Evidence for communication between nerve growth factor and protein tyrosine phosphorylation

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Nerve growth factor (NGF) stimulation of PC12 cells activated two myelin basic protein (MBP) kinase activities >10-fold within 5 min, which were resolved by chromatography on Mono Q. Each enzyme phosphorylated MBP on threonine and was inactivated by incubation with either CD45, a protein tyrosine phosphatase, or protein phosphatase 2A (PP2A), a serine/threonine phosphatase. The effects of CD45 and PP2A were prevented by vanadate and okadaic acid, respectively. Activation of the MBP-kinases provides a mechanism for communication between NGF and intracellular protein tyrosine phosphorylation.

Nerve growth factor; Tyrosine phosphorylation; Protein phosphatase; Okadaic acid; Protein kinase; PC12 cell

1. INTRODUCTION

Nerve growth factor (NGF) is a polypeptide required for the survival and development of sympathetic and sensory nervous systems. However, unlike the receptors for epidermal growth factor, platelet derived growth factor and insulin, the NGF receptor is not a protein tyrosine kinase [1] and the signalling system(s) through which it operates has not yet been elucidated. The rat pheochromocytoma cell line PC12 has become the premier model for studying the mechanism of action of NGF, because although responsive to this growth factor it will survive without it, unlike primary cultures of sympathetic neurons. NGF promotes the neuronal differentiation of PC12 cells, which respond by their morphological conversion from chromaffin-like cells to those with a sympathetic neuron-like phenotype that includes neurite outgrowth, electrical excitability and cessation of growth [2,3]. Here we demonstrate that exposure of PC12 cells to NGF rapidly stimulates a protein serine/threonine kinase that can be inactivated by a protein tyrosine phosphatase, demonstrating communication between NGF and intracellular protein tyrosine phosphorylation.

2. METHODS

2.1. *Materials*

Supplies for tissue culture were purchased from Northumbria Biologicals (Cramlington, Northumberland, UK), myelin basic protein and sodium orthovanadate from Sigma (Poole, Dorset, UK), and [γ-^{32}P]ATP from Amersham International (Bucks, UK). Protein phosphatase 2A (PP2A) was purified from rabbit skeletal muscle [4] and the protein tyrosine phosphatase CD45 from human spleen [5]. A 20 residue peptide corresponding to the active fragment (residues 5-24] of the specific protein inhibitor of cyclic AMP-dependent protein kinase (PKI) [6] was made in Dundee on an Applied Biosystems 431A Peptide Synthesizer by Dr David Campbell. NGF and okadaic acid were generous gifts from Dr Janet Winter (Sandoz Institute for Medical Research, London) and Dr Yasumasa Tsukitani (Fujisawa Pharmaceutical Co., Tokyo), respectively.

2.2. *Culture of PC12 cells and stimulation by NGF*

Cells were cultured at 37°C in a 95% air/5% CO_2 water-saturated atmosphere in 75 cm^2 collagen-coated flasks (Costar, Cambridge, MA, USA) using Dulbecco's modified Eagle's medium containing 10% heat inactivated horse serum, 5% heat inactivated foetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells from a single flask were transferred to 60 mm collagen-coated dishes (Corning, Stone, Staffs, UK) and incubated in the same culture medium for 3 days at a density of 3×10^6 cells per dish. The cells were washed once with 2 ml of prewarmed Krebs-Ringer-Hepes (KRH) buffer (125 mM NaCl, 4.8 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulphonic acid] (Hepes), 5.6 mM α-D(+)-glucose, pH 7.4) and then incubated with 4 ml of KRH buffer containing 50 ng/ml NGF. At various times, the medium was removed and 0.4 ml of ice cold lysis buffer added. This comprised 50 mM Tris/acetate pH 8.0, 0.27 M sucrose, 1 μM okadaic acid, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1% Triton X-100, 4 μg/ml leupeptin, 1 mM benzamidine and 0.1% (v/v) 2-mercaptoethanol. After 15 min on ice, the lysate was removed, centrifuged (4°C; 13 000 $\times g$; 5 min) and the supernatant assayed for protein kinase activity.

