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Detection of Nerve Growth Factor and Epidermal Growth Factor-Regulated Protein Kinases in PC12 Cells with Synthetic Peptide Substrates

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SUMMARY

Synthetic peptide substrates specific for cAMP-dependent protein kinase, protein kinase C, ribosomal S6 kinase, and Ca^{2+}/calmodulin-dependent protein kinases were used to monitor regulation of these protein kinases in digitonin-permeabilized PC12 cells following treatment with nerve growth factor (NGF) and epidermal growth factor (EGF). cAMP-dependent protein kinase was not activated by NGF and EGF. In addition, neither the Ca^{2+}/calmodulin-dependent nor -independent activity of a protein kinase similar to Ca^{2+}/calmodulin kinase II was affected by growth factor treatment. However, protein kinase C was rapidly and transiently activated and ribosomal S6 kinase activity was persistently elevated. Maximal protein kinase C activity was observed after 2 to 5 min of treatment and, subsequently, returned to control levels within 30 to 40 min. In contrast, S6 kinase activity was maximal within 15 min of NGF and EGF addition and was stably maintained for at least 24 hr. In addition to protein kinase C and S6 kinase, NGF and EGF regulated a protein kinase that was maximally elevated after 15 to 30 min and returned to control levels within 3 to 5 hr. This kinase (~100 kDa) failed to bind to a calmodulin affinity column and eluted from a cation exchange column as a single major species that was distinct from S6 kinase activity, which eluted as multiple peaks. The findings indicate that at least three protein kinases are rapidly activated in PC12 cells following treatment with NGF and EGF. The distinct durations of activation of each kinase implicates significantly different roles for each in growth factor signalling in PC12 cells.

Protein serine/threonine kinases are implicated in the transmission of growth factor-generated signals from the plasma membrane to specific intracellular target proteins (1). In PC12 pheochromocytoma cells, where NGF induces neuronal differentiation and EGF elicits a mitogenic response (2, 3), proteins whose levels of phosphorylation increase following binding of NGF or EGF include tyrosine hydroxylase (4, 5), ribosomal protein S6 (4, 6), synapsin 1 (7), histone H1 (4), and an unidentified cytoskeleton-associated protein (8). Several protein kinases have been proposed to be involved in the actions of NGF and EGF. Based on observations that extracellularly applied cyclic nucleotides and phorbol esters mimic some of the NGF- and EGF-induced phosphorylation events, workers have proposed roles for cAMP-dependent protein kinase and protein kinase C in growth factor activation of specific regulatory pathways (4, 9–11). Furthermore, data demonstrating increased levels of inositol phosphates (12) and Ca^{2+} (13) in growth factor-treated PC12 cells suggest that Ca^{2+}/calmodulin-dependent protein kinases might play a role as well. However, specific serine/threonine protein kinases regulated by NGF and EGF are still largely undefined.

To directly assess the activity of different protein kinases in NGF- and EGF-treated PC12 cells, we have employed synthetic peptides that serve as specific protein kinase substrates and inhibitors. By permeabilizing PC12 cells with digitonin after growth factor treatment, these peptides and [γ-^{32}P]ATP can be introduced into the cells for selective assay of various kinases.

Using this strategy, we have assayed the activity of cAMP-dependent protein kinase, protein kinase C, ribosomal protein S6 kinase, and a Ca^{2+}/calmodulin-dependent protein kinase that is highly similar to Ca^{2+}/calmodulin kinase II. Whereas cAMP-dependent protein kinase and Ca^{2+}/calmodulin kinase II do not appear to be activated following growth factor treatment, protein kinase C is rapidly and transiently activated and ribosomal S6 kinase is persistently activated. In addition, we describe an NGF- and EGF-regulated protein kinase that is distinct from protein kinase C and S6 kinase on the basis of

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ABBREVIATIONS: NGF, nerve growth factor; EGF, epidermal growth factor; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethyleneglycol bis (β-aminoethyl ether)*N*,*N*,*N'*,*N'*-tetraacetic acid; TPA, tetradecanoylphorbol acetate; PMSF, phenylmethanesulfonylfluoride, peptides are abbreviated with the single letter codes for amino acids.

chromatographic behavior, substrate specificity, and kinetics of activation. The findings indicate that, minimally, at least three distinct protein kinases are activated in PC12 cells within minutes after treatment with NGF or EGF. The onset and duration of activation for each kinase, however, is distinctly different, even in the continuous presence of growth factor. The results implicate significantly different roles for each kinase in growth factor signalling in PC12 cells.

Experimental Procedures

Cell culture. PC12 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, 5% Opti-MEM I (GIBCO, Grand Island, NY), 100 units/ml penicillin, and 100 μg/ml streptomycin. For assay of protein kinases in permeabilized cells, PC12 cells were seeded into poly-L-lysine-coated 96-well microtiter plates at a density of 10,000 to 25,000 cells/well. The cells were maintained in regular growth medium for 1 to 3 days before experiments.

