Regulation of Protein Kinase C by Nerve Growth Factor, Epidermal Growth Factor, and Phorbol Esters in PC12 Pheochromocytoma Cells*

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We have used a permeabilized cell assay and a synthetic peptide substrate (KRTLRR) to specifically monitor the activity of protein kinase C in PC12 cells preincubated with nerve growth factor (NGF), epidermal growth factor (EGF), or phorbol esters. Pretreatment of PC12 cells with 1 μM 12-O-tetradecanoylphorbol 13-acetate or 1 μM phorbol dibutyrate stimulated the rate of KRTLRR peptide phosphorylation 4.8- and 2.6-fold, respectively. Furthermore, pretreatment of cells with NGF or EGF transiently increased the KRTLRR peptide kinase activity. Peak stimulations of KRTLRR peptide kinase (1.3–2-fold) were observed after 1–5 min of growth factor treatment and returned to control levels within 15–20 min. The KRTLRR peptide kinase activity fulfilled two criteria of protein kinase C: A synthetic peptide inhibitor of protein kinase C inhibited both growth factor- and phorbol ester-stimulated KRTLRR peptide kinase activity. In addition, growth factors and phorbol esters failed to stimulate KRTLRR peptide kinase activity in cells rendered protein kinase C-deficient by long-term treatment with 1 μM 12-O-tetradecanoylphorbol 13-acetate. In contrast to the transient activation of protein kinase C, ribosomal S6 kinase, assayed with the synthetic peptide RRLSSLRA, was persistently activated by NGF and EGF. The findings indicate that protein kinase C serves as an early and transient signal in the molecular actions of NGF and EGF in PC12 cells.

The PC12 pheochromocytoma cell line is a useful system for studying molecular actions of NGF† and EGF. Exposure of PC12 cells to NGF initiates a program that leads to neuronal differentiation (1) while EGF exerts a modest mitogenic action (2). Despite the apparent difference in their cellular actions, the two growth factors initiate a similar set of early biochemical and cellular events including protein oncoprotein transcription (3), ion fluxes (4), and protein phosphorylation (5, 6) and dephosphorylation (7, 8).

In order to understand key signals generated upon binding of NGF and EGF to their respective cell surface receptors, we have begun to identify protein serine/threonine kinases that NGF and EGF acutely regulate in PC12 cells. Recently, we described a strategy whereby growth factor-treated PC12 cells are permeabilized with digitonin in the presence of [γ-32P]ATP and various synthetic peptide substrates in order to monitor specific kinase activities (9). Using this approach, three distinct NGF- and EGF-regulated kinase activities were detected using synthetic peptides derived from ribosomal protein S6, smooth muscle myosin light chain, and the EGF receptor. The S6 peptide kinase activity was very similar to the S6 kinase activity previously described in PC12 cell extracts (10), while the myosin light chain-derived peptide detected a distinct, apparently novel kinase that was unrelated to Ca2+/calmodulin-dependent kinases that also phosphorylate this peptide. The EGF receptor-depended peptide (KRTLRR), which contains a major protein kinase C phosphorylation site within the EGFR receptor (11), detected a second, distinct kinase activity that we proposed to be protein kinase C.

In this report, we have examined the specificity of the KRTLRR peptide and found it to be highly specific for protein kinase C. By using this peptide in the permeabilized cell assay, marked activation of protein kinase C in response to phorbol esters is detected while considerably more modest and transient stimulations of protein kinase C are observed following treatment of PC12 cells with NGF or EGF. The transient stimulation of protein kinase C contrasts with the persistent stimulation of S6 kinase observed in response to these growth factors. The results suggest that protein kinase C serves as an early, transient signal for both NGF and EGF action 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), 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained on Primaria dishes (Falcon) and were grown in poly-L-lysine-coated 96-well plates (10,000–25,000 cells/well) for experiments.

**Preparation of Cell-free Extracts**—Attached PC12 cells (~5 million/100-mm dish) were scraped from the dish in 1 ml of ice-cold buffer consisting of 100 mM NaCl, 25 mM β-glycerophosphate (pH 7.2), 10 mM NaF, 25 μM sodium vanadate, 0.1% Triton X-100, 10 mM magnesium chloride, 1 mM EGTA, 1 mM DTT, 50 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 0.2 unit/ml aprotinin. The cells were vortexed briefly, and the homogenate was cleaned of nuclei and cell debris by a 1-min centrifugation in a microcentrifuge. The supernatant was then centrifuged at 100,000 × g for 20 min at 4°C.

