_2, phospholipase C-gl and phospholipase D through distinguishable signaling mechanisms. *Mol Cell Biochem* **198**, 19-30.

Sapirstein, A., Spech, R. A., Witzgall, R. and Bonventre, J. V. (1996) Cytosolic phospholipase A_2 (PLA_2), but not secretory PLA_2, potentiate hydrogen peroxide cytotoxicity in kidney epithelial cells. *J. Bio Chem.* **271**, 21505-21513.

Schlessinger, J. and Ullrich, A. (1992) Growth Factor Signaling by Receptor Tyrosine Kinases. *Neuron* **9**, 383-391.

Server, A. E. and Shooter, E. M. (1977) Nerve growth factor. *Adv. Protein Chem.* **31**, 339-409.

Sjursen, W., Brekke, O. L. and Johansen, B. (2000) Secretory and cytosolic phospholipase A(2) regulate the long-term cytokine-induced eicosanoid production in human keratinocytes. *Cytokine* **12**, 1189-1194.

Smalheiser, N. R., Dissanayake, S. and Kapil, A. (1966) Rapid Regulation of Neurite Outgrowth and Retraction by Phospholipase A_2-derived Arachidonic Acid and its Metabolites. *Brain Research* **721**, 39-48.

Taniuchi, M. Clark, H. B. and Johnson, E. M. Jr. (1986) Induction of nerve growth factor receptor in Schwann cells after axotomy. *Proc.NatlAcad. Sci. USA* **83**, 4094-4098.

Thornburn, G. (1985) *Growth and Maturation Factors* **3** 175 (G. Guroff, ed) John Wiley & Sons, NY.

Traynor, A. E. (1984) The relationship between neurite extension and phospholipid metabolism in PC12 cells. *Dev. Brain Res.* **14**, 205-210.

Tsukada, Y., Chiba, K., Yamazaki, M. and Mohri, T. (1994) Inhibition of the Nerve Growth Factor-Induced Neurite Outgrowth by Specific Tyrosine Kinase and Phosphoripase Inhibitors. *Biol. Pharm. Bull.* **17**, 370-375.

Vandeheijning, B. J. M. and Greidanus, T. B. V. (1994) The effect on neurohypophyseal hormone release, diuresis and electrolyte output of mu-and kappa-opioid agonists. *Neurosci. Res. Commun.* **15**, 11-20.

Van Willigen, G., Gorter, G. and Akkerman, J. W. (2000) Thrombopoietin in increases platelet sensitivity alpha-thrombin via activation of the ERK2-$cPLA_2$ pathway. *Thromb Haemost.* **83**, 610-616.

Vetter, M. L., Martin-Zanca, D., Parada, L. F., Bishop, J. M. and Kaplan, D. R. (1991) Nerve growth factor rapidly stimulates tyrosine phosphorylation of phospholipase C-γl by a kinase activity associated with the product of the trkprotooncogene. *Proc. Natl. Acad. USA* **88**, 5650-5654

York, R. D., Yao, H., Dillon, T., Ellig, C. L., Eckert, S. P., McCleskey, E. W. and Stork, J. S. (1998) Rapl mediateds sustained MAP kinase activation induced by nerve growth factor. *Nature* **392**, 622-626.