Assay of protein kinases in permeabilized cells. Growth medium was aspirated and the cells were rinsed with 200 μl of a buffered salt solution consisting of 137 mM NaCl, 5.4 mM KCl, 0.3 mM sodium phosphate, 0.4 mM potassium phosphate, 1 mM calcium chloride, 1 mg/ml glucose, and 20 mM HEPES (pH 7.2 at 30°). The cells were incubated for indicated times in 40 μl of the buffered salt solution supplemented with hormones. To permeabilize the cells and initiate protein kinase assays, hormone solutions were aspirated and replaced with 40 μl of the buffered salt solution lacking calcium chloride and supplemented with 50 μg/ml digitonin, 10 mM $MgCl_2$, 25 mM β-glycerophosphate, 100 μM [γ-^{32}P]ATP (~5000 cpm/pmol), and peptides. Further additions for assay of specific kinases were as follows: cAMP-dependent protein kinase, 100 μM Kemptide (14), 0.5 mM EGTA with or without 3 μM cAMP or 25 μg/ml IP-20 (TTYADFIASGRTGRRNAIHD; Ref. 15); protein kinase C, 300 μM KRTLRR (16), 5 mM EGTA, 1 mM $CaCl_2$ (~100 nM free Ca^{2+}); ribosomal S6 kinase, 250 μM RRLSSLRA (17), 0.5 mM EGTA, 10 mM NaF, 20 μM sodium vanadate, 25 μg/ml IP-20; Ca^{2+}/calmodulin kinases, 100 μM KKRPQRATSNVFS (18, 19), 10 mM NaF, 20 μM sodium vanadate, 25 μg/ml IP-20, 1 mM EGTA with or without 1.1 mM $CaCl_2$ and 10 μg/ml bovine brain calmodulin. Kinase reactions were allowed to proceed for 3 to 10 min at 30° and then terminated with 10 μl of 25% (w/v) trichloroacetic acid. Aliquots (45 μl) of the acidified reaction mixtures were spotted on 2 × 2 cm phosphocellulose strips (Whatman P-81) and phosphopeptides were quantified as previously described (20). The efficiency with which phosphopeptides in the trichloroacetic acid-treated assay mixtures bound to the phosphocellulose filters was 92%, compared with the efficiency obtained when reactions were terminated by simply spotting assay mixtures on the phosphocellulose filters and immersing them in the 75 mM phosphoric acid wash solution. Filters spotted with aliquots from wells treated with trichloroacetic acid before addition of kinase reaction mixture (blanks) retained less than 0.025% of the added radioactivity.

Preparation of cell-free extracts. PC12 cells (~5 × 10^6 cells/100-mm dish) were incubated with hormones in the buffered salt solution described above. The incubations were terminated by aspirating the buffer and lysing the cells in 0.5–1 ml of ice-cold buffer consisting of 100 mM NaCl, 25 mM β-glycerophosphate (pH 7.2), 10 mM NaF, 20 μM sodium vanadate, 0.1% Triton X-100, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 50 μM PMSF, 10 μg/ml leupeptin, and 0.2 trypsin inhibitor units/ml aprotinin. The cells were scraped from the dishes with a rubber policeman and vortexed briefly. The extract was cleared of nuclei and cell debris by a 1-min centrifugation in a microfuge and then centrifuged at 100,000 × *g* for 20 min at 4°.

Assay of cell-free extracts and column fractions for protein kinase activity. Aliquots (20 μl) of cell-free extracts or column fractions were mixed with 20 μl of various peptide kinase assay solutions yielding (final concentration) 25 mM β-glycerophosphate, 10 mM NaF, 20 μM sodium vanadate, 10 mM magnesium chloride, 100 μM [γ^{32}P]ATP (5000 cpm/pmol), and synthetic peptide substrates and kinase modulators as described above. After a 5–10-min incubation at 30°, the reaction was terminated with trichloroacetic acid and phosphopeptides were quantitated.

Miscellaneous procedures. Protein was dissolved in 0.2 N NaOH and assayed as described by Bradford (21). Glycogen phosphorylase *a* was assayed in the presence of 0.5 mM caffeine and total phosphorylase in the presence of 3 mM AMP (22).

Materials. Peptides were synthesized with an automated Applied Biosystems solid phase peptide synthesizer. Purity and quantification of the peptides were assessed by amino acid analysis. [γ^{32}P]ATP (~3000 Ci/mmol; Amersham, Arlington Heights, IL) was acidified with 10 mM phosphoric acid and centrifuged through a phosphocellulose spin-column to adsorb radioactive contaminants that elevated assay blanks. NGF (2.5 S) was purified from mouse submaxillary glands (23). EGF (receptor grade) was purchased from Collaborative Research (Bedford, MA) and bovine brain calmodulin and ionomycin (stored as a 5 mM stock in dimethylsulfoxide) were purchased from Calbiochem (La Jolla, CA).

Results

For specific analysis of protein kinases in PC12 cells, we utilized four synthetic peptides whose protein kinase substrate properties have been previously described. The peptide LRRASLG, known as Kemptide, is phosphorylated by cAMP-dependent protein kinase (14), whereas the peptide identical to the *N*-terminal 20 amino acids of the heat-stable inhibitor of cAMP-dependent protein kinase, referred to as IP-20, is a potent inhibitor of cAMP-dependent protein kinase (15). Thus, cAMP-dependent protein kinase activity was defined as the Kemptide kinase activity inhibited by IP-20. The peptide KRTLRR, based on the sequence surrounding a major protein kinase C phosphorylation site within the EGF receptor (16), was used as a substrate for protein kinase C. We have observed that KRTLRR peptide is not phosphorylated by cAMP-dependent or Ca^{2+}/calmodulin-dependent protein kinases in PC12 cells.[1] The ribosomal protein S6-derived peptide RRLSSLRA has been previously shown to be specific for mitogen-activated S6 kinase, provided that IP-20 and EGTA are included to inhibit cAMP-dependent protein kinase and protein kinase C, respectively (17). The peptide KKRPQRATSNVFS, referred to in this report as MLC peptide and corresponding to residues 11–23 of the chicken gizzard myosin light chain molecule, was used as a substrate for smooth muscle myosin light chain kinase (18) and Ca^{2+}/calmodulin kinase II (19). Although MLC peptide is a poor substrate for cAMP-dependent protein kinase,[1] IP-20 was included in kinase assays to completely inhibit residual cAMP-dependent kinase activity. Due to the lengthy list of protein kinases identified to date, it is difficult to exhaustively examine the kinase specificity of each peptide substrate. However, by assaying the cells and extracts under the various conditions described below, we have been able to discriminate the activities of five distinct protein kinases.