**Assay of Protein Kinases in Permeabilized Cells**—Growth medium was aspirated from the wells of the 96-well plates and the attached cells were rinsed with 100 μl of Dulbecco’s modified Eagle’s medium buffered with 20 mM HEPES (pH 7.2). The cells were incubated at 30°C in 40 μl of HEPES-buffered DME for indicated times with or without phorbol esters or growth factors. These solutions were then aspirated and replaced with 40 μl of a salt solution (137 mM NaCl, 5.4 mM KCl, 0.8 mM sodium phosphate, 0.4 mM potassium phosphate, 1 mg/ml glucose, and 20 mM HEPES (pH 7.2)) supplemented with

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‡50% NGF, nerve growth factor; diC8, sn-1,2-diacyl glycerol; DTT, dithiothreitol; EGF, epidermal growth factor; EGTA, N,N,N',N'-pentamethylenediamine-N,N'-bis(2'-hydroxyphenylboronic acid); TPA, 12-O-tetradecanoylphorbol 13-acetate; MLC, myosin light chain; peptides are abbreviated with the single letter amino acid code.
50 μg/ml digitonin, 10 mM magnesium chloride, 25 mM β-glycerophosphate, and 100 μM [γ-32P]ATP (3000 cpm/pmole). Further additions for specific assay of protein kinase C were 5 mM EGTA, 2.5 mM calcium chloride, and 300 μM KRTLRR peptide. Additional reagents for assay of ribosomal S6 kinase and MLC peptide kinase were 1 mM EGTA, 200 mM sodium vanadate, 25 μg/ml IP-20 (12), and either 500 μM RRLSSLR peptide (13) or 100 μM KKRQPRATSNVFS (MLC peptide, Ref. 14). Kinase reactions were terminated after a 10-min incubation at 30 °C with 10 μl of 25% (w/v) trichloroacetic acid. As described in detail elsewhere, with the cell densities, peptide, and ATP concentrations employed in these experiments, the rates of the peptide kinase activities were linear for 20-30 min (9). Aliquots (45 μl) of the acidified reaction mixtures were spotted on 2 x 2 cm phosphocellulose squares (Whatman P-81) and washed batchwise as previously described (15) in three changes (500 ml each) of 75 mM phosphoric acid and one change of 75 mM sodium phophate (pH 7.5). Due to their basicity, phosphorylated KRTLRR peptide, RRLSSLR peptide, and MLC peptide were retained on the phosphocellulose filter at neutral pH while a contaminant of the [γ-32P]ATP that bound to the filters at acidic pH was removed, thus lowering the assay blanks.

Miscellaneous Procedures—Protein was dissolved in 0.2 N NaOH and assayed as described by Bradford (18).

Materials—All peptides were synthesized with an automated solid-phase peptide synthesizer and were verified as to structure by gas-phase sequencing and amino acid analysis. Phorbol esters were purchased from Sigma, nerve growth factor (2.5 S) was purified from mouse submaxillary glands (17), and epidermal growth factor (receptor grade) was purchased from Collaborative Research (Bedford, MA).

RESULTS

Substrate Properties of the KRTLRR Peptide—We have used a synthetic peptide substrate, KRTLRR, whose sequence is based on the major site phosphorylated by protein kinase C in the EGF receptor (11) as an alternative to protein kinase C substrates such as histones that serve as substrates for many kinases. Advantages of this peptide substrate include a positive charge at acidic to neutral pH, thus allowing the simple and rapid assay of peptide phosphorylation by the method of Roskoski (15), a single threonine phosphorylation site, and most importantly, a peptide sequence which confers selectivity as a substrate for protein kinase C.

