Nerve Growth Factor- and Epidermal Growth Factor-stimulated Phosphorylation of a PC12 Cytoskeletally Associated Protein In Situ

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ABSTRACT   Nerve growth factor (NGF) and epidermal growth factor (EGF) produce stable alterations in PC12 cells that persist in the detergent-insoluble cytoskeleton, resulting in the phosphorylation of a 250,000-mol-wt cytoskeletally associated protein in situ. Treatment of PC12 cells with NGF or EGF, followed by detergent lysis of the cells and incubation of the resulting cytoskeletons with γ-^{32}P-ATP, permitted detection of hormonally stimulated, energy-dependent events, which result in the enhanced phosphorylation of a cytoskeletally associated protein as an immediate consequence of receptor occupancy. These events were elicited only upon treatment of intact cells at physiological temperatures. The NGF- and EGF-stimulated events occurred rapidly; however, they were a transient effect of hormone action. NGF and EGF were found to act through independent mechanisms to stimulate the in situ phosphorylation of the 250,000-mol-wt protein, as the effects of NGF, but not EGF, were blocked by methyltransferase inhibitors. The 250,000-mol-wt protein was phosphorylated on serine and threonine residues in response to both NGF and EGF although in somewhat different proportions. The data suggest that the hormone-stimulated labeling of the 250,000-mol-wt protein may be the result of either the direct activation of a protein kinase, the redistribution of the kinase relative to its substrates as a consequence of hormone action, or the coincident occurrence of these events.

Although it is well known that the binding of peptide hormones to cell surface receptors results in various metabolic changes in the target cells, the mechanisms subserving these events are not well understood. Several hypotheses have been advanced to explain the myriad effects of peptide hormones, including the involvement of second messengers generated on hormone binding (1), internalization of the hormone and subsequent transport to the nucleus (2), and the activation of hormonally sensitive protein kinases (3). There is reasonably good evidence that at least some of the biological effects, including the stimulation of DNA synthesis, are the result of receptor aggregation within the plasma membrane (4–6).

The role of protein kinases in hormone action is of particular interest, given the recent discovery that the membrane receptors for several peptide hormones possess an intrinsic protein kinase activity (7–12). The binding of hormone to its receptor immediately activates the protein kinase, resulting in autophosphorylation of the receptor itself as well as the phosphorylation of other cellular proteins (3, 7, 12–14). It is presently uncertain what the significance of these modifications are, although it has been suggested that they are involved in control of cellular proliferation (15, 16). The receptor kinases examined so far specifically phosphorylate tyrosine residues, a property hitherto believed to be limited to retroviral gene products. Both classes of tyrosine kinases phosphorylate cytoskeletal proteins (reference 17 and Landreth, G. E., G. D. Rieser, and L. K. Williams, submitted for publication).

A clonal rat pheochromocytoma, PC12, has proven to be a valuable model for neuronal differentiation. These cells respond to nerve growth factor (NGF)[1] by cessation of division and development of a number of properties characteristic of mature sympathetic neurons (18). These cells possess cell surface receptors for both NGF and epidermal growth factor (EGF). The NGF receptors display heterogeneous binding characteristics (19, 20), with one class of receptors being

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[1] *Abbreviations used in this paper.* EGF, epidermal growth factor; NGF, nerve growth factor.

associated with the cytoskeleton (20).

Protein phosphorylation has been suggested to play a role in NGF action in PC12 cells (18, 21–23). NGF stimulates the phosphorylation of a number of proteins and the dephosphorylation of at least one protein (22). Some of the effects of NGF on protein phosphorylation occur within the first 15 min of hormone exposure, whereas others require longer periods, indicating that NGF modifies the activity of protein kinases or of phosphoprotein phosphatases both acutely and chronically (21).

We have approached the role of protein phosphorylation in hormone action in a somewhat different manner, by postulating that hormone treatment of PC12 cells produces stable alterations in protein kinase activity or distribution relative to its substrates. These experiments involve treating the cells with hormone and then lysing the cells with detergent under conditions where the cytoskeletal structure of the cell is preserved and spatial relationships between proteins are retained (24, 25). Protein phosphorylation is then carried out in situ. The phosphoprotein labeling reflects the activity of immobilized protein kinases acting on protein substrates that are also associated with structural elements of the cell. Using such a system, we have detected the phosphorylation in situ of a 250,000-mol-wt cytoskeletally associated protein in PC12 cells following treatment of the cells with either NGF or EGF.

