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Cross Talk Among Tyrosine Kinase Receptors in PC12 Cells: Desensitization of Mitogenic Epidermal Growth Factor Receptors by the Neurotrophic Factors, Nerve Growth Factor and Basic Fibroblast Growth Factor

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We have studied the effects of nerve growth factor (NGF) and basic fibroblast growth factor (bFGF) on epidermal growth factor (EGF) binding to PC12 cells. We show that NGF and bFGF rapidly induce a reduction in ^{125}I-EGF binding to PC12 cells in a dose-dependent manner. This decrease amounts to 50% for NGF and 35% for bFGF. Both factors appear to act through a protein kinase C(PKC)-independent pathway, because their effect persists in PKC-downregulated PC12 cells. Scatchard analysis indicates that NGF and bFGF decrease the number of high affinity EGF binding sites. In addition to their effect on EGF binding, NGF and bFGF activate in intact PC12 cells one or several serine/threonine kinases leading to EGF receptor threonine phosphorylation. Using an in vitro phosphorylation system, we show that NGF- or bFGF-activated extracellular regulated kinase 1 (ERK1) is able to phosphorylate a kinase-deficient EGF receptor. Phosphoamino acid analysis indicates that this phosphorylation occurs mainly on threonine residues. Furthermore, two comparable phosphopeptides are observed in the EGF receptor, phosphorylated either in vivo after NGF treatment or in a cell-free system by NGF-activated ERK1. Finally, a good correlation was found between the time courses of ERK1 activation and ^{125}I-EGF binding inhibition after NGF or bFGF treatment. In conclusion, in PC12 cells the NGF- and bFGF-stimulated ERK1 appears to be involved in the induction of the threonine phosphorylation of the EGF receptor and the decrease in the number of high affinity EGF binding sites.

INTRODUCTION

The epidermal growth factor (EGF)[1] receptor is a 170-kDa transmembrane glycoprotein, possessing a tyrosine kinase domain in its intracellular region. Binding of EGF induces receptor dimerization and increased receptor tyrosine kinase activity, which leads to receptor autophosphorylation and tyrosine phosphorylation of cellular substrates, such as the phospholipase C-γ and the GTPase activating protein (Ullrich and Schlessinger, 1990). Recent studies have demonstrated that specific binding sites for these substrates are generated after EGF receptor autophosphorylation (Margolis *et al.*, 1990; Rotin *et al.*, 1992). In addition to this activation, the EGF receptor is subjected to several negative regulations. One of them, induced by EGF, is responsible for receptor downregulation because of an increase in internalization and degradation of receptors (Stoscheck and Carpenter, 1984). EGF also causes an inhibition of its receptor tyrosine kinase activity by at least two distinct mechanisms: the activation of protein kinase C (PKC), which phosphorylates the threonine 654 residue of the EGF receptor (Davis, 1988), and the activation of the cal-

[1]Abbreviations used: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; ERK1, extracellular regulated kinase 1; bFGF, basic fibroblast growth factor; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid; IL-1, interleukin 1; MAP kinase, mitogen-activated protein kinase; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TNFα, tumor necrosis factor α; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

modulin-dependent protein kinase II, CAM kinase II, which phosphorylates the receptor serine residues 1046/47 (Countaway *et al.*, 1992). Finally, EGF and other growth factors induce a rapid decrease in high affinity EGF binding by two different pathways. The first one involves PKC activation and is observed after treatment with bombesin and phorbol esters such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (Lee and Weinstein, 1978; Zachary *et al.*, 1986). The second one, induced by EGF, platelet-derived growth factor (PDGF), acidic fibroblast growth factor, basic fibroblast growth factor (bFGF), interleukin 1 (IL-1), and tumor necrosis factor α (TFNα), appears to be independent of PKC activation (Olashaw *et al.*, 1986; Bird and Saklatvala, 1989; Hicks *et al.*, 1989; Tartare *et al.*, 1992). Moreover, it has been demonstrated that EGF receptor serine/threonine phosphorylation plays a crucial role in EGF binding inhibition induced by EGF (Tartare *et al.*, 1992), PDGF (Olashaw *et al.*, 1986; Davis and Czech, 1987; Countaway *et al.*, 1989), IL-1, and TNFα (Bird and Saklatvala, 1990), but the kinase(s) involved is unknown.

The pheochromocytoma cell line PC12, derived from a rat adrenal tumor (Greene and Tischler, 1976), responds to various growth factors including nerve growth factor (NGF), bFGF, and EGF. The effects of bFGF are mediated through a specific tyrosine kinase receptor stimulated by ligand binding (Burgess and Maciag, 1989). In PC12 cells, NGF interacts with two distinct molecular species, $p75^{NGFR}$ and $p140^{trk}$. $p75^{NGFR}$ is a cysteine-rich glycoprotein having a relatively low affinity for NGF ($K_d = 10^{-9}$ mol/l). The trk proto-oncogene product $p140^{trk}$ is a receptor tyrosine kinase activated by NGF (Kaplan *et al.*, 1991), which is characterized by a high affinity for NGF ($K_d = 10^{-11}$ mol/l). To date, the exact composition of the biologically active receptor remains a matter of debate. Although Hempstead *et al.* (1991) favor the idea that the biologically relevant receptor is formed by the association of $p75^{NGFR}$ and $p140^{NGFR}$, Klein *et al.* (1991) have suggested that $p140^{NGFR}$ alone is the high affinity functional receptor.

