Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 2353–2357, April 1986
Biochemistry

Protein kinase C as a component of a nerve growth factor-sensitive phosphorylation system in PC12 cells

TOKIKO HAMA*, KUO-PING HUANG†, AND GORDON GUROFF*

Sections on *Growth Factors and on †Metabolic Regulation, National Institute of Child Health and Human Development, Bethesda, MD 20892

Communicated by Bernhard Witkop, December 12, 1985

ABSTRACT **The treatment of PC12 cells with either nerve growth factor or phorbol 12-myristate 13-acetate caused a decrease in the phosphorylation of a soluble 100-kDa protein (Nsp100). After treatment with nerve growth factor, the activity of Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C) in the cytosol was increased. When the cytosol from untreated PC12 cells was preincubated with purified protein kinase C and its cofactors, the phosphorylation of Nsp100 was decreased. The preincubation of cytosol from nerve growth factor-treated PC12 cells with protein kinase C did not decrease Nsp100 phosphorylation further. Moreover, preincubation of partially purified Nsp100 kinase with protein kinase C decreased its ability to phosphorylate Nsp100. These results suggest that the binding of nerve growth factor to its receptor on PC12 cells causes an increase in the activity of protein kinase C in the cytosol and phosphorylation of Nsp100 kinase, which in turn lowers its ability to phosphorylate Nsp100.**

Nerve growth factor (NGF) is required for the growth and development of the vertebrate sensory and sympathetic nervous systems (1–3). The rat pheochromocytoma cell line PC12 has become the premier model for the study of its actions (4–6). NGF induces neurite outgrowth (7) and a variety of metabolic changes in these cells and, overall, causes their biochemical and morphological conversion into a phenotype very similar to that of sympathetic neurons. These actions are initiated by the binding of NGF to specific receptors on the surface of PC12 cells. Even though NGF is internalized (8), internalization is apparently not involved in its action on its target cells (9). It is generally thought that some kind of second messenger system mediates the cytoplasmic and nuclear effects of NGF. Specific messengers, however, have not been identified.

The regulation of intracellular events by extracellular signals such as NGF often involves reversible covalent modification of proteins. One of the most intensively studied of these modifications is protein phosphorylation. Indeed, studies in this and other laboratories have shown that NGF does regulate the intracellular phosphorylation of several proteins.

Among these proteins are tyrosine hydroxylase (10), a 30-kDa nuclear protein (11, 12), a 100-kDa soluble protein (13, 14), a 70-kDa protein (15), the ribosomal protein S6 (ref. 10; Y. Matsuda, N. Nakanishi, G. Dickens, and G.G., unpublished work), and a 250-kDa cytoskeletal protein (17). One of these proteins, the 100-kDa soluble protein, is designated Nsp100. Its phosphorylation is decreased in PC12 cells (13, 14) and superior cervical ganglia of neonatal rats (18) after treatment with NGF. This phosphorylation has been investigated in cell-free preparations, and it has been found that the substrate (Nsp100) and its kinase are separate and separable molecules (14). Moreover, the decrease in Nsp100 phosphorylation has been shown to be due to a decrease in the activity of the kinase (14). Since this phosphorylation system may be part of a second messenger chain mediating NGF action, it is important to know the molecular mechanism by which NGF decreases the activity of Nsp100 kinase.

One available clue is that treatment of PC12 cells with phorbol 12-myristate 13-acetate (PMA) also decreases the phosphorylation of Nsp100 (13). PMA is known to activate Ca^{2+}, phospholipid-dependent protein kinase (protein kinase C). In this study, we have investigated the effect of protein kinase C on Nsp100 phosphorylation.

MATERIALS AND METHODS

Materials. PMA, histone (V-S), phosphatidylserine, diolein, and *Staphylococcus aureus* protease V8 were obtained from Sigma. [γ-^{32}P]ATP in aqueous solution was from ICN. Medium, serum, and antibiotics were purchased from GIBCO. DEAE-Sephacel was from Pharmacia. Proteins as standards for gel electrophoresis were from Bethesda Research Laboratories. NGF was prepared by the method of Bocchini and Angeletti (19).