2.3. *Assay of myelin basic protein kinase activity*

The incubations (0.05 ml) were carried out at 30°C and comprised 25 mM Tris/HCl pH 7.0, 0.1 mM EGTA, 0.1 mM sodium orthovanadate, 1 μM okadaic acid, 1 μM PKI, myelin basic protein (0.33 mg/ml), PC12 extract or Mono Q purified MBP kinase, 10 mM magnesium acetate and 0.1 mM [γ-^{32}P]ATP(~ 10^6 cpm/nmol). Reactions were initiated with ATP and 0.04 ml aliquots removed after 10

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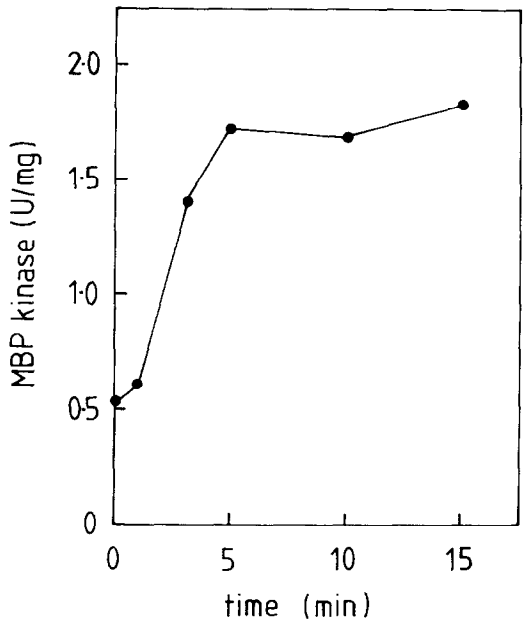

Fig. 1. Stimulation of myelin basic protein kinase in PC12 cells by NGF. The experiment was performed as described in section 2.2 and protein was measured according to Bradford [19]. A different 60 mm dish of cells was used for each time point.

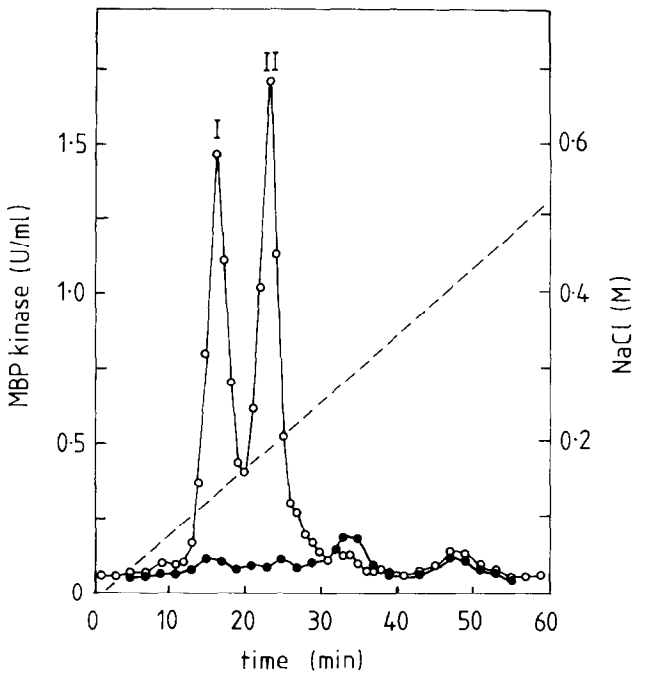

Fig. 2. Separation of two NGF-stimulated myelin basic protein kinase activities by chromatography on Mono Q. PC12 cells were stimulated for 15 min with NGF and the lysates from 5 dishes diluted 5-fold in 50 mM sodium glycerophosphate pH 7.3, 1.5 mM EGTA, 0.15 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM benzamidine and 4 μg/ml leupeptin (Buffer A) and applied to a 5 × 0.5 cm Mono Q column equilibrated in buffer A. The column was developed at 1 ml/min with a 40 ml linear salt gradient from 0–0.7 M NaCl in buffer A and fractions of 0.5 ml were collected. The figure shows the profile of MBP kinase activity obtained from NGF stimulated cells (○) and control cells that had not been exposed to NGF (•). The salt gradient is denoted by the broken line. Two major MBP kinases, termed I and II, were detected in the NGF stimulated cells.

or 20 min and pipetted onto 2 × 1 cm squares of Whatman P81 paper, followed by immersion in 0.5% phosphoric acid (10 ml/assay). After washing four times with phosphoric acid to remove ATP and once in acetone to remove phosphoric acid, the P81 papers were dried and counted. PC12 extracts and Mono Q purified enzyme were assayed at final dilutions of 100-fold and 10-fold, respectively. Under these conditions, phosphorylation was linear with time up to 20 min. One unit of activity (U) was that amount which catalysed the incorporation of 1 nmol of phosphate into MBP in one min.