The three polypeptides, EGF, NGF, and bFGF, induce a common array of biological responses in PC12 cells; however, they have distinct effects on the final destiny of these cells. Whereas both NGF and bFGF lead to differentiation of PC12 cells into sympathetic neuron-like cells (Greene and Tischler, 1982; Rydel and Greene, 1987), EGF exerts a moderate mitogenic effect without displaying any differentiating action (Huff *et al.*, 1981).

Interestingly, we have recently provided evidence that the neurotrophic action of NGF might be due to a robust and sustained activation of the serine/threonine mitogen-activated protein (MAP) kinase extracellular regulated kinase 1 (ERK1) accompanied by nuclear translocation of the enzyme. In contrast, EGF was found to induce only a short-lived activation of ERK1 without nuclear translocation (Nguyen *et al.*, 1993). In an attempt to understand the molecular basis of the strikingly different actions of NGF and bFGF compared with EGF on PC12 cells' destiny, we looked for receptor–cross talk and the possible involvement of ERK1 in this process. First, we analyzed the effects of NGF and bFGF on EGF binding, and we found that both factors induce a rapid and dose-dependent decrease in ^{125}I-EGF binding. This effect appears to be independent of PKC activation and could be because of a transformation of high affinity binding sites into low affinity ones. Next, we studied the phosphorylation state of the EGF receptor in intact PC12 cells after treatment with NGF or bFGF. We found that the EGF receptor is phosphorylated mainly on threonine residues. Moreover, we demonstrated that NGF- or bFGF-activated MAP kinase, ERK1, obtained from PC12 cells, phosphorylates in vitro a kinase-deficient EGF receptor on threonine residues. Finally, we observed that after NGF- or bFGF-stimulation, the time course of ERK1 activation closely follows the time course of the decrease in EGF binding. Taken together, our results suggest that phosphorylation of the EGF receptor by MAP kinase ERK1 might play a key role in the process leading to the decrease in high affinity EGF binding.

MATERIALS AND METHODS

Mouse "receptor grade" EGF was from Sigma Chemical (St. Louis, MO); 2.5 S NGF was from Promega (Madison, WI). bFGF was from D. Gospodarowiz (University of California, San Francisco, CA). EGF was iodinated as described using the chloramine T method (specific activity = 150 μCi/μg) (Honegger *et al.*, 1987). [^{32}P]orthophosphate was obtained from Amersham (Bucks, UK). TPA, Triton X-100, protein A-Sepharose, and bovine serum albumin (BSA) (A7030) were from Sigma Chemical. K-252a was from Calbiochem (San Diego, CA). TPA was solubilized in ethanol, which was used at a final concentration of 0.1% (vol/vol); K-252a was dissolved in dimethyl sulfoxide, which was present at a final concentration of 1% (vol/vol). All reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) were from Bio-Rad Laboratories (Richmond, CA). mAb108 is a monoclonal antibody (mAb) directed against the extracellular domain of the human EGF receptor (Honegger *et al.*, 1989), and anti-C is a rabbit antiserum generated against a synthetic peptide from the COOH terminus of the human EGF receptor (residues 1176–1186) (Honegger *et al.*, 1989). Both antibodies were provided by Professor J. Schlessinger (New York University, New York). Anti-ERK1 is a rabbit antiserum directed against a synthetic peptide from the carboxy-terminal tail of the MAP kinase ERK1 (peptide C: residues 356–367) (Scimeca *et al.*, 1991).

Cell Culture

PC12 cells were cultured in RPMI medium containing 10% horse serum and 5% fetal calf serum (FCS). Cells were plated at 2.5×10^4 cells/cm^2 and grown to confluence. Before incubation with the effectors, PC12 cells were starved for 2 h in RPMI medium with 0.2% BSA. Details concerning the IHE2 cell line, expressing a kinase-deficient human EGF receptor after a mutation of the ATP binding site, have been previously described (Tartare *et al.*, 1991). IHE2 cells were cultured to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and starved for 2 h in DMEM/0.2% BSA.

^{125}I-EGF Binding to PC12 Cells

After the serum starvation period, PC12 cells in 12-well culture dishes were incubated at 37°C with the different effectors at the indicated concentration and for the indicated time. Then the cells were rapidly cooled on ice and the binding of a subsaturating concentration of ^{125}I-EGF (0.1 nM) to cell surface receptors was measured by incubation of the cells at 4°C for 3 h in RPMI medium with 0.2% BSA/20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.5). For Scatchard analysis, the cells were incubated for 3 h at 4°C with increasing concentrations of ^{125}I-EGF (from 10 to 10 000 pM) in the same binding medium. Nonspecific binding was estimated in incubations containing a 100-fold excess of unlabeled EGF. Cells were rinsed three times with ice-cold RPMI medium and solubilized in 0.2 N NaOH. The radioactivity was determined in a gamma-radiation counter. Binding parameters were calculated from Scatchard analysis by a homemade computer program.