MATERIALS AND METHODS

Materials: Dulbecco's modified Eagle's medium and fetal calf serum were from Gibco Laboratories (Grand Island, NY) and horse serum from KC Biological, Inc. (Lenexa, KS). EGF was purchased from Collaborative Research Inc. (Lexington, MA). NGF was prepared by the method of Burton et al. (26). Radiolabeled ATP was purchased from ICN Pharmaceuticals, Inc. (Irvine, CA) or synthesized using GammaPrep A from Promega-Biotech Inc. (Madison, WI); [^{32}P]orthophosphate was also from ICN Pharmaceuticals, Inc. Phosphotyrosine standard was generously provided by Dr. M. A. Bothwell (Princeton University, NJ). Cellulose thin layer chromatography plates (MN300) were from Brinkmann Instruments, Inc. (Westbury, NY). Triton X-100 was from Sigma Chemical Co. (St. Louis, MO).

Cell Culture: PC12 cells were grown in Dulbecco's modified Eagle's medium containing 5% fetal calf serum and 10% horse serum in an atmosphere of 88% air, 12% CO_2.

In Situ Phosphorylation: PC12 cells were harvested from tissue culture dishes with calcium- and magnesium-free PBS containing 1 mg/ml BSA and (except where indicated) 1 mg/ml glucose. The cells were washed twice and resuspended at a concentration of 10^6 cells/ml. NGF and EGF were added to aliquots of the cell suspension and incubated at 37°C. The cells were pelleted by centrifugation, gently resuspended in 0.15% Triton X-100, 25 mM HEPES, pH 7.4, 2 mM $MnCl_2$, and 1 mM phenylmethylsulfonyl fluoride (0.5 ml/10^6 cells), and incubated for 2 min at 4°C. The resulting cytoskeletons were centrifuged for 1 min in a Beckman Microfuge B (Beckman Instruments, Inc., Palo Alto, CA) and resuspended in cold detergent-free buffer (0.1 ml/10^6 cells), and γ-^{32}P-ATP was added (10 μM, 55 dpm/fmol ATP). The phosphorylation reaction was carried out for 10 min at 4°C and stopped by the addition of electrophoresis sample buffer and heating to 100°C for 5 min.

SDS PAGE: The cytoskeletal proteins were separated on 4–13% or 5–15% polyacrylamide gradient gels as described by Laemmli (27). The proteins were stained with 0.1% Coomassie Blue and the gels were dried and exposed to Kodak XAR film with or without Lighting Plus intensifying screens (E. I. DuPont de Nemours & Co., Newtown, CT). Autoradiograms were scanned densitometrically using a Beckman DU8 spectrophotometer.

Phosphoamino Acid Analysis: The ^{32}P-labeled 250,000-mol-wt protein was excised from the dried gel, rehydrated in 50 mM NH_4HCO_3, pH 8.0, 0.05% SDS containing 50 μg/ml Proteinase K, and incubated overnight at room temperature. The solubilized protein was collected, lyophilized, and hydrolyzed for 2 h at 110°C in 6 N HCl. The labeled amino acids were combined with a mixture of phosphoamino acid standards and an aliquot was applied to cellulose thin layer plates. The phosphoamino acids were separated in the first dimension by electrophoresis at pH 1.9 for 1 h (1 kV), followed by ascending chromatography in isobutyric acid/0.5 M NH_4OH (5:3) in the second dimension. The chromatogram was then exposed to Kodak XAR film.

RESULTS

Treatment of PC12 cells with NGF or EGF resulted in stable changes within the cells, which persisted upon detergent lysis and resulted in enhanced phosphorylation of a 250,000-mol-wt cytoskeletally associated protein in situ (Fig. 1). The term "cytoskeleton" is operationally defined as those elements of the cell that are insoluble in non-ionic detergent and include, in addition to fibrous structural proteins, the nucleus, surface lamina, and associated proteins. The phosphorylation was due to the action of a protein kinase which was also cytoskeletally associated and in proximity to the 250,000-mol-wt substrate. The phosphorylation reaction occurs under conditions that preserve cytoskeletal structure (except microtubules). Indeed, identical results were obtained using the more stringent buffer conditions of Burr et al. (24) and also with the buffer of Schliwa and van Blerkom (25) which was reported to preserve cellular microtubules. Neither the 250,000-mol-wt protein nor any protein kinase was found in solution in cytoskeletal preparations of PC12 cells, as determined by direct assay of the buffer for protein kinase activity and for labeled 250,000-mol-wt protein released from the cytoskeletal preparation during the phosphorylation reaction. Extensive washing of the cytoskeletons in detergent-free buffer had no effect on the in situ phosphorylation of the 250,000-mol-wt cytoskeletal protein.