Rat brain protein kinase C was purified to homogeneity (K.-P. H., K.-F. J. Chan, T. J. Singh, H. Nakabayashi, and F. L. Huang, unpublished work) by a procedure modified from that of Kikkawa *et al.* (21). One unit of the kinase is defined as the amount of enzyme catalyzing the incorporation of 1 nmol of phosphate from ATP into histone III-S under the standard assay conditions (21).

Culture Conditions. PC12 cells were maintained as monolayers in 150-cm^2 plastic flasks. They were grown in Dulbecco's modified Eagle's medium supplemented with 7% (vol/vol) fetal bovine serum, 7% (vol/vol) horse serum, and 100 μg of streptomycin and 100 units of penicillin per ml. They were kept at 37°C in humidified 6% CO_2/94% air.

Preparation of Cell-Free Extracts from PC12 Cells. The medium was removed by aspiration, and the cells were dislodged by shaking with ice cold 0.32 M sucrose containing 1 mM $MgCl_2$, 1 mM EGTA, and 1 mM sodium phosphate buffer, pH 6.5. All procedures were carried out at 0–4°C. The cells were collected by centrifugation and washed with the same buffer. The washed pellet was taken up in a buffer consisting of 0.1 M Pipes, pH 6.9, containing 1 mM $MgSO_4$, 2 mM EGTA, 0.1 mM Na_2MoO_4, and 1 mM phenylmethylsulfonyl fluoride. The cell suspension was homogenized in a Teflon-glass homogenizer and centrifuged at 100,000 × *g* for 60 min. The supernatant portion was dialyzed overnight at 0°C against 0.1 M Pipes, pH 6.9, containing 1 mM dithiothreitol.

Preparation of Nsp100 and Nsp100 Kinase. The 100,000 × *g* supernatant was fractionated with solid $(NH_4)_2SO_4$ between 40 and 60% saturation. The precipitate was collected and dissolved in 0.1 M Tris·HCl, pH 7.5, containing 1 mM

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: NGF, nerve growth factor; PMA, phorbol 12-myristate 13-acetate.

dithiothreitol. After dialyzing the preparation overnight at 0°C against this buffer, the preparation was applied to a DEAE-Sephacel column equilibrated with the same buffer. The column was eluted with a linear gradient of 0–0.5 M NaCl in 0.1 M Tris·HCl, pH 7.5, containing 1 mM dithiothreitol. Nsp100 appeared in the flow-through fraction, and Nsp100 kinase was eluted at about 0.3 M NaCl as described (14). Each fraction was dialyzed overnight at 0°C against 15 mM Tris·HCl, pH 7.5, containing 1 mM dithiothreitol, assayed, and stored at −70°C.

Phosphorylation of Nsp100. Phosphorylation was carried out in an incubation mixture containing 50 mM Mes, pH 6.2, 10 mM $MgSO_4$, 0.1 mM Na_2MoO_4, and 10 μM [γ-^{32}P]ATP. The sample was incubated at 37°C for 10 min, and the reaction was terminated by the addition of 100% (wt/vol) trichloroacetic acid to a final concentration of 10% (wt/vol). The samples were boiled in $NaDodSO_4$/sample buffer and analyzed by $NaDodSO_4$/PAGE as described by Laemmli (22).