^{32}P-labeling in Intact PC12 Cells

Confluent PC12 cells growing in six-well culture dishes were washed with Krebs Ringer Bicarbonate buffer without phosphate (NaCl 120 mM, KCl 5 mM, $MgSO_4$ 1 mM, $NaHCO_3$ 25 mM, $CaCl_2$ 1 mM, HEPES 20 mM, pH 7.5) and incubated for 3 h in RPMI medium with 0.2% BSA/2 mM glutamine without phosphate and containing [^{32}P]-orthophosphate (1 mCi/ml). At the end of the labeling period, EGF (10^{-7} M), TPA (10^{-7} M), NGF (100 ng/ml), or bFGF (50 ng/ml) were added for the appropriate times. Cells were solubilized for 15 min on ice in buffer A (50 mM HEPES, pH 7.5; 150 mM NaCl; 10 mM EDTA; 10 mM $Na_4P_2O_7$; 2 mM sodium orthovanadate; 100 mM NaF; and protease inhibitors, 100 U/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 20 μM leupeptin) containing 1% Triton X-100. Cell extracts were then incubated for 90 min at 4°C with antibodies to the EGF receptor carboxyl terminus, anti-C, adsorbed on a protein A-Sepharose pellet. After six washes alternating buffer A/1% Triton X-100 and buffer A/0.2% Triton X-100/0.5 M NaCl/0.1% SDS, Laem mli buffer (3% SDS) was added to dried pellets, and proteins were submitted to SDS–PAGE under reducing conditions. The gel was then treated with 1 M KOH for 2 h at 55°C and washed for 45 min twice with 10% acetic acid/10% isopropanol at room temperature. Labeled proteins were visualized by autoradiography.

In Vitro Phosphorylation of Immunopurified EGF Receptor by ERK1

After the starvation period, confluent PC12 cells in 145-cm^2 culture dishes were stimulated with NGF (100 ng/ml) or bFGF (50 ng/ml) for 15 min at 37°C. Cells were solubilized for 15 min on ice in buffer A containing 1% Triton X-100. The lysate obtained was subjected to immunoprecipitation with anti-ERK1 antibodies (as described above). Pellets were washed six times alternating buffer A with 1% Triton X-100 and buffer B (50 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100), and ERK1 was eluted during a 30-min incubation at 4°C in buffer B (supplemented with 10% glycerol) containing 10 μM C peptide and 0.2 mM sodium orthovanadate. Confluent IHE2 cells in 145-cm^2 culture dishes were solubilized in lysis buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100, and protease inhibitors (100 U/ml aprotinin, 1 mM PMSF, and 20 μM leupeptin), and immunoprecipitation was performed with mAb108 antibodies (as described above). After three washes with buffer B, the pellets were incubated with eluted ERK1, and phosphorylation was performed for 1 h at room temperature in the presence of 10 mM magnesium acetate and [γ-^{32}P]ATP (5 μM, 33 Ci/mmol). The pellets were washed four times alternating buffer B/0.5 M NaCl and buffer B, dried, and Laemmli buffer was added. The proteins were analyzed by SDS–PAGE and visualized by autoradiography.

Phosphopeptide and Phosphoamino Acid Analysis of the EGF Receptor

The EGF receptor was phosphorylated in intact cells or in a cell-free system as described above. For phosphopeptide map analysis after SDS–PAGE, the gel was submitted to autoradiography, and the gel pieces corresponding to the ^{32}P-labeled receptors were excised and incubated for 24 h in 50 mM NH_4HCO_3 (pH 8.0) at 37°C. Diphenylcarbamyl chloride-treated trypsin (Sigma) was then added at a final concentration of 60 μg/ml, and the incubation continued for 36 h at 37°C. For each sample, the eluted phosphopeptides were lyophilized and dissolved in 15 mM NH_4OH. Phosphopeptides were separated by two-dimensional analysis on cellulose thin-layer plates as described in Ballotti *et al.* (1989). The plates were then dried and subjected to autoradiography. For phosphoamino acid analysis, after the SDS–PAGE the proteins were transferred to an Immobilon membrane (Immobilon PVDF Millipore, Bedford, MA). The transferred phosphoproteins were localized by autoradiography and membrane pieces corresponding to the ^{32}P-labeled EGF receptor were excised. Receptors were then hydrolyzed in 6 N HCl for 90 min at 110°C; the phosphoamino acids obtained were separated on cellulose thin-layer plates by electrophoresis at pH 3.5 for 2 h at 1000 V and analyzed by autoradiography as described in Cooper *et al.* (1983).

Time-Course of ERK1 Activation by NGF and bFGF

After incubation with NGF or bFGF for different times, PC12 cells were solubilized in buffer A with 1% Triton X-100, and cell extracts were incubated with anti-ERK1 antibodies. The pellets were then washed six times as described above, and the phosphorylation was performed for 1 h at room temperature with myelin basic protein (MBP) at 0.2 mg/ml, 10 mM magnesium acetate, and [γ-^{32}P]ATP (5 μM, 33 Ci/mmol). The reaction was terminated by depositing the samples on 3 MM Chr Whatmann paper (Clifton, NJ) immersed in 10% tetraacetic acid (TCA). After three washes in 5% TCA, the radioactivity was estimated by Cerenkov counting.

RESULTS

Effects of NGF and bFGF on ^{125}I-EGF Binding to PC12 Cells

PC12 cells were incubated with increasing concentrations of NGF or bFGF and thereafter ^{125}I-EGF binding was measured at 4°C. Figure 1 shows that NGF and bFGF provoked a decrease in ^{125}I-EGF binding in a concentration-dependent manner. The maximal NGF-induced inhibition (~50%) was obtained at 100 ng/ml (7 nM). With bFGF, this decrease reached 30–35% at 50 ng/ml (3 nM). The half-maximal effect occurred at similar concentrations for both effectors, which is about 5 ng/ml (0.4 nM for NGF and 0.3 nM for bFGF).