The 250,000-mol-wt protein was observed to be present in small amounts in the PC12 cell cytoskeleton but became highly phosphorylated in situ upon hormone treatment of the cells. The 250,000-mol-wt protein appears to be the only protein whose phosphorylation was dramatically enhanced by hormone treatment; still, we cannot rule out the presence of other lower molecular weight proteins whose labeling was stimulated less markedly. Similar results were obtained using γ-^{32}P-ATP and γ-^{35}S-thiol-ATP. The phosphorylation of the 250,000-mol-wt protein occurred using either Mn^{++} or Mg^{++}. The magnitude of the hormone-stimulated phosphorylation of the 250,000-mol-wt protein varied between experiments from two- to fivefold greater than controls.
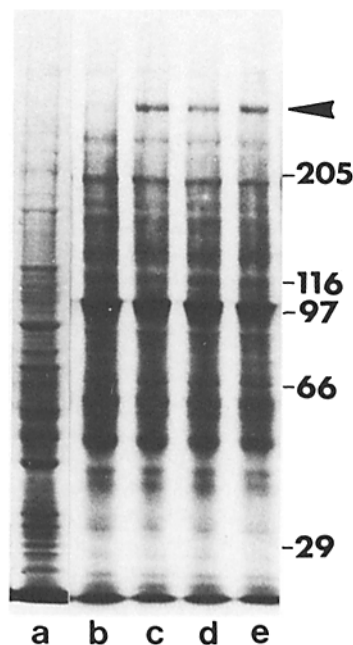

FIGURE 1 NGF- and EGF-stimulated phosphorylation of cytoskeletal protein. PC12 cells were incubated in the presence or absence of 10 ng/ml of either NGF or EGF for 5 min at 37°C. Cytoskeletons were prepared and then incubated with [^{32}P] ATP for 10 min at 4°C. The cytoskeletal proteins were separated on 4–13% SDS polyacrylamide gels. Coomassie Blue-stained cytoskeletal proteins are shown in lane a. Autoradiograms of ^{32}P-labeled phosphoproteins from untreated cells (lane b); and cells treated with NGF (lane c), EGF (lane d), or both NGF and EGF (lane e). The positions of molecular weight markers ($\times 10^{-3}$) are shown.

–205

–116
–97

–66

–29

a b c d e

NGF and EGF stimulated the in situ phosphorylation of the 250,000-mol-wt protein by independent mechanisms. The effects of NGF on PC12 cells can be blocked by methyltransferase inhibitors whereas those of EGF are unaffected (28). PC12 cells were preincubated with 3 mM 5'-S-methyl adenosine for 30 min before the addition of either NGF or EGF. The methyltransferase inhibitor blocked the NGF-stimulated in situ phosphorylation of the 250,000-mol-wt protein but had no effect on the phosphorylation produced by EGF (Fig. 2). These data indicate that the two hormones act through mechanistically distinct pathways to produce the in situ phosphorylation of the 250,000-mol-wt cytoskeletally associated protein. This conclusion is supported by two additional lines of evidence. First, the addition of NGF and EGF (with each at concentrations that maximally stimulate the response) together produced phosphorylation of the 250,000-mol-wt protein at a level greater than that produced by the individual hormones alone (Table I and Fig. 1). Although the effects of the hormones were not additive, these observations suggest that the two hormones act independently to produce the response. Second, acid hydrolysis of the 250,000-mol-wt protein and separation of the labeled phosphoamino acids revealed only ^{32}P-labeled phosphoserine and phosphothreonine residues. NGF and EGF stimulated the labeling of both residues although in different proportions, indicating that the two hormones produce protein phosphorylation, but with different specificities (Fig. 3). In addition, the data demonstrate that, at least for EGF, the protein phosphorylation was not the result of the EGF-receptor kinase activity, since this enzyme specifically phosphorylates tyrosine residues (10).

The ability of both NGF and EGF to stimulate the phosphorylation of the 250,000-mol-wt protein required treatment of intact cells (Fig. 4). In vitro exposure of cytoskeletons to hormone has no effect on protein phosphorylation, indicating either that the relevant enzymes were not activated under these circumstances, that the substrates were not positioned near the kinases, or that the hormone receptors were lost on detergent lysis. It was also possible that the changes resulting in the in situ phosphorylation required an integrated cellular response, some aspect of which was lost on detergent treatment.
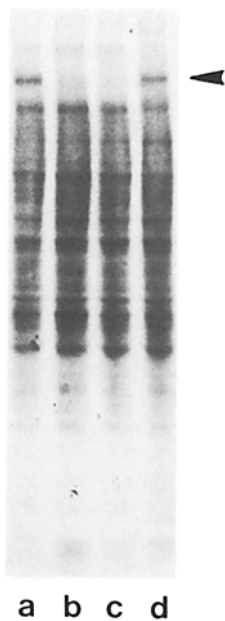
Figure 2 Effect of methyltransferase inhibitors on the phosphorylation of the 250,000-mol-wt cytoskeletal protein. PC12 cells were incubated in the absence (lane a) or presence (lanes b–d) of 3 mM 5'-S-methyl adenosine for 30 min at 37°C; NGF (lanes a and c) or EGF (lane d) was then added (50 ng/ml) and the incubation was carried out for 5 min more. Cytoskeletons prepared from these cells were then incubated with [^{32}P]ATP for 10 min at 4°C. Autoradiograms of the ^{32}P-labeled phosphoproteins are shown; the position of the 250,000-mol-wt protein is indicated.