Assay for Protein Kinase C. PC12 cells were homogenized in 0.25 M sucrose solution containing 5 mM EGTA, 2 mM EDTA, 50 mM 2-mercaptoethanol, 2.5 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, and 20 mM Tris·HCl, pH 7.5, using a Teflon-glass homogenizer. This homogenate was centrifuged at 100,000 × *g* for 60 min, the supernatant portion was collected, and the protein concentration was measured (23). This supernatant was applied to a DEAE-Sephacel column equilibrated with 20 mM Tris·HCl, pH 7.5, containing 50 mM 2-mercaptoethanol, 5 mM EGTA, 2 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride. In a given experiment, equal amounts of protein from each supernatant were applied to the same DEAE-Sephacel column. Proteins were eluted with a linear gradient of 0–0.15 M NaCl in the same buffer, and each fraction was dialyzed against 20 mM Tris·HCl, pH 7.5, containing 50 mM 2-mercaptoethanol and 0.5 mM phenylmethylsulfonyl fluoride. The assay for protein kinase C was carried out in a 200-μl volume containing 5 mM magnesium acetate, 10 μM [^{32}P]ATP, 0.5 mM $CaCl_2$, 5 μg/ml phosphatidylserine, and 0.5 μg/ml diolein. Histone (type V-S, 20 μg) was used as substrate. To measure basal kinase activity 0.1 mM EGTA was used instead of $CaCl_2$, phosphatidylserine, and diolein. After incubation at 30°C for 2 min the reaction was terminated with trichloroacetic acid and analyzed by $NaDodSO_4$/PAGE. The band corresponding to histone was cut out, and its radioactivity counted.

Treatment with Protein Kinase C. Cytosol from PC12 cells or partially purified Nsp100 or Nsp100 kinase was incubated with 10 μM unlabeled ATP under conditions for the assay of kinase C in the presence or absence of protein kinase C (2.5 milliunits). After preincubation at 30°C for 1 min, the mixture was put on ice and then incubated with [γ-^{32}P]ATP under conditions described above for the phosphorylation of Nsp100 at 37°C as described above. In the case of partially purified Nsp100 kinase, Nsp100 was added as a substrate.

One-Dimensional Peptide Mapping of Radioactive Nsp100. One-dimensional peptide mapping was carried out according to the procedure described by Cleveland *et al.* (24) using *Staphylococcus aureus* protease V8. After phosphorylation of PC12 extracts under the standard conditions and analysis by $NaDodSO_4$/PAGE, the band corresponding to Nsp100 was excised and treated with 2 μg of protease at room temperature for 5 hr.

RESULTS

Effect of PMA on Nsp100 Phosphorylation. When PC12 cells were incubated with PMA (100 ng/ml; 0.16 μM) for 2 hr, the subsequent cell-free phosphorylation of Nsp100 was decreased; this decrease was similar to that observed after NGF treatment for 2 hr (Fig. 1). To determine whether the decrease in phosphorylation due to treatment with PMA is similar to that due to treatment with NGF, one-dimensional peptide mapping was done (Fig. 2). Very similar patterns were observed when Nsp100 from NGF-treated cells was compared with that from PMA-treated cells. This suggests that