Assay of peptide kinases in permeabilized cells. In order to rapidly analyze protein kinase activity after exposure of PC12 cells to NGF or EGF, we developed an assay in which the cells were permeabilized with digitonin in the presence of [γ^{32}P]ATP and a synthetic peptide substrate. Cell permeabilization allowed rapid detection of kinase stimulations using

[1] Heasley and Johnson, unpublished observation.

various peptide substrates and inhibitors under conditions that minimally perturb the cells. The data in Fig. 1A demonstrate the digitonin-dependent phosphorylation of Kemptide, KRTLRR peptide, and MLC peptide using attached PC12 cells. Maximal Kemptide, KRTLRR peptide, and MLC peptide kinase activity regulated by cAMP, TPA, and Ca^{2+} and calmodulin, respectively, was observed when PC12 cells were permeabilized with 50 to 100 μg/ml digitonin. This concentration range of digitonin did not detach the PC12 cells or significantly alter cell morphology.

The data in Fig. 1B demonstrate that phosphorylation of the MLC peptide measured in the presence or absence of Ca^{2+} and calmodulin proceeded without a detectable lag, suggesting that permeabilization was essentially instantaneous. Furthermore, the kinase assay was linear for at least 20 min. Similar assay time courses were observed for cAMP-stimulated Kemptide phosphorylation and TPA-enhanced KRTLRR phosphorylation (data not shown). In the absence of peptide substrates, the amount of radioactivity that bound to the phosphocellulose filters represented only 3–7% of the peptide kinase activity and was unaffected by inclusion of Ca^{2+} and calmodulin (Fig. 1B) or cAMP and phorbol esters (data not shown), demonstrating that the contribution of phosphorylated cellular proteins to the radioactivity on the filters was minimal.

Regulation of peptide kinase activity by growth factors. Having established the conditions for assaying protein kinase activity in permeabilized PC12 cells, we measured phosphorylation of each peptide substrate following treatment of cells for various times with NGF and EGF.

NGF and EGF increased the rate of phosphorylation of the peptides specific for protein kinase C, S6 kinase, and Ca^{2+}/calmodulin-dependent protein kinases (Fig. 2 A–C). In contrast the activity of cAMP-dependent protein kinase was unaffected after treatment with NGF or EGF (Table 1). Pretreatment of cells with 2-chloroadenosine, an adenosine analog that is known to stimulate adenylate cyclase in PC12 cells (24), increased the activity of cAMP-dependent protein kinase ~3-fold (Table 1). These data strongly argue that cAMP-dependent protein kinase plays little or no direct role in NGF or EGF signalling in PC12 cells.

Phosphorylation of the peptide KRTLRR was rapidly and transiently increased in cells pretreated with NGF and EGF (Fig. 2A). Maximal activation was observed within 2 to 5 minutes of growth factor treatment, and subsequently, returned to control levels by 30 to 40 min. In addition, the phorbol ester TPA produced a marked and prolonged elevation of KRTLRR kinase activity. Thus, the permeabilized cell assay using the peptide KRTLRR provides a direct demonstration of NGF- and EGF-regulated protein kinase C activity. A detailed report of this protein kinase C assay will be presented elsewhere.

In contrast to the transient activation of protein kinase C (Fig. 2A), S6 peptide kinase activity, measured in the presence of IP-20 and EGTA, to inhibit cAMP-dependent protein kinase and protein kinase C was maximal within 15 min of NGF and EGF addition and was stably maintained for the 30-min assay period (Fig. 2B). These data suggest that ribosomal S6 kinase was rapidly regulated by NGF and EGF, a finding that has also been noted by Blenis and Erikson (6).

In addition to protein kinase C and S6 kinase, NGF and EGF also induced a 2- to 3-fold increase in the rate of MLC peptide phosphorylation measured in the presence of EGTA (Fig. 2C). MLC peptide kinase activity that was independent of Ca^{2+} and calmodulin was also enhanced after treatment of the cells with the Ca^{2+} ionophore ionomycin (Fig. 2C). However, the MLC peptide kinase activity that was detectable when Ca^{2+} and calmodulin were included in the assay was unaffected by either growth factors or ionomycin.

NGF and EGF stimulated the Ca^{2+}/calmodulin-independent MLC peptide kinase activity in a concentration-dependent manner (Fig. 3). Again, note the lack of effect of NGF and EGF on the MLC peptide kinase activity that was strictly dependent upon the presence of Ca^{2+} and calmodulin in the assay. Stimulation of the Ca^{2+}/calmodulin-independent MLC peptide kinase activity, as well as protein kinase C and S6 kinase, by NGF and EGF required pretreatment of intact PC12 cells, inasmuch as addition of the growth factors directly to the kinase assay was ineffective (data not shown).

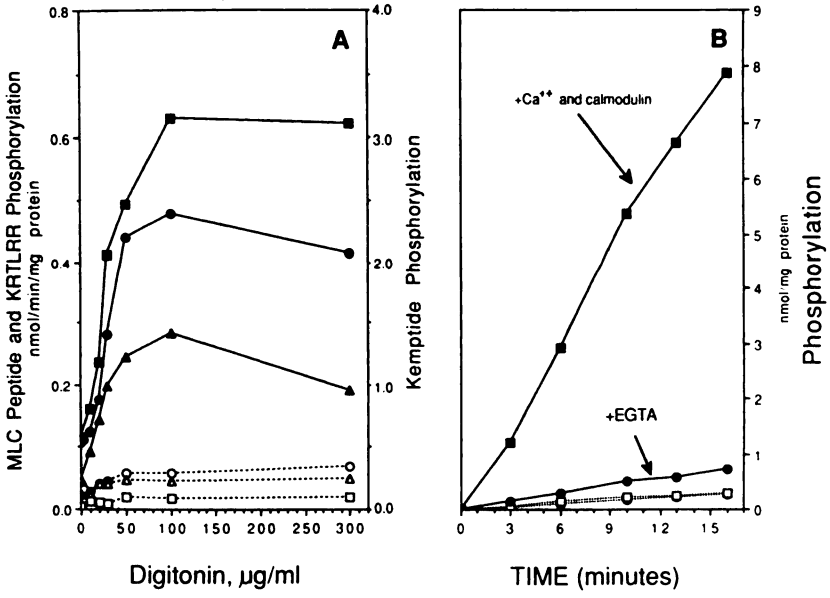

Fig. 1. Characterization of the digitonin dependence and time course of synthetic peptide phosphorylation in permeabilized PC12 cells. A, Peptide phosphorylation was assayed in the presence of increasing concentrations of digitonin. Kemptide phosphorylation was assayed with (■) or without (□) 3 μM cAMP and MLC peptide phosphorylation was assayed with (●) or without (○) 100 μM excess Ca^{2+} and 10 μg/ml calmodulin. KRTLRR peptide phosphorylation was assayed in control cells (△) or cells pretreated for 10 min with 1 μM TPA (▲). B, Kinase activity was measured in PC12 cells permeabilized with 50 μg/ml digitonin with (●, ■) or without (○, □) 100 μM MLC peptide in the presence (□, ■) or absence (○, ●) of Ca^{2+} and calmodulin.