a b c d

TABLE I

Effect of NGF and EGF Alone or Combined on the Phosphorylation of the 250,000 mol-wt Protein

	Radioactivity incorporated
Untreated	1.00
NGF	1.82 (0.10)
EGF	1.69 (0.23)
NGF + EGF	2.18 (0.61)

PC12 cells were incubated with 10 ng/ml NGF, EGF, or both hormones for 5 min at 37°C. Cytoskeletons were prepared and incubated for 10 min at 4°C with ^{32}P-ATP. The cytoskeletal proteins were separated on 4–13% polyacrylamide gels and autoradiograms were prepared. The autoradiograms were scanned densitometrically and radioactivity in the 250,000-mol-wt protein was determined. The radioactivity present in the 250,000-mol-wt band as a percentage of total cytoskeletally incorporated radioactivity was determined, with data from hormonally stimulated samples expressed relative to untreated cells. The data are the mean (± SD) of five separate experiments.
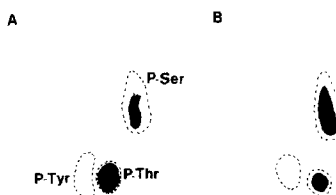
Figure 3 Phosphoamino acid analysis of the 250,000-mol-wt cytoskeletal protein. The 250,000-mol-wt PC12 cytoskeletal protein from cells treated for 5 min at 37°C with 10 ng/ml NGF (A) or EGF (B) was subjected to acid hydrolysis, and the phosphoamino acids were separated by electrophoresis at pH 1.9 (vertical dimension) followed by ascending chromatography in isobutyric acid/NH₄OH (horizontal dimension). The positions of phosphoamino acid standards are indicated. These data are representative of three separate experiments.

At low temperature (4°C), the ability of NGF and EGF to stimulate 250,000-mol-wt protein phosphorylation was arrested (Fig. 4). This was not due to any lack of hormone binding, since both NGF (20) and EGF (29) binding occurs at low temperature, although at diminished levels. The experiments were performed using hormone concentrations (50 ng/ml) sufficient to result in receptor occupancy at levels known to maximally stimulate the response.

When PC12 cells were incubated in the presence of glucose, the in situ phosphorylation of the 250,000-mol-wt protein was measurably enhanced compared with cells in glucose-free medium (Fig. 5); however, if PC12 cells were pretreated with 10 mM sodium azide, the phosphorylation of the 250,000-mol-wt protein was blocked. Azide treatment resulted in a 70% reduction in intracellular ATP content compared with cells incubated in glucose-containing medium (5.5 and 18.2 fmol ATP/cell, respectively), which indicates that the events required for 250,000-mol-wt protein phosphorylation require metabolic energy. In the azide-treated cells, the in situ labeling of other cytoskeletal phosphoproteins was uniformly greater than in untreated cells. This latter effect is likely to be due to back-phosphorylation as a result of lower ATP levels in vivo, which would diminish protein phosphorylation but leave cellular phosphatase activities unimpaired, thus generating unoccupied phosphorylation sites that are subsequently phosphorylated in situ.