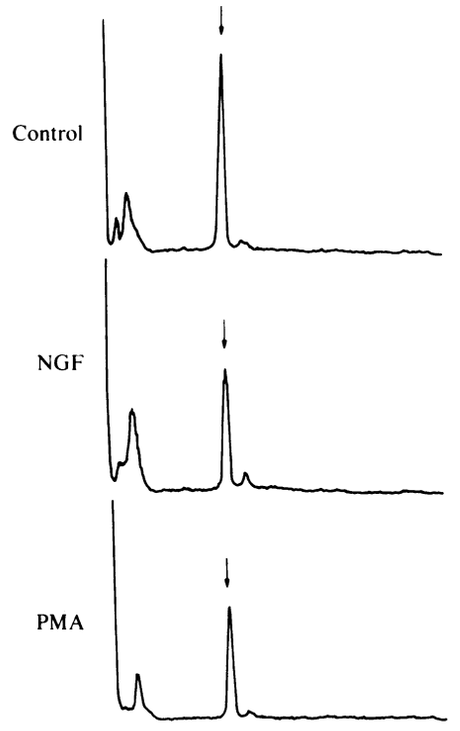

FIG. 1. Effect of PMA on the phosphorylation of Nsp100 in PC12 cells. PC12 cells were treated with NGF (50 ng/ml) or PMA (100 ng/ml) for 2 hr. The cells were collected, cytosol fractions were prepared, and the phosphorylation of Nsp100 was assayed. Each assay contained the same amount of protein. Proteins were separated on 5.5% $NaDodSO_4$/polyacrylamide gels, autoradiograms were prepared, and densitometer tracings were made. The arrows indicate the position of Nsp100. Integration of the densitometer tracings indicated that the Nsp100 peak had the following relative densities: control, 100; NGF, 62; PMA, 52.

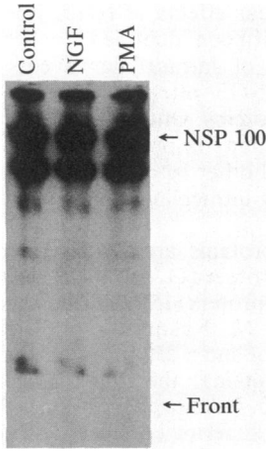

FIG. 2. One-dimensional peptide mapping of phosphorylated Nsp100. Cell-free extracts from control PC12 cells and from cells treated with NGF (50 ng/ml) or with PMA (100 ng/ml) for 2 hr were phosphorylated and analyzed on 5.5% $NaDodSO_4$/polyacrylamide gels. The bands corresponding to Nsp100 were excised and digested with 2 μg of *S. aureous* protease V8 for 5 hr at room temperature. The resulting peptides were separated on an 11.5% $NaDodSO_4$/polyacrylamide gel.

the mechanism of the PMA-induced decrease is similar to that of the NGF-induced decrease. Since PMA is known to affect protein kinase C, it seemed possible that NGF also acts on Nsp100 phosphorylation by activation of protein kinase C.

Protein Kinase C Activity of PC12 Cells After Treatment with NGF. To determine if protein kinase C activity in PC12 cytosol is changed after treatment of the cells with NGF, protein kinase C activity was measured using DEAE-Sephacel column chromatography and NaDodSO$_4$/PAGE. The cytosol fractions from cells treated with NGF for 0, 15, 30, and 60 min were collected, and equal amounts of protein were applied to a DEAE-Sephacel column. The activity was expressed as radioactivity in the bands corresponding to histone; basal kinase activity was subtracted from each point. As shown in Fig. 3, while protein kinase C activity in the cytosol of control PC12 cells was very low, it was increased 4- to 5-fold in 30–60 min by treatment with NGF. The activity returned to control levels within 7 hr and remained there for at least the next 11 hr, even in the continued presence of NGF.

Treatment of PC12 Cell Cytosol with Protein Kinase C. Since protein kinase C activity in PC12 cytosol was increased by treatment of the cells with NGF, it seemed reasonable to ask if protein kinase C *in vitro* can mimic NGF treatment of whole cells. Accordingly, the cytosol from untreated PC12 cells was preincubated with kinase C cofactors and unlabeled ATP and with or without protein kinase C. After that, the phosphorylation of Nsp100 was assayed with [^{32}P]ATP in the usual way. When the cytosol was preincubated with protein kinase C, the subsequent phosphorylation of Nsp100 was decreased (Fig. 4*A*), while cytosol from PC12 cells treated with NGF for 2 hr showed no further decrease of Nsp100 phosphorylation when it was preincubated with protein kinase C (Fig. 4*B*). Addition of protein kinase C and its appropriate cofactors, but without unlabeled ATP, had no effect on the subsequent phosphorylation of Nsp100 in cytosol from untreated cells. Addition of kinase C and its appropriate cofactors to the Nsp100 phosphorylation mixture directly and without preincubation had no effect on Nsp100 phosphorylation.