3. RESULTS

Incubation of PC12 cells with NGF (50 ng/ml) caused an increase in protein kinase activity towards myelin basic protein (MBP). The effect ranged from 2.5-fold to 4-fold and was maximal after about 5 min (Fig. 1). Inclusion of both the protein serine/threonine phosphatase inhibitor okadaic acid (reviewed in [7]) and the protein tyrosine phosphatase inhibitor sodium orthovanadate in the homogenisation buffer was essential to detect stimulation (data not shown), suggesting that activation of the MBP kinase involved a phosphorylation event. Extracts were prepared, in the presence of phosphatase inhibitors, from PC12 cells that had been incubated with NGF and subjected to anion exchange chromatography on Mono Q. This pro-

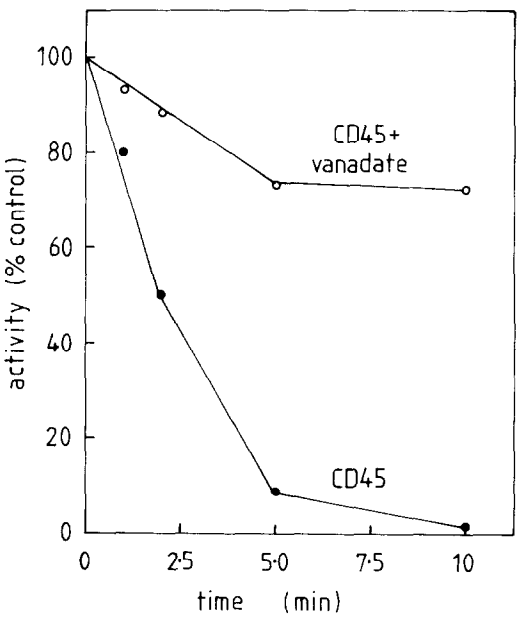

Fig. 3. Inactivation of MBP kinase I by incubation with the protein tyrosine phosphatase CD45. A 0.1 ml aliquot of MBK kinase I from Fig. 2 was centrifuge-desalted through Sephadex G50 Superfine equilibrated at 4°C in 50 mM Tris/HCl pH 7.0, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, bovine serum albumin 1 mg/ml (buffer B), in order to remove the protein phosphatase inhibitors sodium glycerophosphate and vanadate. An aliquot (0.05 ml) was then incubated at 30°C with 5 μl of CD45 (10 U/ml) and 5 μl of either buffer B (•) or 2.4 mM sodium orthovanadate in buffer B (○). At the indicated times, 5 μl aliquots were withdrawn and assayed for MBP kinase activity. One unit of CD45 activity was that amount which catalysed the dephosphorylation of 1 nmol of ^{32}P-phosphotyrosyl-lysozyme in 1 min [13].

cedure resolved two major peaks of MBP kinase activity, termed MBK kinase I and MBP kinase II, eluting at ~0.15 M NaCl and ~0.2 M NaCl respectively (Fig. 2). Both peaks were greatly reduced or absent in extracts prepared from PC12 cells that had not been stimulated by NGF (Fig. 2), demonstrating that MBP kinase-I and MBP-kinase-II are both activated at least 10-fold by NGF and that the smaller effects in extracts reflect the presence of other (NGF insensitive) MBP kinases. Both MBP kinase I and MBP kinase II phosphorylated a threonine residue(s) in MBP (data not shown), indicating that they are protein serine/threonine kinases and not protein tyrosine kinases. Neither MBP kinase was inhibited by the specific peptide inhibitor of cyclic AMP-dependent protein kinase, which was included routinely in the assays at 1 μM.