Possible Involvement of PKC and the p140^{trk} NGF Receptor

To assess the role of PKC in this process, we compared the effect of NGF and bFGF on EGF binding in untreated and PKC-downregulated PC12 cells. Cells were incubated or not with 10^{-6} M TPA for 24 h at 37°C, and thereafter the effectors were added (Figure 2). In the TPA-pretreated cells, addition of TPA for 15 min had no effect on ^{125}I-EGF binding, whereas it induced a 70% decrease in untreated cells. In contrast, NGF and bFGF provoked the same ^{125}I-EGF binding inhibition

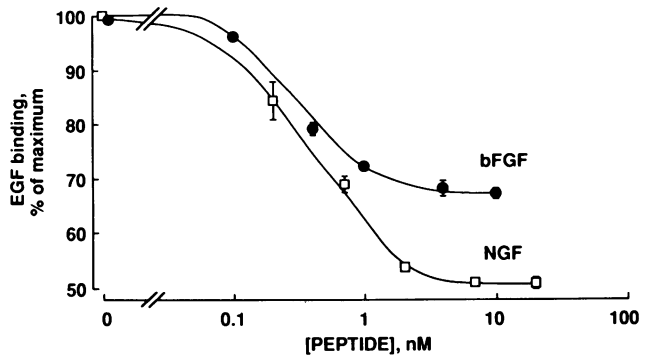

Figure 1. Effect of increasing concentrations of NGF or bFGF on ^{125}I-EGF binding to PC12 cells. PC12 cells in 12-well dishes were incubated for 15 min at 37°C in the presence of the indicated concentrations of NGF (0.2–20 nM) (□ —— □) or bFGF (0.1–10 nM) (● —— ●). Specific ^{125}I-EGF binding to PC12 cell was determined at 4°C as described in MATERIALS AND METHODS. The results are presented as the mean ± SEM of at least three determinations and are expressed as a percentage of specific EGF binding measured in absence of NGF or bFGF.

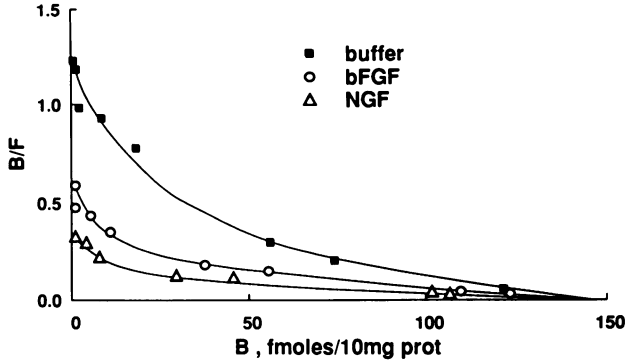

Figure 3. Scatchard analysis of ^{125}I-EGF binding in buffer and NGF- or bFGF-treated PC12 cells. PC12 cells in 12-well plates were incubated for 20 min at 37°C with one of the following: buffer (■), 100 ng/ml NGF (△), or 50 ng/ml bFGF (○). Specific ^{125}I-EGF binding (from 10 to 10 000 pM) to cell surface receptors was determined at 4°C. The data were analyzed by the method of Scatchard. Similar results were obtained in three separate experiments.

in both conditions. These results indicate that the mechanism by which NGF and bFGF induce a desensitization of ^{125}I-EGF binding to PC12 cells is independent of PKC activation, at least a PKC that is TPA-regulated.

To determine whether the NGF effect involves the p140^{trk} NGF receptor, we used K-252a (final concentration 2×10^{-6} M), which inhibits NGF action by decreasing the p140^{trk} tyrosine kinase activity (Berg *et al.*, 1992). PC12 cells were incubated for 20 min at 37°C with TPA, bFGF, NGF in the presence of K-252a, or carrier only. ^{125}I-EGF binding was then measured as described before. As shown in Figure 2, no inhibition of ^{125}I-EGF binding was observed in cells treated with both NGF and K-252a, whereas NGF alone induced a 50% decrease as expected. In contrast, K-252a did not modify the inhibitory effect of TPA and bFGF on ^{125}I-EGF binding. These data indicate that the action of NGF on EGF binding is mediated by the p140^{trk} proto-oncogene tyrosine kinase.

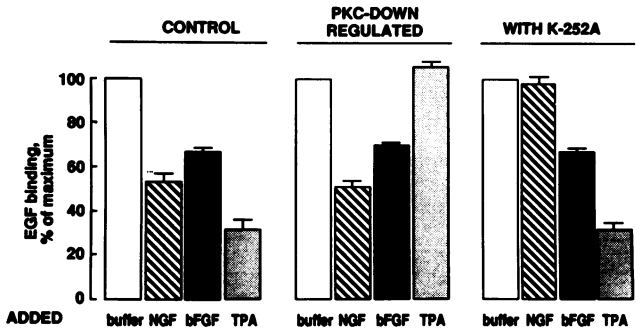

Figure 2. Involvement of PKC and p140^{trk} in EGF binding desensitization induced by NGF and bFGF. To downregulate PKC, PC12 cells in 12-well plates were exposed to 1 μM TPA for 24 h at 37°C. Untreated PKC-downregulated cells and K-252A (2×10^{-6M}) cells were then incubated for 15 min with the following: buffer (□), 100 ng/ml NGF (▨), 50 ng/ml bFGF (■), and 0.1 μM TPA (▩). Specific ^{125}I-EGF binding was measured at 4°C. Results are expressed as a percentage of specific binding obtained in buffer-treated cells. One hundred percent of specific binding corresponded to 10 480 ± 329, 8366 ± 173, and 10 049 ± 197 cpm, respectively, in control, PKC-downregulated, and K-252a–treated cells. The results are presented as the mean ± SEM of at least three determinations and are expressed as the percentage of EGF binding.