Two important conclusions can be drawn from this experiment: (a) inclusion of glucose in the incubation medium enhances in situ phosphorylation of the 250,000-mol-wt protein; and (b) azide blocks this event. These data demonstrate that metabolic energy is required to elicit the cellular events
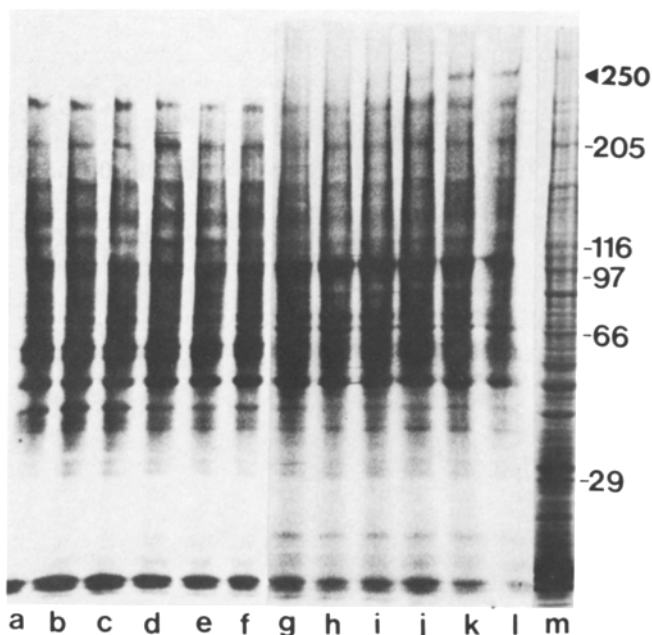
FIGURE 4 In situ phosphorylation of the 250,000-mol-wt cytoskeletal protein requires hormone treatment of intact cells at physiological temperatures. Cytoskeletons were prepared from naive PC12 cells (lanes a–f) and then received either no additions (lanes a and d) or 50 ng/ml of NGF (lanes b and e) or EGF (lanes c and f); they were incubated for 5 min at 37°C (lanes a–c) or 4°C (lanes d–f) and then again for 10 min more at 4°C with [^{32}P]ATP. Intact PC12 cells were incubated alone (lanes g and j) or with 50 ng/ml of either NGF (lanes h and k) or EGF (lanes i and l) at 4°C (lanes g–i) or 37°C (lanes j–l) for 5 min; cytoskeletons were then prepared and incubated for 10 min with [^{32}P]ATP. An autoradiogram of the labeled cytoskeletal phosphoproteins is shown. The Coomassie Blue-stained cytoskeletal proteins are shown (m), and the positions of molecular weight markers (× 10^{-3}) are indicated.

leading to the phosphorylation of the 250,000-mol-wt protein in situ.

A concern in these experiments was that the hormone may have stimulated in vivo phosphorylation or dephosphorylation of the 250,000-mol-wt protein, influencing the level of ^{32}P labeling observed subsequently to be incorporated in situ, due to altered levels of occupancy of phosphorylation sites with phosphate from endogenous ATP pools. To test directly if hormone treatment modified the in vivo phosphorylation of the 250,000-mol-wt protein, we preincubated the cells with [^{32}P]orthophosphate and followed this by addition of hormone and incubation at 37°C. NGF treatment of PC12 cells resulted in a small stimulation of labeling of the 250,000-mol-wt cytoskeletal protein (Fig. 6). This result was variable in magnitude and inconsistently obtained. The significance of this observation was that in no case was there a diminution of labeling of the 250,000-mol-wt protein in vivo, demonstrating that the 250,000-mol-wt protein was not specifically dephosphorylated as a consequence of hormone treatment. These data argue that the enhanced in situ phosphorylation was not a consequence of phosphoprotein phosphatase activity in vivo, resulting in the dephosphorylation of the 250,000-mol-wt protein, which would generate unoccupied phosphorylation sites that could subsequently be phosphorylated in situ.

The effect of NGF and EGF on the in situ phosphorylation

of the 250,000-mol-wt protein was dose dependent (Fig. 7), with half-maximal stimulation occurring below 1 ng/ml for both NGF and EGF (38 and 165 pM, respectively). This is similar to the concentration dependence of other effects of the hormones on these cells, indicating that maximal effects occur at low levels of receptor occupancy.

There was a dramatic difference in the in situ labeling of the 250,000-mol-wt protein with different periods of hormone exposure of the PC12 cells (Fig. 8). Both EGF and NGF maximally stimulated the in situ phosphorylation of the 250,000-mol-wt protein within 5 min of hormone exposure. The amount of radioactivity incorporated into the 250,000-mol-wt protein in situ decreased with longer exposure of the cells to NGF. These data demonstrated that the initial hormone-stimulated phosphorylation occurred with a time course that closely paralleled the association of hormones with their cell surface receptors (19, 30), which indicates that the hormone-mediated changes resulting in the phosphoryla-
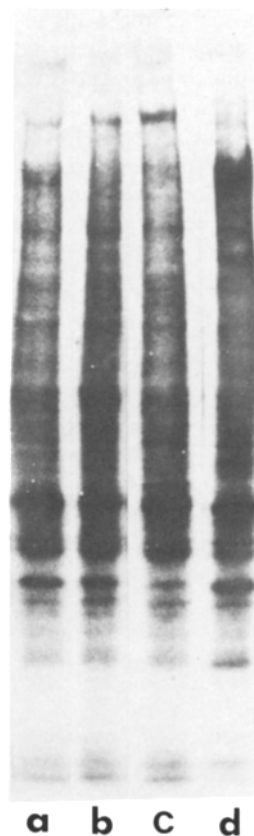