The MBP kinase I was incubated with CD45, a protein phosphatase which dephosphorylates tyrosine residues exclusively [8], and PP2A which dephosphorylates serine and threonine residues (reviewed in [9]). Incubation with CD45 rapidly inactivated MBP kinase-I, an effect that was largely inhibited by sodium orthovanadate (Fig. 3). Incubation with PP2A also inactivated the MBP kinase, an effect inhibited by okadaic acid (Fig. 4) which is an inhibitor of PP2A (reviewed in [7]). The slight inactivation in the presence of vanadate is explained by the presence of traces of PP2A activity in the partially purified MBP kinase. Incubation of MBP kinase II with CD45 or PP2A gave similar results to those obtained with MBP kinase I.

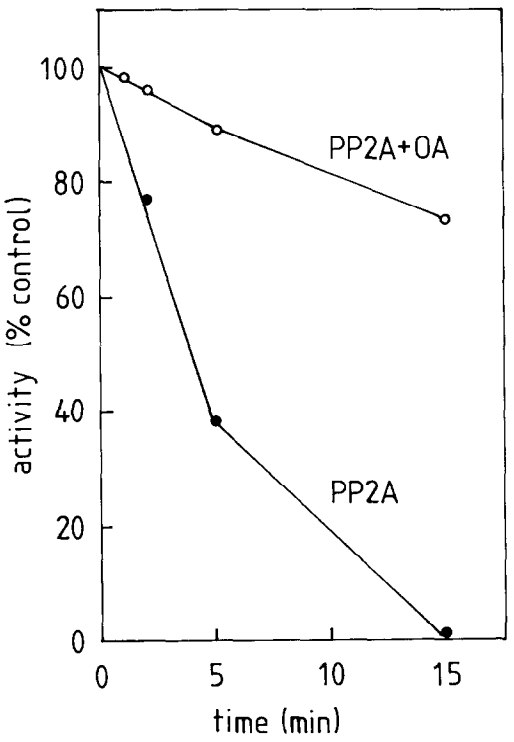

Fig. 4. Inactivation of MBP kinase I by incubation with protein phosphatase 2A. The experiment was carried out as described in the legend to Fig. 3, except that PP2A (final concentration 20 mU/ml) replaced CD45, and okadaic acid (OA) (final concentration 5 μM) replaced sodium orthovanadate. Incubations with PP2A were performed in the absence (•) and presence (○) of OA. One unit of PP2A was that amount which catalysed the dephosphorylation of 1 μmol of phosphorylase in one min [4].

4. DISCUSSION

A protein kinase has been identified in several cell types that is transiently activated in response to a variety of hormones and mitogens, including insulin, PDGF, EGF and phorbol esters (reviewed in [10–12]). This protein kinase was originally detected as an enzyme capable of phosphorylating microtubule-associated protein 2 (MAP2) and therefore termed MAP-kinase, but MBP was subsequently shown to act as an alternative substrate [13]. The active form of MAP-kinase was found to contain both phosphotyrosine and phosphothreonine [14], and it was recently demonstrated that it could be inactivated by incubation with either the protein tyrosine phosphatase CD45 or the protein serine/threonine phosphatase PP2A [13]. Studies using ^{32}P-labelled MAP-kinase established that CD45 and PP2A dephosphorylated tyrosine and threonine residues, respectively [13]. It is therefore assumed that two distinct protein kinases are required to activate MAP kinase in vivo, one specific for tyrosine and the other for threonine residues.

The present work has demonstrated the presence of a protein kinase in PC12 cells that closely resembles the mitogen-activated MAP-kinase found in other cells in that it phosphorylates myelin basic protein (Figs 1 and 2) and can be inactivated by incubation with either CD45 or PP2A (Figs 3 and 4). Most importantly, however, the results provide one of the first pieces of evidence which connects NGF action to a protein tyrosine phosphorylation event. To our knowledge, the only previous indication that NGF may stimulate protein tyrosine phosphorylation was presented by Maher [15], who reported that four proteins could be detected on SDS/polyacrylamide gels by immunoblotting with antibodies to phosphotyrosine after exposure of PC12 cells to NGF. Their apparent molecular masses of 150, 120, 60 and 55 kDa do not correspond to that of the mitogen-stimulated MBP/MAP-kinase, which has an apparent molecular mass of ~40 kDa in other cells. This suggests that the antibodies either do not recognise MAP-kinase or are not specific for phosphotyrosine containing proteins. It has been reported that some monoclonal antibodies to phosphotyrosine also recognise phosphohistidine-containing proteins [16].