Scatchard Analysis of ^{125}I-EGF Binding to PC12 Cells Preexposed to NGF or bFGF

To further characterize the NGF- and bFGF-induced inhibition of the EGF receptor, binding was performed with increasing concentrations of ^{125}I-EGF, and the data were analyzed by the method of Scatchard (Figure 3). In buffer-treated cells, we obtained a curvilinear Scatchard plot, which for the EGF receptor is considered to reflect the existence of two populations of EGF binding sites. Binding parameters are summarized in Table 1. We observed that NGF and bFGF induced a decrease in the number of high affinity binding sites. In NGF- and bFGF-treated cells, there was, respectively, an approximately fourfold and an approximately threefold reduction in the number of high affinity binding sites without significant change in the high affinity dissociation constant (K_d = 20–30 pM) and in the total number of ^{125}I-EGF binding sites. Note that an approximately twofold increase in the K_d value for low affinity receptors was observed. In summary, in PC12 cells NGF and bFGF lead to a decrease in high affinity EGF binding sites without detectable change in the amount of receptors expressed at the cell surface.

Table 1. Binding parameters of ^{125}I-EGF binding in control and NGF- or bFGF-treated PC12 cells

	Low affinity		High affinity	
	Bmax sites/cell	$K_d \times 10^9$	Bmax sites/cell	$K_d \times 10^{11}$
Buffer	14 000 ± 2 000	0.7 ± 0.02	2 000 ± 320	2.4 ± 0.6
NGF	15 400 ± 2 200	1.6 ± 0.2	550 ± 80	2.9 ± 0.4
bFGF	15 600 ± 2 600	1.2 ± 0.1	620*	2.9 ± 0.4

The dissociation constant (K_d) and maximum binding capacity (Bmax) of low- and high-affinity EGF receptors of untreated and NGF- or bFGF-treated PC12 cells were calculated from the Scatchard analysis of three different experiments done in triplicate.

* Mean of two experiments.

Effect of NGF and bFGF on EGF Receptor Phosphorylation in PC12 Cells

Decreases in high affinity EGF binding sites have been correlated with changes in the phosphorylation state of the receptors (Davis and Czech, 1987; Countaway *et al.*, 1989; Bird and Saklatvala, 1990; Tartare *et al.*, 1992). Hence, we were interested in evaluating the effect of NGF and bFGF on EGF receptor phosphorylation in PC12 cells. The PC12 cells were labeled with [^{32}P]orthophosphate and exposed to the different effectors. After solubilization, proteins were subjected to immunoprecipitation with antibodies to EGF receptor, and the immune precipitates were analyzed by SDS–PAGE followed by autoradiography. In doing so, we found a strong basal phosphorylation and no, or little, stimulation. In contrast, when the gel was submitted to KOH treatment to eliminate the serine phosphorylation, increased EGF receptor phosphorylation was observed after stimulation with EGF, TPA, NGF, and bFGF (Figure 4, top). Phosphoamino acid analysis was performed after the same experiment but without prior KOH treatment (Figure 4, bottom). EGF induced an increase in phosphotyrosine because of EGF receptor autophosphorylation and a weaker increase in phosphothreonine (Figure 4, lane 2). TPA only stimulated phosphothreonine (Figure 4, lane 3), which is in agreement with results obtained in other cell types (Davis and Czech, 1985). Exposure to NGF and bFGF for 15 min also resulted in an increase in phosphothreonine (Figure 4, lanes 4 and 6). Finally, in the basal state and in all stimulated conditions tested, a strong but comparable serine phosphorylation was observed. These results suggest that binding of NGF and bFGF to their receptors leads to the activation of one or several serine/threonine kinase(s), which in turn phosphorylate(s) the EGF receptor on threonine residues. Because NGF and bFGF have been previously described as strong activators of the MAP kinase ERK1 in PC12 cells (Tsao *et al.*, 1990), we wished to evaluate the role of ERK1 in the NGF- or bFGF-stimulated phosphorylation of the EGF receptor.

In vitro Phosphorylation of the EGF Receptor by Immunopurified ERK1

To see whether the EGF receptor is a potential substrate for ERK1, we used a human EGF receptor, which is kinase-deficient because of a mutation of the ATP binding site. This receptor, being unable to induce its autophosphorylation, makes it easier to detect receptor phosphorylation by other kinases. In brief, proteins were solubilized from PC12 cells treated with NGF or bFGF; ERK1 was immunoprecipitated by anti-ERK1 antibodies, eluted, and added to immunoprecipitated kinase-deficient EGF receptors. Finally, phosphorylation was performed in presence of [γ-^{32}P]ATP for 1 h at room temperature, and the phosphoproteins were analyzed by SDS–PAGE under reducing conditions. When the EGF receptor was incubated alone or with nonstimulated ERK1, no phosphorylation could be detected (Figure 5A, lanes 1 and 2). In contrast, in the presence of NGF- or bFGF-activated ERK1, EGF receptor was strongly phosphorylated. NGF-induced phosphorylation was more marked than that induced by bFGF (Figure 5A, lanes 3 and 4). Phosphoamino acid analysis demonstrated that EGF receptor phosphorylation by activated-ERK1 occurs mainly on threonine residues and, to a