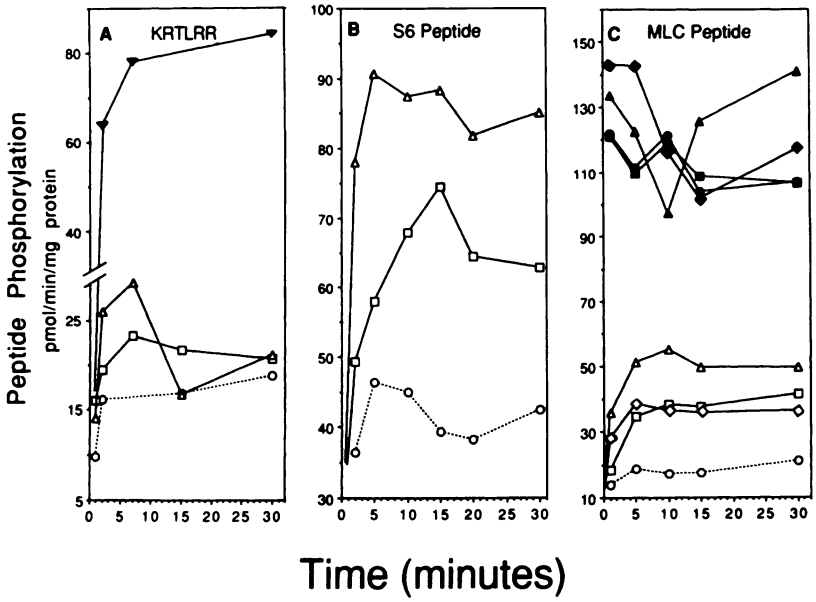

Fig. 2. Time course of activation of KRTLRR, S6, and MLC peptide kinases by NGF and EGF. PC12 cells were treated for 1–30 min at 30° without (○) or with 100 ng/ml NGF (□, ■), 10 ng/ml EGF (△, ▲), 1 μM TPA (▼), or 5 μM ionomycin (◇, ◆). The cells were permeabilized and assayed for kinase activity with 300 μM KRTLRR peptide (A), 250 μM S6 peptide (B), or 100 μM MLC peptide in the absence (○, □, △, ◇) or presence (●, ■, ▲, ◆) of Ca^{2+} and calmodulin. The *data points* in the experiment shown are the means of duplicate determinations and are representative of three independent experiments. Note that the ordinates are non-zero.

TABLE 1

Effects of NGF, EGF, and 2-chloroadenosine on cyclic AMP-dependent protein kinase and glycogen phosphorylase

Cyclic AMP-dependent protein kinase was assayed as described in Experimental Procedures. Basal cAMP-dependent protein kinase activity was defined as the Kemptide kinase activity inhibited by 25 μg/ml IP-20 whereas total cAMP-dependent kinase was defined as that activity stimulated by 3 μM cAMP but inhibited by 25 μg/ml IP-20. The data are the average ± standard error of the means of duplicate determinations from three or four independent experiments. For analysis of glycogen phosphorylase *a* activity, PC12 cells (~ 1 × 10^6 cells/60-mm dish) were incubated for 10 min at 30° in 3 ml of the HEPES-buffered salt solution (see Experimental Procedures) with or without hormones and growth factors. After the buffer was aspirated, the cells were quick-frozen in liquid nitrogen, homogenized, and assayed for total phosphorylase and phosphorylase *a*. The results, shown as percentage phosphorylase *a*, are the means of duplicate determinations from two independent experiments.

Treatment	cAMP-Dependent protein kinase			Glycogen phosphorylase *a*
	Basal	+cAMP	−cAMP/+cAMP	
	nmol/min/mg of protein			%
Control	0.19 ± 0.02	0.67 ± 0.06	0.29 ± 0.03	13.8, 47.4
NGF (100 ng/ml)	0.19 ± 0.02	0.67 ± 0.06	0.28 ± 0.03	28.9, 54.6
EGF (10 ng/ml)	0.22 ± 0.03	0.77 ± 0.17	0.29 ± 0.03	25.1, 67.8
2-Chloroadenosine (10 μM)	0.58 ± 0.10	0.74 ± 0.12	0.79 ± 0.05	58.4, 80.5

Cell-free assay and chromatographic properties of hormone-stimulated MLC peptide kinase activity. Combined, the preceeding data indicate that NGF and EGF rapidly increased the activity of protein kinase C, S6 kinase, and a MLC peptide kinase that can be measured in the absence of Ca^{2+} and calmodulin. This latter result, also observed in response to ionomycin, could be explained by a mechanism analogous to that described *in vitro* for Ca^{2+}/calmodulin kinase II, whereby the kinase autophosphorylates and becomes independent of Ca^{2+} and calmodulin for activity (25, 26). Alternatively, the growth factors could activate a protein kinase that was independent of Ca^{2+} and calmodulin before treatment. To distinguish between these possibilities, we turned to a cell-free system in order to characterize the MLC peptide kinase activity.

The majority (~75%) of the Ca^{2+}/calmodulin-dependent MLC peptide kinase activity, as well as the Ca^{2+}/calmodulin-independent MLC peptide kinase activity regulated by growth factors, was recovered in the soluble fraction of PC12 cells (Table 2). Treatment of cells with growth factors and ionomycin increased the soluble Ca^{2+}/calmodulin-independent MLC peptide kinase activity 2- to 4-fold and, to a lesser extent (~2-fold), the activity present in the particulate fraction. However, total Ca^{2+}/calmodulin-dependent MLC peptide kinase activity in either soluble or particulate fractions of growth factor-treated PC12 cells was not different from activity in extracts from control cells (Table 2).