We observed that protein kinase C purified from bovine retina (18) readily phosphorylated (Km ~ 130 μM) the KRTLRR peptide in a Ca2+-, diacylglycerol- and phospholipid-dependent manner (data not shown). However, addition of cAMP, cGMP, or calmodulin to the permeabilized cell kinase assay previously characterized in detail (9) and described below did not increase the rate of phosphorylation of this peptide (data not shown), indicating that the KRTLRR peptide is a poor substrate for cyclic nucleotide- and Ca2+/calmodulin-dependent protein kinases. Additionally, as shown in Table I and Fig. 1, the KRTLRR peptide did not appear to be a substrate for two growth factor-regulated kinases that we recently described in PC12 cells (9). As shown in Table I, cell-free extracts from NGF-treated PC12 cells exhibited a 2-4-fold increase in kinase activity when assayed with synthetic peptides whose sequences are derived from smooth muscle myosin light chain (MLC peptide, KKRQPRATSNVFS; Ref. 14) or ribosomal protein S6 (S6 peptide, RRLSSLR; Ref. 13). However, under these same assay conditions (absence of added Ca2+, phospholipid, and diacylglycerol) where protein kinase C is in an inactive state, phosphorylation of the KRTLRR peptide by cell-free extracts from NGF-treated cells was not elevated compared to extracts from control cells. This finding demonstrated that the MLC peptide kinase and S6 peptide kinase did not recognize the KRTLRR peptide as a phosphorylation substrate (Table I). Addition of Ca2+, phospholipid, and diacylglycerol was required to activate protein kinase C in extracts from both the control and NGF-treated cells. Note that an effect of NGF on cellular protein kinase C activity would not be observed in this experiment because lysis of the cells with buffer containing 0.1% Triton X-100 (see "Experimental Procedures") extracted both the activated and inactive forms of protein kinase C. In contrast, the activation of S6 peptide and MLC peptide kinases was readily apparent in detergent extracts.

Fractionation of cell-free extracts from control and NGF-treated cells on a cation exchange column showed that NGF-stimulated MLC peptide kinase activity eluted as a single major peak at fraction 13 (Fig. 1, panel A), while two major peaks of NGF-regulated activity eluting at fractions 13 and 18 were detected with the S6 peptide (Fig. 1, panel B). Assay of the column fractions under conditions identical to those in Fig. 1, panels A and B, revealed that the KRTLRR peptide was not phosphorylated by the peak of MLC peptide kinase activity or either peak of S6 peptide kinase activity (Fig. 1, panel C). However, inclusion of Ca2+, diacylglycerol, and phospholipid in the KRTLRR peptide kinase assay revealed a large peak of protein kinase activity that eluted in fractions 3-7 (Fig. 1, panel D). Thus, the KRTLRR peptide is readily phosphorylated by protein kinase C, but is not recognized by S6 kinase, MLC peptide kinase, cyclic nucleotide-dependent kinases, or calmodulin-dependent kinases. Protein kinase C is also readily resolved from these kinases by Protein Pak SP 5PW cation exchange chromatography.

Assay of Phorbol Ester-regulated Protein Kinase C in Permeabilized PC12 Cells—Protein kinase C appears to be converted by diacylglycerol or phorbol esters from a loosely bound, extrinsic membrane protein to a form that exhibits a higher affinity for the plasma membrane (19). Estimates of the cellular activity of protein kinase C have been largely based on the observation that in the presence of EGTA, the inactive, loosely membrane-bound form of protein kinase C fractionates with the cytosol while the activated form remains associated with the plasma membrane. However, assays based on this apparent translocation can be susceptible to homogenization artifacts and the affinity of protein kinase C for the plasma membrane may differ depending on whether the enzyme is activated with phorbol esters, membrane-permeable diglycerides, or endogenously formed diacylglycerol (20). We have used low concentrations of digitonin to permeabilize

<table>
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<tr>
<th>Treatment</th>
<th>KRTLRR peptide</th>
<th>MLC peptide</th>
<th>S6 peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>70.6</td>
<td>1305</td>
<td>107.9</td>
</tr>
<tr>
<td>NGF</td>
<td>58.6</td>
<td>1501</td>
<td>442.5</td>
</tr>
</tbody>
</table>

Table I

Soluble peptide kinase activities in extracts from control and NGF-treated PC12 cells