FIGURE 5 Effect of glucose and azide on the in situ phosphorylation of the 250,000-mol-wt cytoskeletal protein. PC12 cells were incubated alone (lane a) or with 20 ng/ml of NGF (lanes b–d) in PBS (lanes a, b, and d), or supplemented with 1 mg/ml glucose (lane c). One group of cells (lane d) was preincubated with 10 mM sodium azide for 30 min before hormone addition. After a 5-min incubation at 37°C with NGF, cytoskeletons were prepared and incubated with [^{32}P]ATP for 10 min at 4°C. An autoradiogram of the labeled phosphoproteins is shown.
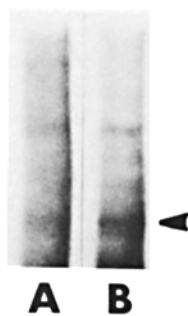

FIGURE 6 In vivo labeling of PC12 cytoskeletal proteins. PC12 cells were incubated with 50 μCi/ml [^{32}P]orthophosphate in phosphate-free growth medium for 3 h at 37°C. The cells were then incubated in the absence (lane A) or presence (lane B) of 20 ng/ml NGF for 10 min. The cells were lysed and the cytoskeletal proteins were separated on 4–13% polyacrylamide gels. An autoradiogram of the labeled phosphoproteins is shown. The position of the 250,000-mol-wt protein (arrow) is shown.

tion of the 250,000-mol-wt protein were an immediate consequence of receptor occupancy. This is one of the most rapid biochemical effects of either hormone yet demonstrated on these cells. The subsequent fall in ^{32}P incorporated into the 250,000-mol-wt protein indicated that the events leading to the in situ phosphorylation were a transient consequence of hormone action.

The NGF- and EGF-mediated events resulting in the phosphorylation of the 250,000-mol-wt protein, once they have occurred, were themselves stable in the cytoskeletal preparations (Fig. 9). PC12 cells were incubated with EGF for 10 min at 37°C and the cytoskeletons were prepared and incubated with radiolabeled ATP for 1-20 min. There was a time-
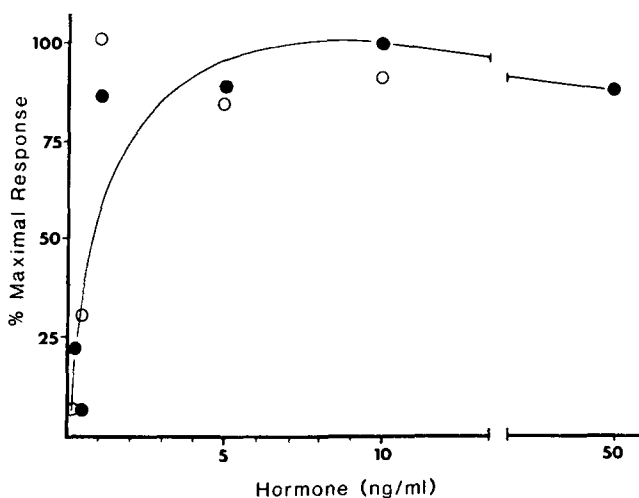
FIGURE 7 Concentration dependence of hormone-stimulated phosphorylation of the 250,000-mol-wt cytoskeletal protein. PC12 cells were incubated with the indicated concentration of either NGF (O) or EGF (●) for 15 min at 37°C. Cytoskeletons were then prepared and incubated with [^{32}P]ATP for 10 min at 4°C. The resulting autoradiograms were densitometrically scanned and the radioactivity in the 250,000-mol-wt protein was determined.

dependent increase in the labeling of the 250,000-mol-wt protein similar to that observed with other cytoskeletal phosphoproteins. Identical results were obtained with NGF. These data indicate that the transient nature of the cellular response is not a consequence of the lability of the hormone-produced events leading to the phosphorylation of the 250,000-mol-wt protein in situ.

Several agents were also tested for their effect on the phosphorylation of the 250,000-mol-wt cytoskeletal protein. PC12 cells or cytoskeletons were incubated, without effect, with 1 mM Ca^{++}, 0.01-1 mM cyclic AMP (or 1 mM dibutryl cyclic AMP for whole cells), 20-50 μM cytochalasin D, 10-80 μM trifluorperazine, and 10 mM dansylcadaverine.

The identity of the 250,000-mol-wt cytoskeletal protein is unknown. Two obvious candidates are fodrin and filamin. We have been unable to immunoprecipitate the phosphorylated 250,000-mol-wt protein with antisera to fodrin or to chicken gizzard filamin (Amersham Corp., Arlington Heights, IL) by using the method of Levine and Willard (31).