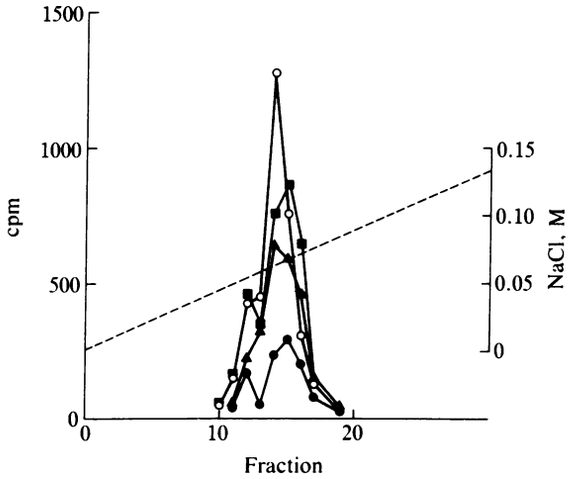

FIG. 3. Protein kinase C activity in PC12 cytosol after treatment with NGF. PC12 cells were treated with NGF (50 ng/ml) for 0, 15, 30, and 60 min. Cells were collected, and the cytosol fractions were prepared. An equal amount of protein from each cytosol was analyzed by DEAE-Sephacel column chromatography. Proteins were eluted with a linear gradient of 0–0.15 M NaCl. Each fraction was dialyzed against 20 mM Tris·HCl, pH 7.5, containing 50 mM 2-mercaptoethanol and 0.5 mM phenylmethylsulfonyl fluoride, and protein kinase C activity was measured using histone (V-S) as a substrate. To measure basal kinase activity, EGTA was added. After NaDodSO$_4$/PAGE, the bands corresponding to histone were excised, and the radioactivity in each was determined. Each point represents the counts minus the basal kinase activity. ●, 0 min; ▲, 15 min; ○, 30 min; ■, 60 min.

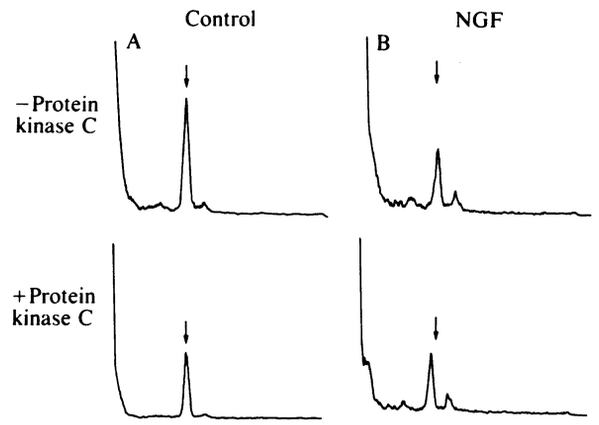

FIG. 4. Effect of protein kinase C on PC12 cytosol. Untreated PC12 cells (*A*) or cells treated with NGF (50 ng/ml) for 2 hr (*B*) were collected, homogenized, centrifuged, and dialyzed overnight at 0°C against 20 mM Tris·HCl, pH 7.5, containing 1 mM dithiothreitol. Equal amounts of protein from each cytosol were preincubated with kinase C cofactors and unlabeled ATP with or without protein kinase C (2.5 milliunits) at 30°C for 1 min. After preincubation, the mixtures were cooled immediately on ice and assayed for Nsp100 phosphorylation in the presence of [γ-^{32}P]ATP. The samples were analyzed using 5.5% NaDodSO$_4$/polyacrylamide gels, autoradiograms were prepared, and densitometer tracings were made. The arrows indicate the position of Nsp100. Integration of the densitometer tracings indicated that the Nsp100 peak had the following relative densities: control (− kinase C), 100; control (+ kinase C), 56; NGF (− kinase C), 52; NGF (+ kinase C), 50.