As our studies were nearing completion, Saltiel and coworkers reported that incubation of PC12 cells with NGF stimulated a MAP2 kinase activity 2- to 4-fold within a few minutes [17]. The protein kinase had an

apparent molecular mass of ~40 kDa on gel-filtration and was inactivated by incubation with alkaline phosphatase [17]. They also demonstrated that MAP-2 kinase increased upon incubation of PC12 cells with okadaic acid and concluded that the enzyme was activated by a serine/threonine phosphorylation event [18]. Their MAP2 kinase is likely to be identical to the MBP kinase reported here, since chromatography of the extracts on Mono Q yielded two peaks of MAP2 kinase activity similar to the doublet observed in Fig. 2 [18].

Further research is required to elucidate the molecular mechanism by which NGF stimulates the MBP/MAP-kinase in PC12 cells. Although the interaction of NGF with its plasma membrane-bound receptor might trigger the activation of an associated protein tyrosine kinase and thereby initiate a protein tyrosine kinase cascade that leads to activation of the MBP kinase, other explanations are possible. For example, interaction of NGF with its receptor might stimulate an associated protein serine/threonine kinase that activates the MBP kinase, or inhibit a protein serine/threonine phosphatase or a protein tyrosine phosphatase which inactivates MBP kinase.

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REFERENCES

[1] Radeke, M.J., Misko, T.P., Hsu, C., Herzenberg, L.A. and Shooter, E.M. (1987) Nature 325, 593–597.
[2] Greene, L.A. and Tischer, A.S. (1976) Proc. Natl. Acad. Sci. USA 73, 2424–2428.
[3] Dichter, M.A.A., Tischer, A.S. and Green, L.A. (1977) Nature 268, 501–504.
[4] Cohen, P., Alemany, S., Hemmings, B.A., Resink, T.J., Stralfors, P. and Tung, H.Y.L. (1988) Methods Enzymol. 159, 390–408.
[5] Tonks, N.K., Charbonneau, H., Diltz, C.D., Fischer, E.H. and Walsh, K.A. (1988) Biochemistry 27, 8695–8701.
[6] Scott, J.D., Glaccum, M.B., Fischer, E.H. and Krebs, E.G. (1986) Proc. Natl. Acad. Sci. USA 83, 1613–1616.
[7] Cohen, P., Holmes, C.F.B. and Tsukitani, Y. (1990) Trends Biochem. Sci. 15, 98–102.
[8] Tonks, N.K., Diltz, C.D. and Fischer, E.H. (1990) J. Biol. Chem. 265, 10674–10680.
[9] Cohen, P. (1989) Annu. Rev. Biochem. 58, 453–508.
[10] Ray, L.B. and Sturgill, T.W. (1988) J. Biol. Chem. 263, 12721–12727.
[11] Rossomondo, A.J., Payne, D.M., Weber, M.J. and Sturgill, T.W. (1989) Proc. Natl. Acad. Sci. USA 86, 6940–6943.
[12] Haystead, T.A.J., Weiel, J.E., Litchfield, D.W., Tsukitani, Y., Fischer, E.H. and Krebs, E.G. (1990) J. Biol. Chem. 265, in press.
[13] Anderson, N.G., Maller, J.L., Tonks, N.K. and Sturgill, T.W. (1990) Nature 343, 651–653.
[14] Ray, L.B. and Sturgill, T.W. (1988) Proc. Natl. Acad. Sci. USA 85, 3753–3757.
[15] Maher, P.A. (1988) Proc. Natl. Acad. Sci. USA 85, 6788–6791.
[16] Frackelton, A.R., Ross, A.H. and Eisen, H.N. (1983) Mol. Cell. Biol. 3, 1343–1352.
[17] Miyasaka, T., Chao, M.V., Sherline, P. and Saltiel, A.R. (1990) J. Biol. Chem. 265, 4730–4735.
[18] Miyasaka, T., Miyasaka, J. and Saltiel, A.R. (1990) Biochem. Biophys. Res. Commun. 168, 1237–1243.
[19] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.