Gel exclusion analysis of the soluble fractions of control and growth factor-treated cells showed that the Ca^{2+}/calmodulin-dependent MLC peptide kinase activity eluted almost exclusively from a Sephacryl S-300 column with a Stokes radius that corresponds to a globular protein of molecular mass greater than 500 kDa (Fig. 4A). A minor peak of activity eluted as a protein of ~100 kDa. In addition to the MLC peptide, the high molecular weight Ca^{2+}/calmodulin-dependent kinase also readily phosphorylated synapsin I (data not shown). Thus, based on its large molecular size (>500 kDa) and its ability to phosphorylate both MLC peptide and synapsin I, this protein kinase is similar to Ca^{2+}/calmodulin kinase II (27).

The Ca^{2+}/calmodulin-independent MLC peptide kinase activity regulated by growth factors and ionomycin was clearly resolved from the peak of Ca^{2+}/calmodulin kinase II, eluting with a Stokes radius corresponding to a globular protein of approximately 100 kDa (Fig. 4B). Thus, a Ca^{2+}/calmodulin-independent form of Ca^{2+}/calmodulin kinase II similar to that described *in vitro* (25, 26) was not detected in extracts from PC12 cells treated with growth factors or ionomycin. Besides resolution by molecular size, the growth factor-regulated MLC peptide kinase activity failed to bind to a column of calmodulin-agarose under conditions in which Ca^{2+}/calmodulin-dependent

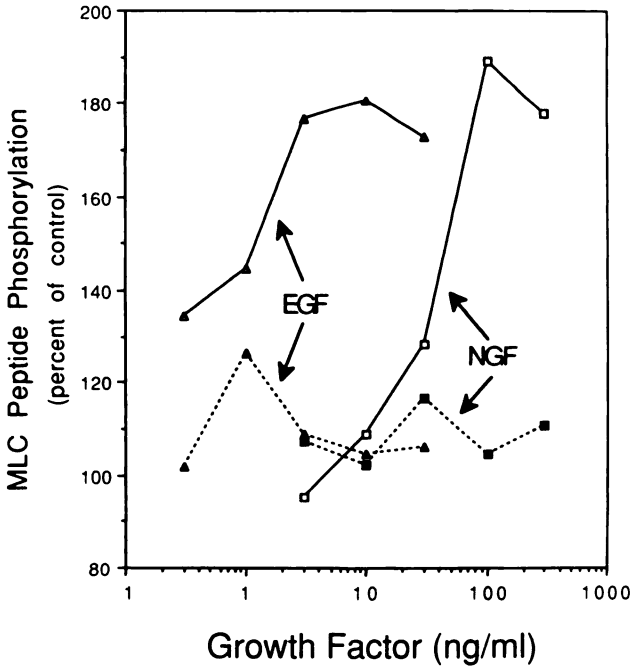

Fig. 3. Concentration dependence for stimulation of Ca^{2+}/calmodulin-independent MLC peptide kinase activity by NGF and EGF. PC12 cells were incubated for 10 min at 30° with various concentration of NGF (□, ■) or EGF (△, ▲) and then assayed for MLC peptide kinase activity in the absence (□, △) or presence (■, ▲) of Ca^{2+} and calmodulin. Control activity in the absence and presence of Ca^{2+} and calmodulin was 34.0 and 153.2 pmol/min/mg, respectively. The data are representative of two independent experiments.

TABLE 2

Effects of NGF, EGF, and ionomycin on Ca^{2+}/calmodulin-dependent and -independent MLC peptide kinase activity measured in broken cell fractions

PC12 cells (~5 × 10^6 cells/100-mm dish) were incubated at 30° for 10 min in 7 ml of buffered salt solution with or without growth factors or ionophore. Cell-free fractions were prepared as described in Experimental Procedures and diluted 5-fold into a buffer consisting of 20 mM β-glycerophosphate (pH 7.0), 90 mM NaCl, 10 mM NaF, 20 μM sodium vanadate, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol. MLC peptide kinase activity was measured in the absence or presence of 100 μM excess $CaCl_2$ plus 10 μg/ml bovine brain calmodulin. The data are means ± standard errors of four independent experiments.

Treatment	Cellular fraction	MLC peptide phosphorylation	
		Ca^{2+}/Calmodulin-independent	Ca^{2+}/Calmodulin-dependent
		nmol/min/mg	
Control	Soluble	0.11 ± 0.01	1.59 ± 0.27
	Particulate	0.11 ± 0.01	0.74 ± 0.14
NGF (100 ng/ml)	Soluble	0.31 ± 0.02[a]	1.67 ± 0.32
	Particulate	0.18 ± 0.03	0.64 ± 0.15
EGF (10 ng/ml)	Soluble	0.40 ± 0.05[a]	1.47 ± 0.28
	Particulate	0.19 ± 0.02	0.78 ± 0.17
Ionomycin (5 μM)	Soluble	0.19 ± 0.03[b]	1.59 ± 0.26
	Particulate	0.19 ± 0.05	0.56 ± 0.23

[a] $p < 0.001$ compared with control.
[b] $p < 0.005$.

MLC peptide kinase activity was completely retained (Fig. 4, C and D). Thus, the NGF- and EGF-regulated MLC peptide kinase is clearly Ca^{2+}- and calmodulin-independent.