Treatment of Nsp100 Kinase with Protein Kinase C. In previous studies, we have shown that NGF treatment alters the activity of Nsp100 kinase, not the levels of Nsp100 (14). To determine if protein kinase C also acts on Nsp100 kinase and not on its substrate Nsp100, partially purified Nsp100 kinase and Nsp100 were used. After Nsp100 kinase fraction was preincubated with protein kinase C and unlabeled ATP, it was mixed with Nsp100 and [^{32}P]ATP and incubated in the usual way. The Nsp100 kinase that was preincubated with protein kinase C showed lower Nsp100 phosphorylation (Fig. 5*A*). Preincubation of protein kinase C with Nsp100 itself did not change the eventual phosphorylation of the Nsp100 by Nsp100 kinase (Fig. 5*B*).

DISCUSSION

The data presented here strongly suggest that protein kinase C is a participant in the phosphorylative alterations initiated by treatment of PC12 cells with NGF. That evidence is as follows: (*i*) PMA, a known activator of protein kinase C, mimics the action of NGF in decreasing the phosphorylation of Nsp100. (*ii*) NGF treatment causes an increase in the cytoplasmic activity of protein kinase C. (*iii*) The addition of purified protein kinase C to cell-free supernatant preparations from untreated PC12 cells decreases the phosphorylation of Nsp100, but a comparable addition to supernatant preparations from NGF-treated cells does not. (*iv*) Pretreatment of purified Nsp100 kinase with protein kinase C lowers its ability to phosphorylate Nsp100.

Treatment of PC12 cells with PMA has some other NGF-like effects. PMA elicits increases in the incorporation of [^{3}H]choline, causes an induction of ornithine decarboxylase and increases the phosphorylation of a 30-kDa nuclear protein (25). In other systems, PMA also has been seen to have NGF-like effects. PMA induces neurite outgrowth in mouse cerebellar cell cultures (26), in neuroblastoma clones (27, 28), and in chicken embryo sensory ganglia (29). Indeed,

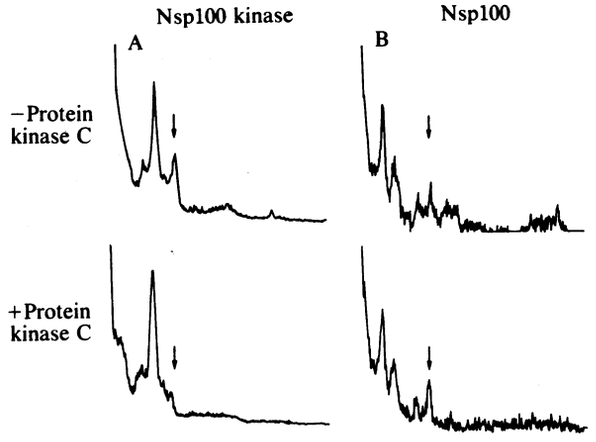

FIG. 5. Effect of protein kinase C on Nsp100 kinase. Nsp100 and its kinase from PC12 cells were separated using DEAE-Sephacel. Nsp100 kinase fractions were concentrated and dialyzed overnight at 0°C against 20 mM Tris·HCl, pH 7.5, containing 50 mM 2-mercaptoethanol. The dialyzed Nsp100 kinase (*A*) was preincubated with kinase C cofactors and unlabeled ATP with or without protein kinase C at 30°C for 1 min, put on ice, mixed with Nsp100 under standard conditions, and incubated with [γ-^{32}P]ATP at 37°C for 8 min. Proteins were analyzed using 5.5% NaDodSO$_4$/polyacrylamide gels. Comparable experiments were done using purified Nsp100 in the preincubation and partially purified Nsp100 kinase in the incubation (*B*). Autoradiograms were prepared, and densitometer tracings were made. The arrows indicate the position of Nsp100. Integration of the densitometer tracings indicated that the Nsp100 peak had the following relative densities: Nsp100 kinase (− kinase C), 100; Nsp100 kinase (+ kinase C), 42; Nsp100 (− kinase C), 87; Nsp100 (+ kinase C), 87.

PMA can modulate development in many systems, inhibiting differentiation in some, inducing differentiation in others (30).