PC12 cells (5 million cells per dish) were incubated in 7 ml of 137 mM NaCl, 5.4 mM KCl, 0.3 mM sodium phosphate, 0.4 mM potassium phosphate, 1.0 mM calcium chloride, 1 mg/ml glucose, 20 mM HEPES (pH 7.2 at 30 °C) with or without 100 mg/ml NGF. Cell-free extracts were prepared as described under "Experimental Procedures," and a portion was diluted 5-fold into 25 mM β-glycerophosphate (pH 7.2), 10 mM sodium fluoride, 20 μM sodium vanadate, 1 mM EGTA, and 1 mM DTT. Aliquots (20 μl) of the diluted extracts were mixed with an equal volume of various kinase assay solutions. KRTLRR peptide kinase was assayed in the absence (−PS, DAG, Ca2+) or in the presence (+PS, DAG, Ca2+) of cocsonicated phosphatidyserine and diolein (326 and 19 μg/ml, respectively) and 0.1 mg CaCl2 in excess of EGTA. Following a 15-min incubation at 30 °C, the reactions were terminated with 10 μl of 25% trichloroacetic acid, and phosphopeptides were quantified.
FIG. 1. Cation-exchange chromatography of MLC, S6, and KRTLRR peptide kinase activities. Extracts (0.4 ml, 500 μg of protein) from control PC12 cells (C) or cells incubated for 15 min with 100 ng/ml NGF (D) 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 DTT (Buffer A) and applied to a Protein Pak SP 5PW column (Waters, 7.5 mm × 7.5 cm) equilibrated in Buffer A. The column was developed at 1 ml/min with a linear gradient of 0-500 mM NaCl in Buffer A. Fractions (1 ml) were collected, and aliquots (20 μl) were assayed for kinase activity with MLC peptide (panel A), S6 peptide (panel B), or KRTLRR peptide in the absence (panel C) or presence (panel D) of Ca²⁺, phosphatidylserine, and diacylglycerol. Note that the kinase activity has been divided by 1000 in panel D. This experiment is representative of two independent experiments.

attached PC12 cells in the presence of [γ-32P]ATP and the KRTLRR peptide to rapidly and specifically assay protein kinase C in phorbol ester- or growth factor-pretreated cells. While digitonin permeabilization has been used previously in protocols for assessing hormonal regulation of protein kinase C (20, 21), the procedure described in this report provides a rapid and direct measurement of protein kinase C activity that does not involve cell homogenization and estimation of the distribution of the enzyme based on fractionation of membranes and cytosol by centrifugation. Using the digitonin permeabilization procedure, little protein kinase C escapes the cells, and direct measurement of activity is possible after exposure of cells to agonists.

Characterization of the protein kinase C-mediated phosphorylation of the KRTLRR peptide following treatment of PC12 cells with phorbol esters is shown in Fig. 2. Digitonin-dependent phosphorylation of KRTLRR peptide was observed with attached PC12 cells that had been pretreated for 10 min with or without 100 nM TPA (Fig. 2, panel A). Maximal TPA-stimulated activity (3.4-fold) was observed in cells permeabilized with 50 μg/ml digitonin, a concentration that did not alter cell morphology or adhesion. The increased KRTLRR peptide kinase activity observed in TPA-treated cells was retained in the cell monolayer after digitonin treatment. This finding indicated that the kinase activity represented membrane-associated protein kinase C which, as predicted from the tight association of protein kinase C with membranes after TPA treatment, was not released from the cells by digitonin permeabilization (data not shown).

The ability of phorbol esters to lead to increased KRTLRR peptide kinase activity corresponded to their known ability to activate protein kinase C (Fig. 2, panel B). Pretreatment of PC12 cells with two active phorbol esters, TPA, or phorbol dibutyrate, increased KRTLRR peptide kinase activity in permeabilized cells in a concentration-dependent manner (EC₅₀ ~42 nM). The maximal stimulation by TPA was approximately twice that achieved with phorbol dibutyrate. Pretreatment of cells with an inactive phorbol ester, 4-α-phorbol
that inhibits calmodulin-dependent kinases nor was it synergistic of calcium chloride phosphorylation in buffer containing 5 mM experiments. 

duplicate determinations and are representative of three independent actions of Ca" was not inhibited by a synthetic peptide (22) due to Ca²⁺-activated protein kinase C, and not a calmodulin-dependent kinase activity was assayed (10 min, 30 °C, 300 μM KRTLRR peptide) in control and TPA-treated PC12 cells in the presence of various concentrations of digitonin. Panel B, PC12 cells were treated for 10 min with various concentrations of the indicated phorbol ester (TPA, 12-O-tetradecanoylphorbol 13-acetate; PDB, phorbol dibutyrate; 4α-PDD, 4α-phorbol didecanoate), and assayed for KRTLRR peptide kinase activity. Panel C, control and TPA-treated cells were assayed for KRTLRR peptide kinase activity in the presence of various concentrations of protein kinase C19-36 peptide (PKC19-36 peptide). All data points are the means of duplicate or triplicate determinations and are representative of two (panels B and C) and four (panel A) independent experiments.