As an indicator of the activity of another Ca^{2+}-regulated protein kinase, we measured the activity of glycogen phosphorylase, an enzyme whose physiologic regulation appears to occur solely by way of phosphorylase kinase (28). Phosphorylase kinase, in turn, can be activated either by Ca^{2+} binding to the δ-subunit (calmodulin) or through phosphorylation by cAMP-dependent protein kinase (1). Treatment of attached PC12 cells with NGF and EGF significantly increased the activity ratio of phosphorylase, 1.4- and 1.5-fold, respectively (Table 1). Because this activation of glycogen phosphorylase occurred without an increase in the activity of cAMP-dependent protein kinase (Table 1), the data imply that phosphorylase kinase was regulated through the Ca^{2+}-dependent mechanism. 2-Chloroadenosine, an agent that clearly stimulated cAMP-dependent protein kinase (Table 1), activated glycogen phosphorylase 2.3-fold. Thus, NGF and EGF apparently activated phosphorylase kinase and glycogen phosphorylase in a cAMP-independent manner but did not induce persistent activation of Ca^{2+}/calmodulin kinase II.

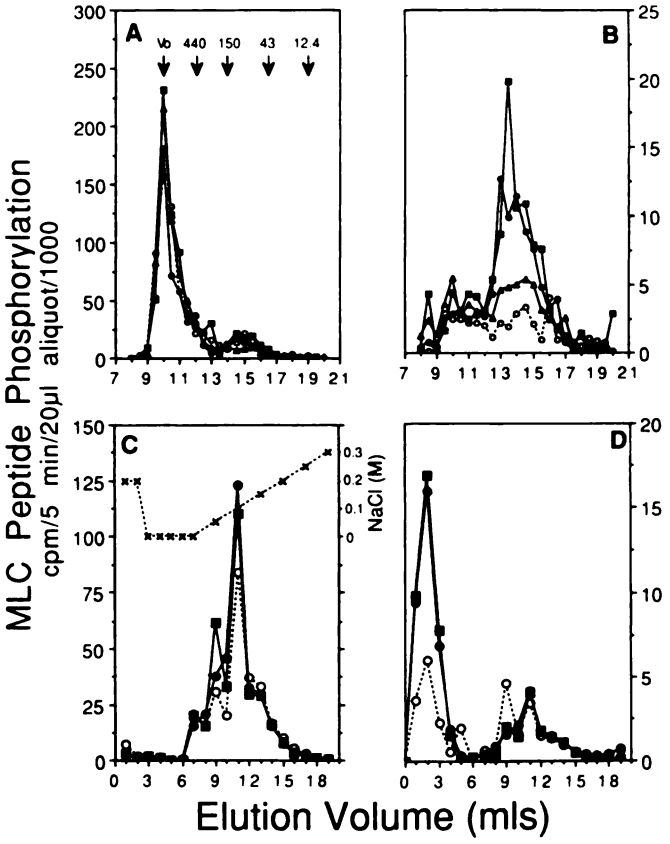

Fig. 4. Sephacryl S-300 and calmodulin-agarose chromatography of MLC peptide kinase activities in soluble extracts from growth factor-treated PC12 cells. A and B, Soluble fractions (0.5 ml, 500 μg of protein) from PC12 cells incubated for 10 min at 30° without (○) or with NGF (●), EGF (■), or ionomycin (△) were applied to a sephacryl S-300 column (25 × 0.9 cm) equilibrated in 20 mM β-glycerophosphate (pH 7.0), 10 mM NaF, 20 μM sodium vanadate, 90 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol. The column was eluted at a flow rate of ~0.2 ml/min and 0.5-ml fractions were collected and assayed for MLC peptide kinase activity in the presence (A) or absence (B) of Ca^{2+} and calmodulin. Elution positions of ferritin, alcohol dehydrogenase, ovalbumin, and cytochrome *c* are indicated in kilodaltons. The void volume (V_o) was estimated with blue dextran. C and D, soluble fractions (0.9 ml, 500 μg of protein) from treated and untreated cells were adjusted to 100 μM excess $CaCl_2$ and 0.2 M NaCl and applied without flow for 10 min to 1 ml calmodulin-agarose columns (Sigma) that had been equilibrated in 25 mM β-glycerophosphate (pH 7.5), 10 mM NaF, 20 μM sodium vanadate, 0.2 M NaCl, 0.2 mM $CaCl_2$, 1 mM dithiothreitol, and 50 μg/ml PMSF (Buffer A). The columns were washed with 1 ml of Buffer A and 3 ml of buffer A without NaCl and then eluted with a linear gradient of 0–300 mM NaCl in Buffer A in which 2 mM EGTA was substituted for $CaCl_2$. Fractions (1 ml) were collected and assayed for MLC peptide kinase activity in the presence (C) or absence (D) of Ca^{2+} and calmodulin.

Gel filtration chromatography of a soluble extract from NGF-treated PC12 cells on a Sephacryl S-300 column revealed that the MLC peptide and S6 peptide kinase activities essentially coeluted (Fig. 5). The similar gel exclusion profiles for the two peptide kinase activities suggested that the NGF- and EGF-regulated MLC peptide kinase activity was simply S6 kinase. However, partial resolution of the MLC peptide kinase and S6 peptide kinase activities was achieved following Protein Pak SP-5PW cation exchange chromatography (Fig. 6). S6 peptide kinase activity in extracts from PC12 cells treated for 15 min with NGF eluted as multiple peaks. The activity eluting between 160 and 200 mM NaCl appeared to chromatograph as two partially resolved peaks (Fig. 6) whereas the activity eluting at 320 mM NaCl consistently chromatographed as a single peak. In contrast, MLC peptide kinase activity eluted almost exclusively at 160 mM NaCl, with a minor peak of activity at 320 mM NaCl (Fig. 6). Although the major peak of MLC peptide kinase activity and the heterogeneous peak of S6 peptide kinase activity eluted at similar salt concentrations on the cation exchange column (Fig. 6), the MLC peptide kinase activity consistently eluted earlier than the S6 peptide kinase activity, suggesting that the two activities represented distinct protein kinases. Cation exchange chromatography of extracts from EGF-treated PC12 cells yielded results that were essentially identical to those observed with NGF (data not shown).