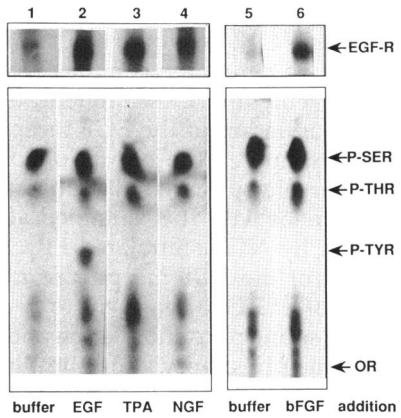

Figure 4. Top: EGF receptor phosphorylation in intact PC12 cells. PC12 cells in 6-well dishes were incubated with [^{32}P]orthophosphate for 3 h at 37°C. At the end of the labeling, cells were incubated with buffer, 0.1 μM EGF (5 min), 0.1 μM TPA (45 min), 100 ng/ml NGF (45 min), or with 50 ng/ml bFGF for 15 min. Proteins were then solubilized and submitted to precipitation by antibodies to EGF receptor. The precipitates were analyzed by SDS-PAGE under reducing conditions and submitted to KOH treatment before autoradiography. Bottom: phosphoamino acid analysis of EGF receptor phosphorylated in intact PC12 cells. The EGF receptors phosphorylated in the conditions described above were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane pieces corresponding to the receptor were hydrolyzed in 6 N HCl for 90 min at 110°C. The phosphoamino acids were separated on a cellulose plate by electrophoresis and the plate was autoradiographed.

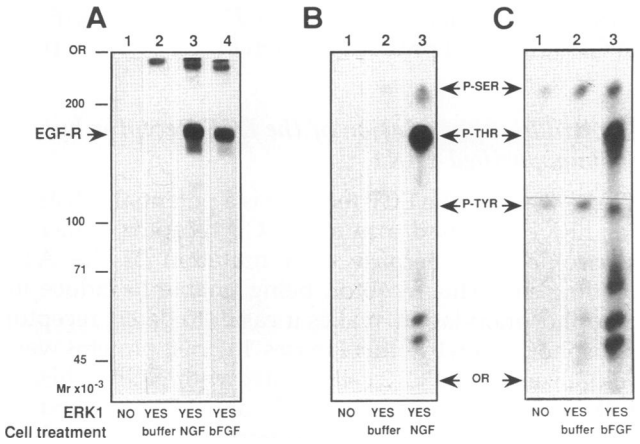

Figure 5. In vitro phosphorylation of the EGF receptor by immunopurified ERK1. PC12 cells were incubated at 37°C for 15 min with 100 ng/ml NGF or 50 ng/ml bFGF. The proteins were solubilized and submitted to precipitation by anti-ERK1 antibodies. After elution by C peptide, eluates were added to immunoprecipitated kinase-deficient EGF receptor, and the phosphorylation was performed as described in MATERIALS AND METHODS. Phosphorylated proteins were analyzed by SDS-PAGE gel under reducing conditions. (A) Autoradiograph of the gel. (B and C) Phosphoamino acid analysis of the EGF receptor phosphorylated as described in A.

much lesser extent, on serine residues (Figure 5, B and C). It should be mentioned that in some experiments phosphotyrosine was detected, probably because of the coprecipitation of the endogenous EGF receptor. In summary, in a cell-free system, NGF- and bFGF-activated ERK1 from PC12 cells phosphorylates the EGF receptor, mainly on threonine residues. To further establish the involvement of ERK1 in neurotropic factor-induced phosphorylation of the EGF receptor in intact PC12 cells, we compared the phosphopeptide map of the EGF receptor phosphorylated in intact PC12 cells after NGF treatment with that obtained after cell-free phosphorylation by NGF-stimulated ERK1. Phosphopeptide analysis of the EGF receptor from intact cells was performed after KOH treatment to avoid interference with strong serine phosphorylation and to visualize only the threonine phosphorylation sites. In both cases (in vitro and in vivo phosphorylation), we found three major phosphopeptides. Two of them displaying a poor chromatographic mobility were found to be comparable in both conditions (Figure 6). These results suggest that ERK1 is likely to be responsible for the EGF receptor threonine phosphorylation in NGF-treated PC12 cells.

Correlation Between the NGF- and bFGF-induced Time Courses of ERK1 Activation and of ^{125}I-EGF Binding Inhibition

To measure ERK1 activation, we examined the ability of activated ERK1 to phosphorylate MBP, which is an excellent substrate for ERK1 activity in vitro. With NGF, maximal ERK1 activation was obtained after 5 min and was maintained for ≥60 min (Figure 7A). NGF-induced EGF binding inhibition was maximal at 15 min and remained stable for ≥60 min (Figure 7B). In contrast, with bFGF, maximal ERK1 activation was reached within 5 min and was followed by a rapid decrease and a plateau for ≥60 min. A similar profile was observed for the bFGF effect on EGF binding. Thus, the maximal effect was obtained at 15 min and then a moderate decrease and a plateau followed. The biphasic effect of bFGF on EGF binding was less pronounced than the one observed for bFGF-induced ERK1 activity. Nevertheless, statistic analysis using paired Student *t*-test confirmed that in bFGF-treated cells, the EGF binding inhibition at 40 min ($p < 0.05$) and at 60 min ($p < 0.01$) was significantly lower than the binding inhibition at 20 min. These results show that the profiles of the curves are similar and that the magnitude of the effect on EGF binding can be correlated with ERK1 activity. This suggests that in PC12 cells ERK1 may be involved in the process of ^{125}I-EGF binding inhibition induced by NGF and bFGF.