DISCUSSION

We have shown that hormone treatment of PC12 cells resulted in stable cellular changes that persisted in the cytoskeleton following extraction of the cells in non-ionic detergent and led to the phosphorylation of a 250,000-mol-wt cytoskeletally associated protein in situ. The experiments were performed on cytoskeletons prepared from hormonally treated cells under conditions where cytoskeletal structure (except microtubules) is preserved; thus, the protein phosphorylation occurred between immobilized enzyme and substrate (25). These experiments have demonstrated that NGF and EGF rapidly, but transiently, produced cellular events as an immediate consequence of receptor occupancy, resulting in the enhanced phosphorylation of a single cytoskeletally associated protein. This type of hormonally mediated change has not previously been observed, and it provides a new measure of hormone
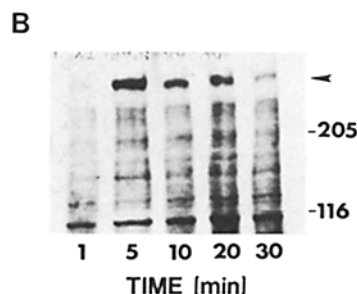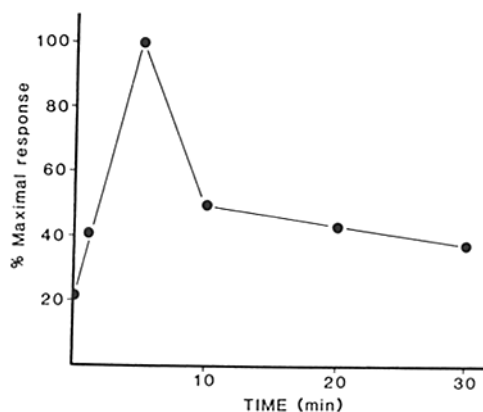
FIGURE 8 Time course of hormone treatment of PC12 cells on the in situ phosphorylation of the 250,000-mol-wt cytoskeletal protein. (A) PC12 cells were incubated with 10 ng/ml EGF for the indicated periods and then cytoskeletons were prepared and incubated with [^{32}P]ATP for 10 min at 4°C. Autoradiograms of the labeled phosphoproteins were scanned densitometrically and the radioactivity in the 250,000-mol-wt cytoskeletal protein was determined. The amounts of ^{32}P incorporated into the 250,000-mol-wt protein in untreated controls and from cells treated with EGF for 5 min were 1.0 and 4.9%, respectively. The data are representative of three separate experiments. (B) Autoradiogram of cytoskeletal phosphoproteins from PC12 cells treated with 10 ng/ml NGF for the indicated time periods. The cytoskeletal proteins were phosphorylated by a 10-min incubation with ^{35}S-thiol-ATP. The data are representative of seven separate experiments.
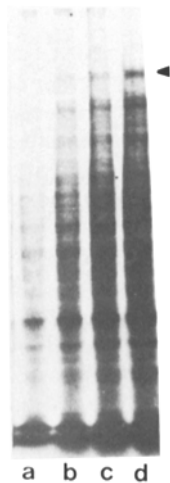
a b c d

FIGURE 9 Stability of the hormone-stimulated phosphorylation of the 250,000-mol-wt protein. PC12 cells were treated with NGF (10 ng/ml) for 5 min at 37°C, and then cytoskeletons were prepared and incubated with [^{32}P]ATP for 1 (lane a), 5 (lane b), 10 (lane c), or 20 min (lane d). An autoradiogram of the labeled phosphoproteins is shown.

action, permitting the investigation of the primary events in the mechanism of NGF and EGF action.

The hormonally enhanced protein labeling in situ can arise from either (a) direct activation of a cytoskeletally associated protein kinase, (b) hormonally induced cytoskeletal attachment of either the protein kinase or its substrates, (c) redistribution of the enzyme in proximity to its substrates, or (d) the coincident occurrence of any of these events.

The hormonally stimulated phosphorylation of the 250,000-mol-wt protein may be the result of direct kinase activation. As we have no direct evidence for this, experiments are currently in progress to test this possibility. Halegoua and Patrick (21) reported that both NGF and EGF independently stimulated the phosphorylation of a number of different cellular proteins in PC12 cells.

The hormonally stimulated phosphorylation of the 250,000-mol-wt cytoskeletal protein may be the result of active redistribution of either the enzyme or its substrate by an energy-requiring process. Two types of reorganization may be involved. Hormone treatment of PC12 cells may result in the transfer of the enzyme or its substrate from the cytoplasm to cytoskeletal elements or, alternatively, may promote the attachment of membrane proteins to structural proteins of the "surface lamina" (32). There is evidence that the latter type of interaction occurs with the NGF receptors on these cells (20).