An extended time course of action of NGF and EGF revealed significantly different durations of activation of the kinases that phosphorylate the MLC peptide and S6 peptide (Fig. 7). Growth factor-activated S6 peptide kinase was maximal after 15 min of treatment. Following a partial decline (20–50%), S6

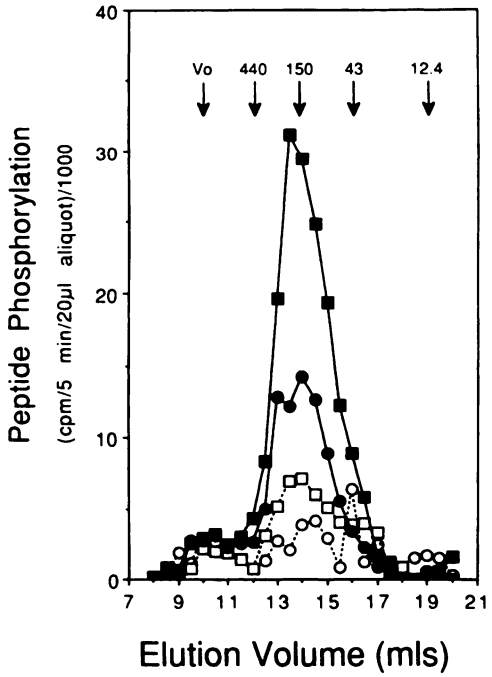

Fig. 5. Sephacryl S-300 chromatography of MLC peptide and S6 peptide kinases in soluble fractions of NGF-treated PC12 cells. Soluble fractions (0.5 ml, 500 µg of protein) from PC12 cells treated without (○, □) or with (●, ■) 100 ng/ml NGF for 10 min at 30° were chromatographed as described in Fig. 4. Aliquots (20 µl) of fractions were assayed for kinase activity with 250 µM S6 peptide (□, ■) or 100 µM MLC peptide (○, ●) in the presence of 1 mM EGTA. Assay of S6 kinase activity in the presence of Ca^{2+} and calmodulin revealed that the S6 peptide was not a substrate for Ca^{2+}/calmodulin kinase II (data not shown).

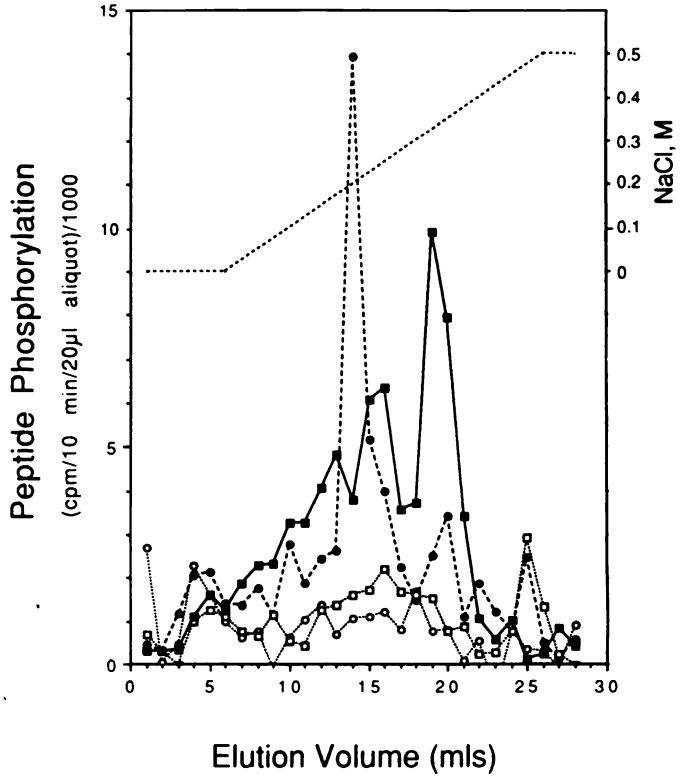

Fig. 6. Cation-exchange chromatography of S6 peptide and MLC peptide kinase activities. Lysates (0.4 ml, 600 µg of protein) from control PC12 cells (○, □) or cells incubated at 37° for 15 min with 100 ng/ml NGF (●, ■) in complete growth medium were diluted to 1 ml with 25 mM β-glycerophosphate (pH 7.5), 10 mM NaF, 20 µM sodium vanadate, 1 mM EGTA, 1 mM dithiothreitol (Buffer B) and applied to a Protein Pak SP 5PW column (Waters; 7.5-mm × 7.5-cm) equilibrated in Buffer B. The column was developed at 1 ml/min with a linear gradient of 0–500 mM NaCl in Buffer B. Fractions (1 ml) were collected and aliquots (20 µl) were assayed for MLC peptide (○, ●) and S6 peptide (□, ■) kinase activities. Similar results were obtained in four independent experiments. Phospholipid- and diacylglycerol-dependent KRTLRR kinase activity was recovered entirely in the flow-through fractions (4–8) whereas Ca^{2+}/calmodulin-dependent MLC peptide kinase activity distributed equally in the flow-through and at 250 mM NaCl (data not shown).

peptide kinase activity remained stably elevated, compared with control cells, for at least 24 hr. In contrast, growth factor-regulated MLC peptide kinase activity was maximal after 15 to 30 min of growth factor exposure, and subsequently, returned completely to control levels within 3 to 5 hr, even in the continuous presence of growth factor. Thus, NGF and EGF transiently activated the MLC peptide kinase activity, although for a significantly longer duration than that of protein kinase C, which returns to control within ~30 min. S6 kinase, in contrast, is persistently activated. These data provide additional evidence to those in Fig. 6 that the MLC peptide kinase activity and S6 peptide kinase activity represent distinct protein kinases.