DISCUSSION

We show here in PC12 cells that NGF and bFGF are able to induce a decrease in EGF binding amounting to 50 and 35%, respectively. This action persists with the same magnitude in PKC-downregulated cells, indicating that both effectors act through a pathway that is independent of a TPA-sensitive PKC. Such a phenomenon has been reported to occur in the homologous EGF receptor desensitization induced by the ligand (Tartare

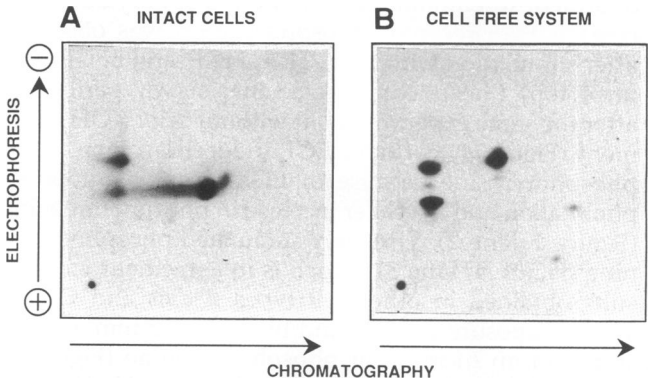

Figure 6. Phosphopeptide map analysis. EGF receptor phosphorylated in intact PC12 cells after NGF treatment (A) or in cell-free system by NGF-activated ERK1 (B) as described in the legends to Figure 4 (top) and 5 were separated by SDS-PAGE. The phosphoproteins corresponding to the EGF receptor were eluted and digested with trypsin (60 μg/ml) in 50 mM NH_4HCO_3 (pH 8.0) for 36 h at 37°C. The eluted peptides were lyophilized and resuspended in 15 mM NH_4OH. Phosphopeptides were separated on cellulose plates by electrophoresis followed by chromatography. The plates were then autoradiographed.

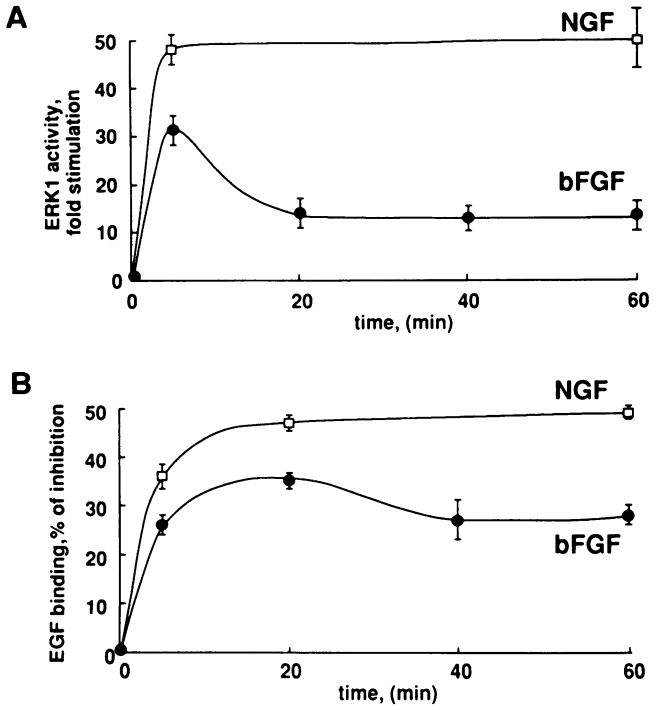

Figure 7. Time course of the effects of NGF or bFGF on ^{125}I-EGF binding and on ERK1 activity. PC12 cells in 12-well plates were incubated in the presence of 100 ng/ml NGF (□) or 50 ng/ml bFGF (●) for 5, 20, 40, or 60 min at 37°C. (A) ERK1 activity. Proteins were solubilized, and ERK1 was immunoprecipitated with specific antibodies. MBP was then added to the pellets and phosphorylated as described in MATERIALS AND METHODS. (B) Measurement of ^{125}I-EGF binding. Specific ^{125}I-EGF binding to PC12 cells was determined at 4°C as described in MATERIALS AND METHODS.

et al., 1992) and in the heterologous desensitization seen with PDGF, IL-1, and TNFα (Olashaw *et al.*, 1986; Bird and Saklatvala, 1989). The reduction in EGF binding induced by NGF and bFGF is rapid (maximal within 15 min) and dose dependent (EC_{50} = 5 ng/ml). Using K-252a originally described as a PKC inhibitor (Kase *et al.*, 1987) and recently also shown to inhibit the p140^{trk} tyrosine kinase activity, we observed a specific and complete disappearance of the NGF effect on EGF binding. This demonstrates the involvement of the p140^{trk} NGF receptor. Interestingly, K-252a does not interfere with the action of TPA on EGF binding. Similarly, a lack of K-252a effect on the activation of ornithine decarboxylase by TPA has been reported in PC12 cells by Koizumi *et al.* (Koizumi *et al.*, 1988).

Scatchard analysis of binding data showed that NGF and bFGF induce a decrease in the number of high affinity EGF binding sites. This could be explained at least by the two following mechanisms, a transformation of high affinity sites into low affinity ones and/or an internalization of the high affinity receptors. The first hypothesis is compatible with our data, indicating that NGF and bFGF do not change the total number of EGF receptors present on the surface of PC12 cells. Further, we did not observe an effect of monensine (a drug that blocks retroendocytosis) on the NGF-induced inhibition of EGF binding, confirming that the action of NGF is not mediated through an increase in EGF receptor internalization. Similarly, Brown and Carpenter (1991) showed that the NGF-induced acute decrease in EGF binding to PC12 cells is not accompanied by demonstrable differences in EGF receptor degradation or synthesis.