The mechanism by which NGF increases the activity of protein kinase C remains to be determined. It has been reported that NGF treatment causes an increased turnover of phosphatidylinositol in PC12 cells (31) and rat superior cervical ganglia (32). Phosphatidylinositol provides diacylglycerol, a cofactor of protein kinase C. Also one of its metabolites, inositol-1,4,5-triphosphate, regulates Ca^{2+} release from an intracellular compartment (33–37). Clearly, then, treatment of PC12 cells with NGF leads to the availability in the cytosol of cofactors for protein kinase C. Whether the increased availability of these cofactors is sufficient to produce an increase in the activity of existing enzyme or the NGF causes a translocational alteration in the distribution of the enzyme between membrane and cytosol is not yet known for sure. Our preliminary data indicate the latter; the increase in cytosolic protein kinase C activity is matched by a comparable decrease in membrane protein kinase C activity. A similar membrane–cytosol shift has been reported in corticotropin-treated adrenal cortical cells (38). The use of the DEAE-Sephacel column assay for the separation of protein kinase C, and the consequent addition of exogenous cofactors to the relevant fractions, also strongly suggests the latter possibility.

The present data argue for a role for calcium in NGF-induced phosphorylation. The role of calcium in NGF action, in general, has been a subject of some controversy. It was originally suggested by Schubert *et al.* (39) who presented data indicating that NGF caused a change in calcium efflux from the cells. Landreth *et al.* (40) were unable to confirm the effects of NGF on calcium efflux and so argued against a role of calcium in the actions of NGF. In other experiments, Koike (16) has implicated Ca^{2+} in the NGF-induced growth of neurites from PC12 cells. Hashimoto *et al.* (20) have shown that pretreatment of PC12 cells for 60 min with EGTA prevents the actions of NGF on Nsp100 phosphorylation. The conclusion from all these experiments may be that, although there is no NGF-induced change in calcium movement in or out of the cell, Ca^{2+} levels in the soluble portion of the cell must be above a certain level for NGF to act. The mobilization of calcium stores may be required for NGF action, and this can be prevented by depletion of overall cellular calcium by pretreatment of the cell with EGTA.

Thus, information is accumulating that allows a suggestion as to the exact steps involved in the NGF-induced decrease in Nsp100 phosphorylation. NGF activates phospholipid metabolism producing cofactors for protein kinase C, some of which act to mobilize cellular calcium stores. At the same time, protein kinase C levels in the cytoplasm rise, perhaps because some kinase is released from the membrane due to alterations in intracellular calcium concentrations (21). Protein kinase C, then, phosphorylates Nsp100 kinase, inhibiting its ability to phosphorylate Nsp100. Since the identity of Nsp100 is not yet known, it is not possible to speculate on the relationship of its phosphorylation to the other actions of nerve growth factor.