Discussion

The data in this report provide direct evidence for the regulation by NGF and EGF of protein kinase C, S6 kinase, phosphorylase kinase, and a previously unreported protein kinase that is selectively detected with the MLC peptide. In addition, this report defines for the first time the temporal relationship of the regulation of these protein kinases by NGF and EGF; S6

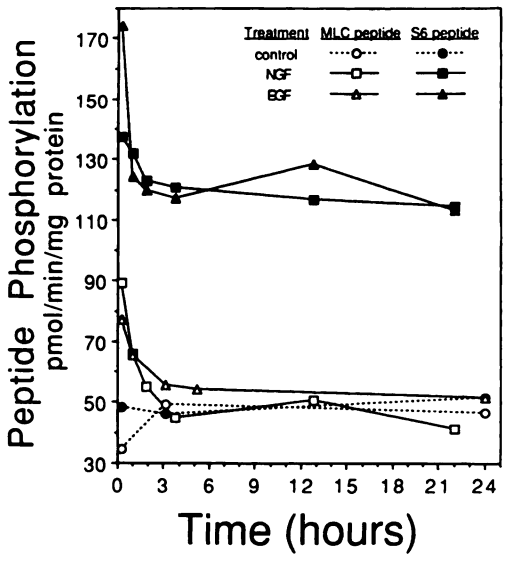

Fig. 7. Kinetics of activation of MLC peptide and S6 peptide kinases by NGF and EGF. PC12 cells were incubated (37°) for 15 min to 24 hr in complete growth medium with or without NGF and EGF. To initiate kinase assays, the growth medium was aspirated and the cells were permeabilized and assayed for S6 peptide kinase activity and MLC peptide kinase activity in the presence of EGTA and IP-20 peptide. The data are representative of three independent experiments.

kinase is persistently activated whereas protein kinase C and the MLC peptide kinase are transiently activated.

The regulation In PC12 cells of protein kinases that phosphorylate ribosomal protein S6 has been previously reported (6, 29). Similar to our finding, Blenis and Erikson (6) observed that S6 kinase activity was resolved into two peaks on a Mono S cation exchange column. In contrast, serum-stimulated S6 kinase from chicken embryo fibroblasts (6) and Swiss 3T3 cells (30) elutes as a single peak of activity at ~0.2 M NaCl on Mono S cation exchange columns. It is presently unknown whether the multiple peaks of S6 kinase activity observed in PC12 cells represent different forms of the same enzyme or, perhaps, distinct kinases.

Using the MLC-derived peptide, we have detected a growth factor-regulated kinase that is activated more slowly than protein kinase C, but whose enhanced activity is maintained for 2 to 3 times longer. This kinase activity is distinct from the major calmodulin-dependent protein kinase in PC12 cells, on the basis of molecular size and its failure to bind to a calmodulin affinity column. Although the MLC peptide kinase elutes similarly to the form of S6 kinase that elutes at lower salt concentrations on the cation exchange column (Fig. 6), it appears to be distinct, based on the kinetics of activation by NGF and EGF (Fig. 7). Recently, Yu *et al.* (31) described a protein kinase isolated from insulin-treated rat adipocytes that phosphorylated the MLC peptide, but not ribosomal protein S6. Although the duration of activation of the kinase was not reported, the adipocyte-derived kinase bears some resemblance to the NGF- and EGF-regulated MLC peptide kinase from PC12 cells and may represent another major growth factor-regulated kinase pathway in addition to S6 kinase.

The information provided in this report on the temporal relationship of protein kinase activation allows us to begin to relate various protein kinases to previously characterized growth factor-elicited responses in PC12 cells. A well characterized early response of PC12 cells to NGF and EGF in which protein kinases may play a role is the transiently increased transcription of c-*fos*, c-*myc*, β-actin, and ornithine decarboxylase genes (32). Increased c-*fos* and β-actin transcription is detectable within 5 min of growth factor treatment, whereas c-*myc* and ornithine decarboxylase increase later, at 1–2 hr. The fact that multiple kinases are activated in PC12 cells in response to growth factors requires that each kinase be carefully evaluated to define its putative role in transcriptional regulation of early and late response genes. Our data demonstrating the transient activation of protein kinase C and the MLC peptide kinase raise the possibility that one or more of these kinases may serve as a switch or relay to transiently regulate the transcription of these genes.

Another early growth factor-elicited response observed in PC12 cells is enhanced phosphorylation of tyrosine hydroxylase (4, 5). Maximal phosphorylation of tyrosine hydroxylase in response to NGF is achieved within ~10–15 min and, apparently, is persistently maintained in the presence of growth factor (4). Using *in vivo* labeling and peptide mapping, Cremins *et al.* (11) have suggested that cAMP-dependent protein kinase and protein kinase C mediate NGF-stimulated tyrosine hydroxylase phosphorylation. However, our data clearly demonstrate that NGF and EGF fail to activate cAMP-dependent protein kinase in PC12 cells. Furthermore, the rather slow and persistent time course with which tyrosine hydroxylase is phosphorylated argues against a role of protein kinase C as the major phosphorylation pathway, because protein kinase C is rapidly and transiently activated (Fig. 2A). Instead S6 kinase or a protein kinase with similar kinetics of activation seems a more likely candidate for the growth factor-regulated protein kinase that maintains persistent phosphorylation of tyrosine hydroxylase. A recent observation by Blenis *et al.* (33) that purified chicken embryo S6 kinase and both peaks of S6 kinase partially purified from PC12 cells phosphorylate tyrosine hydroxylase *in vitro* provides support for this hypothesis. Also, Rowland *et al.* (34) have used tyrosine hydroxylase as a substrate to detect, in lysates from NGF-treated PC12 cells, a protein kinase that is distinct from cAMP-dependent protein kinase, protein kinase C, Ca^{2+}/calmodulin-dependent kinases, and casein kinase II but displays some features of ribosomal S6 kinase.

Despite exerting essentially opposite actions on PC12 cells, NGF and EGF initiate a similar set of biochemical responses. We have recently reported that constitutive expression of an exogenous c-*myc* gene in PC12 cells converts the action of NGF from a differentiation response to a mitogenic response (35). The NGF receptor binding properties and induction of ornithine decarboxylase were normal in c-*myc*-expressing clones, indicating that early signalling events initiated by NGF were similar to those in wild-type cells. This observation combined with our present findings that EGF, which is mitogenic in PC12 cells (3), and NGF activate the same measurable serine/threonine protein kinases suggest that the set of early signals that initiate PC12 cell growth and differentiation largely overlap.

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