Acute inhibition of EGF binding has been reported to occur in cells after treatment by numerous agents such as phorbol esters and growth factors including EGF. In the case of the TPA-induced desensitization, the role of phosphorylation was substantiated by studies indicating that on plasma membrane preparations PKC was able to phosphorylate the EGF receptor and to decrease EGF binding (Fearn and King, 1985). However, the precise mechanism of this desensitization remains unclear, because no general agreement appears to exist. Two reports have shown that mutation of the EGF receptor on threonine 654, corresponding to the major PKC phosphorylation site, does not modify the effect of TPA on EGF binding (Countaway *et al.*, 1989; Friedman *et al.*, 1989). However, two other groups have published that a similar EGF receptor mutation affects the TPA-induced desensitization, suggesting that phosphorylation of threonine 654 by PKC is responsible for the reduction in high affinity EGF binding sites (Lin *et al.*, 1986; Decker *et al.*, 1990). In PKC-independent desensitization induced by PDGF, IL-1, TNFα, and EGF, it also appears that the EGF receptor phosphorylation state plays an important role, but the kinases and the receptor phosphorylation sites have not been identified.

With these observations in mind, we decided to study EGF receptor phosphorylation in intact PC12 cells treated with NGF or bFGF. We show that NGF and bFGF enhance threonine phosphorylation of the EGF receptor, indicating that these two agents induce the activation of one or more serine/threonine kinase(s). The following reasons led us to investigate the involvement of MAP kinases in this phosphorylation: 1) the EGF receptor contains a consensus sequence for phosphorylation by ERK1 (PRO-X-TRH^{669}-PRO) (Gonzales *et al.*, 1991); 2) ERT kinase (EGF receptor Thr^{669} kinase), which phosphorylates the EGF receptor on threonine 669, is related to the MAP kinase family (Northwood *et al.*, 1991); and 3) in PC12 cells ERK1 has been reported to be activated by NGF and bFGF (Tsao *et al.*, 1990). In brief, using immunopurified ERK1, we found that NGF- and bFGF-activated ERK1 phosphorylates the EGF receptor on threonine residues. Furthermore, with NGF two comparable phosphopeptides were visualized in the EGF receptor after phosphorylation either in a cell-free system or in intact PC12 cells, suggesting that the same sites are phosphorylated in both conditions. This supports the idea that in PC12 cells

ERK1 directly phosphorylates the EGF receptor in response to NGF. This view is further strengthened by recent studies in A431 cells, demonstrating a direct association between a newly identified MAP kinase (40 kDa) and the EGF receptor (Sanghera *et al.*, 1992).

The role of MAP kinases in the desensitization process is not clearly established. However, the good correlation we observed between the time courses of the NGF and bFGF effects on ERK1 activity and on EGF binding suggests that ERK1 may play a role in this receptor modulation. The delay of 10 min between both time courses could correspond to the time it takes for ERK1 to phosphorylate a minimal number of receptors before we can detect the inhibition of EGF binding.

As far as the molecular mechanism underlying the decrease in EGF binding is concerned, the ERK1-mediated EGF receptor phosphorylation on intracellular serine/threonine residues could induce a conformational change, which is propagated to the extracellular domain. This would then lead to a loss of high affinity EGF binding sites. Alternatively, the conformational change could be because of the association with, or the dissociation from, the receptor of a cellular component modulating EGF binding sites. This interaction could be regulated by the serine/threonine phosphorylation state of the receptor, as it was previously described for rhodopsin and arrestin (Wilden *et al.*, 1986). However, these hypotheses are difficult to reconcile with other studies addressing PDGF-induced EGF receptor desensitization. Indeed, PDGF also leads to threonine 669 phosphorylation of the EGF receptor, considered as the major phosphorylation site for MAP kinases. The replacement of this threonine by an alanine does not affect the decrease in EGF binding induced by PDGF (Countaway *et al.*, 1990). However, our results indicate that ERK1 phosphorylates residues other than the threonine 669, because we found at least three phosphopeptides when the EGF receptor was phosphorylated in vitro by immunopurified ERK1. Phosphorylation of these unidentified sites may be involved in EGF binding desensitization. Further, we cannot exclude the possibility that the inhibition of EGF binding is caused by the phosphorylation by ERK1 of proteins associated to an intracellular EGF receptor domain. Such a view is supported by Walker and Burgess (1991) showing that EGF binding desensitization induced by PDGF can be correlated with the dissociation of several phosphoproteins from the cell membrane.

In summary, we have shown that in PC12 cells NGF and bFGF induce a decrease in high affinity EGF binding and an increased threonine phosphorylation of the EGF receptor. Furthermore, our results strongly suggest that ERK1 is likely to be responsible for this phosphorylation and for the EGF binding desensitization in these cells. The high affinity binding sites play a critical role in the transduction of the EGF mitogenic response (Defize *et al.*, 1989; Bellot *et al.*, 1990) and their transformation to low affinity binding sites inevitably results in reduced action of the growth factor. The rat pheochromocytoma line PC12 is an extensively used cultured cell system for the analysis of neuronal differentiation and is unique because it allows the study of the action of factors with distinct ultimate effects, i.e., differentiation seen with NGF and bFGF versus mitogenesis seen with EGF. Considering the mounting evidence that MAP kinases are involved in the control of key cellular functions, the distinct pattern of the MAP-kinase, ERK1, activation observed in PC12 cells with differentiating- versus growth-promoting factors represents a provocative but certainly fragmented explanation for the neurotrophic action of those factors. Finally, looking at a more physiological situation where cells such as PC12 cells are exposed simultaneously to several factors, some of which exert opposing actions, the rapid desensitization of the growth-promoting EGF receptor by the differentiating factors, NGF and bFGF, reported here, would provide the PC12 cells with a tight control on the decision-making events terminating in neuronal differentiation.

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