1. Levi-Montalcini, R. & Angeletti, P. U. (1968) *Physiol. Rev.* **48,** 534–569.
2. Greene, L. A. & Shooter, E. M. (1980) *Annu. Rev. Neurosci.* **3,** 353–402.
3. Thoenen, H. & Barde, Y. A. (1980) *Physiol. Rev.* **60,** 1284–1335.
4. Greene, L. A. & Tischler, A. S. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 2424–2428.
5. Greene, L. A. & Rein, G. (1977) *Nature (London)* **268,** 349–351.
6. Dichter, M. A., Tischler, A. S. & Greene, L. A. (1977) *Nature (London)* **268,** 501–504.
7. Burstein, D. E. & Greene, L. A. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 6059–6063.
8. Levi, A., Schecter, Y., Neufeld, E. J. & Schlessinger, J. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 3469–3473.
9. Heumann, R., Schwab, M. & Thoenen, H. (1981) *Nature (London)* **292,** 838–840.
10. Halegoua, S. & Patrick, J. (1980) *Cell* **22,** 571–581.
11. Yu, M. W., Tolson, N. W. & Guroff, G. (1980) *J. Biol. Chem.* **255,** 10481–10492.
12. Nakanishi, N. & Guroff, G. (1985) *J. Biol. Chem.* **260,** 7791–7799.
13. End, D., Tolson, N., Hashimoto, S. & Guroff, G. (1983) *J. Biol. Chem.* **258,** 6549–6555.
14. Togari, A. & Guroff, G. (1985) *J. Biol. Chem.* **260,** 3804–3811.
15. Por, S. B. & Huttner, W. B. (1984) *J. Biol. Chem.* **259,** 6526–6533.
16. Koike, T. (1983) *Brain Res.* **289,** 293–303.
17. Landreth, G. E. & Rieser, G. D. (1985) *J. Cell Biol.* **100,** 677–683.
18. Hama, T. & Guroff, G. (1985) *J. Neurochem.* **45,** 1279–1287.
19. Bocchini, V. & Angeletti, P. U. (1969) *Proc. Natl. Acad. Sci. USA* **64,** 787–794.
20. Hashimoto, S., Iwasaki, C., Kuzuya, H. & Guroff, G. (1986) *J. Neurochem.*, in press.
21. Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S. & Nishizuka, Y. (1982) *J. Biol. Chem.* **257,** 13341–13348.
22. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
23. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
24. Cleveland, P. W., Fischer, S. G., Kirchner, M. W. & Laemmli, U. K. (1977) *J. Biol. Chem.* **252,** 1102–1106.
25. End, D., Tolson, N., Yu, M. W. & Guroff, G. (1982) *J. Cell. Physiol.* **111,** 140–148.
26. Krystosek, A. (1979) *J. Supramol. Struct. Suppl.* **3,** 227 (abstr.).
27. Spinelli, W. & Ishii, D. N. (1983) *Cancer Res.* **43,** 4119–4125.
28. Ishii, D., Recio-Pinto, E., Spinelli, W., Mill, J. F. & Sonnenfeld, K. H. (1985) *Int. J. Neurosci.* **26,** 109–127.
29. Hsu, L., Natyzak, D. & Laskin, J. D. (1984) *Cancer Res.* **44,** 4607–4614.
30. Weinstein, I. B., Lee, L.-S., Fischer, P. B., Mufson, A. &

Yamasaki, M. (1979) *J. Supramol. Struct.* **13,** 195–208.
31. Traynor, A. E. (1984) *Dev. Brain Res.* **14,** 205–210.
32. Lakshmanan, J. (1978) *Biochem. Biophys. Res. Commun.* **82,** 767–775.
33. Streb, H., Irvine, R. F., Berridge, M. J. & Schule, I. (1983) *Nature (London)* **306,** 67–69.
34. Joseph, S. K., Thomas, A. P., Williams, R. J., Irvine, R. F. & Williamson, J. R. (1984) *J. Biol. Chem.* **259,** 3077–3081.
35. Burgess, G. M., Godfrey, P. P., McKinney, J. S., Berridge, M. J., Irvine, R. F. & Putney, J. W. (1984) *Nature (London)* **309,** 63–66.
36. Prentki, M., Biden, T. J., Janjie, D., Irvine, R. F., Berridge, M. J. & Wollheim, C. B. (1984) *Nature (London)* **309,** 562–564.
37. Joseph, S. K., Williams, R. J., Corkey, B. E., Matschinsky, F. M. & Williamson, J. R. (1984) *J. Biol. Chem.* **259,** 12952–12955.
38. Vilgrain, I., Cochet, C. & Chambaz, E. M. (1984) *J. Biol. Chem.* **259,** 3403–3406.
39. Schubert, D., LaCorbiere, M., Whitlock, C. & Stallcup, W. (1978) *Nature (London)* **273,** 718–723.
40. Landreth, G., Cohen, P. & Shooter, E. M. (1980) *Nature (London)* **283,** 202–204.