The second possibility is that hormone treatment promotes a redistribution of membrane proteins, which occur as a result of the rapid and dramatic changes in cell surface morphology. Both NGF and EGF produce an immediate increase in membrane ruffling which persists for ~10 min after hormone exposure and the loss of microvilli in PC12 cells within 15 min (33, 34). Similar events occur on EGF treatment of A431 cells (35). Interestingly, Rous sarcoma virus-infected cells, which possess a membrane-associated tyrosine kinase, also undergo an analogous change in cell surface architecture (36, 37). These cell surface changes are accompanied by alterations in spatial relationships between proteins, and it is not unreasonable that during this process active protein kinases may become associated with their substrates, perhaps transiently.

PC12 cells also undergo a separate class of events involving the distribution of cell surface proteins which accompanies cellular processing of the hormone. Following binding of hormone to cell surface receptors, the receptors translocate within the membrane and aggregate prior to their internalization (38). The aggregation of receptors is arrested at 0°C, while the internalization of the hormone-receptor complex requires metabolic energy.

The nature of the cellular events subserving the hormone-stimulated phosphorylation of the 250,000-mol-wt protein is unclear. Those changes, however, are dependent on metabolic energy as demonstrated by the inability of NGF to elicit the phosphorylation of the 250,000-mol-wt protein in cells whose ATP pools were depleted by 70%.

We believe that it is unlikely that the enhanced in situ phosphorylation of the 250,000-mol-wt protein is due to active dephosphorylation of the protein in vivo by hormonally stimulated phosphoprotein phosphatases. In PC12 cells labeled with ^{32}P-orthophosphate we have been consistently unable to detect the dephosphorylation of the 250,000-mol-wt protein in vivo. These data indicate that hormone-stimulated phosphatase activation is not likely to occur. The variable extent of in vivo labeling of the 250,000-mol-wt protein suggests that its phosphorylation state in vivo may be tightly regulated, and that we are able to detect it in situ because of the removal of active phosphoprotein phosphatases upon detergent treatment of the cells. This suggestion is supported by the recent observation that phosphotyrosyl phosphatases are approximately one hundred-fold more active than the respective kinases (39).

The cellular mechanisms subserving the actions of NGF and EGF appear to be distinct. A broad spectrum of NGF-mediated events is blocked by inhibition of methyltransferase reactions; however, those elicited by EGF are unaffected or enhanced (28). These data suggest that methylation is a very early and obligatory step in the mechanism of action of NGF. The inhibition of NGF-stimulated phosphorylation of the 250,000-mol-wt protein provides additional evidence that the two hormones operate through independent mechanisms to elicit their biological effects. Furthermore, the ability of NGF and EGF, when added simultaneously, to stimulate the phosphorylation of the 250,000-mol-wt protein at levels greater than those produced by the individual hormones alone, and the different ratios of serine and threonine phosphorylation produced by the two hormones, support this hypothesis. The combined effect of NGF and EGF, however, is not additive. This result may be due to low substrate concentration, a possibility difficult to assess given the small amount of the 250,000-mol-wt protein present in the cytoskeleton and the immobilization of both enzyme and substrate.

It is of some interest that Connolly et al. (34) have recently reported that NGF and EGF treatment of PC12 cells resulted in a large increase in coated pit density on the cell surface. This effect occurred maximally within 3 min of hormone treatment. If both hormones were added, the combined treatment resulted in a quantity of coated pits only slightly greater than that produced by the individual hormones.

PC12 cells respond to NGF and EGF by phosphorylating a number of cellular proteins (21). The phosphorylation of several of these proteins was common to both NGF and EGF, but NGF uniquely stimulated labeling of a few other specific proteins. Similarly, both hormones stimulated the phosphorylation of a 30,000-mol-wt nuclear protein in these cells (23).

These observations are of interest in that EGF and NGF share a set of positive pleiotypic actions on these cells and are processed by the cell in a generally similar manner (29, 30). NGF, however, inhibits cell proliferation and directs the acquisition of a neuronal phenotype by these cells. Not unexpectedly, there are specific changes in cellular phosphoproteins associated with differentiating events as demonstrated by the increased abundance of the ^{32}P-labeled microtubule-associated protein MAP-1 (18).

The results here clarify several aspects of hormone action on PC12 cells. It is clear that both NGF and EGF produce stable alterations in these cells which can be detected using a cell-free system. The molecular events responsible for the enhanced labeling of cytoskeletal proteins in situ are initiated immediately on binding of hormone to its receptor and are a transient consequence of hormone action. Whether these changes are directly involved in the generation of the cellular responses to the hormones, or are linked to processing of the hormone by common mechanisms, is presently under investigation.

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