### Table II 

 Ca²⁺ dependence of KRTLRR peptide phosphorylation in control and TPA-treated PC12 cells

<table>
<thead>
<tr>
<th>EGTA:CaCl₂</th>
<th>KRTLRR peptide phosphorylation</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>pmol/min/mg protein</td>
</tr>
<tr>
<td>5:0</td>
<td>16.3</td>
</tr>
<tr>
<td>5:1</td>
<td>16.0</td>
</tr>
<tr>
<td>5:1.5</td>
<td>15.0</td>
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<tr>
<td>5:2.5</td>
<td>15.4</td>
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<tr>
<td>5:5</td>
<td>25.9</td>
</tr>
<tr>
<td>5:5.5</td>
<td>41.1</td>
</tr>
<tr>
<td>5:5.6</td>
<td>53.4</td>
</tr>
</tbody>
</table>

KRTLRR peptide kinase activity was assayed in TPA-pretreated cells was a direct measure of protein kinase C19-36 peptide) that has previously been shown to be a potent and specific inhibitor of protein kinase C (23) inhibited the TPA-stimulated KRTLRR peptide kinase activity in a concentration-dependent manner (IC₅₀ = 3.8 μg/ml; Fig. 2, panel C). Thus, the increased KRTLRR peptide kinase activity assayed in TPA-pretreated cells was a direct measure of protein kinase C that is specifically inhibited by a highly selective pseudosubstrate peptide.

The time course with which TPA stimulated KRTLRR peptide kinase activity is shown in Fig. 3. Maximal stimulation of KRTLRR peptide kinase activity was achieved within 10 min and was maintained without decline for the duration of the experiment. Longer time courses revealed that intermediate concentrations of TPA (100 nM) elevated KRTLRR peptide kinase activity for at least several hours (not shown). In contrast, a cell-permeable diglyceride, sn-1,2-diocanoylglycerol (diCs), produced a small and transient increase in KRTLRR peptide kinase activity (Fig. 3). The transient nature of the response is probably due to the fact that diCs can be rapidly phosphorylated to form phosphatidic acid or hydrolyzed to form monoacylglycerol and fatty acid similar to the metabolism of endogenous diglycerides. Interestingly, diCs stimulated protein kinase C in a manner very similar to that caused by growth factors (shown below). Thus, diCs appears to much more closely mimic growth factor activation of protein kinase C in PC12 cells with respect to magnitude and time course of activation than do phorbol esters.
Assay of Growth Factor-regulated Protein Kinase C in Permeabilized PC12 Cells—Based on observations that phorbol esters can mimic some cellular actions of NGF and EGF, various roles for protein kinase C in NGF and EGF action in PC12 cells have been proposed (7, 24-26). It should be noted, however, that no direct demonstration of the activation of protein kinase C by NGF or EGF in PC12 cells was demonstrated in any of these studies. PC12 cells show a transient 1.6-fold increase in inositol triphosphate within 30 s of exposure to NGF that returns toward basal levels after 1-2 min (27). Consistent with this finding, we have measured a 2-fold elevation of diacylglycerol levels in PC12 cells within 1 min of NGF treatment that returns to control levels by 10 min of incubation. Therefore, PC12 cells exhibit a small and transient elevation of both diacylglycerol and inositol triphosphate in response to NGF treatment. We therefore examined KRTLRR peptide kinase activity in cells treated for various times with NGF or EGF. As shown in Fig. 4, panel A, pretreatment of PC12 cells with NGF or EGF transiently increased the KRTLRR peptide kinase activity. Peak kinase stimulations of 1.3-2-fold were observed after 1-5 min of growth factor treatment. Within 15-20 min of growth factor treatment, KRTLRR peptide kinase activity returned to levels observed in untreated cells. The time course of protein kinase C activation thus correlated strongly with the rapid, transient increase in diacylglycerol levels.

Temporally, regulation of protein kinase C by NGF and EGF contrasted with the time course of activation observed for another growth factor-regulated protein kinase, ribosomal S6 kinase (Fig. 4, panel B). When PC12 cells were treated for various times with NGF or EGF and assayed for ribosomal S6 kinase with the RRLSSLRA peptide, S6 kinase was maximally activated after 10-15 min of treatment compared to approximately 1 min for protein kinase C (Fig. 4, panel A).

Furthermore, S6 kinase activity was maintained without decline for the duration of the experiment. In fact, we have previously shown that S6 kinase remains activated for at least 24 h following treatment with NGF or EGF (9). Thus, NGF and EGF appeared to transiently increase the activity of protein kinase C while, in identically treated cells, S6 kinase remained persistently activated.

Similar to its ability to inhibit phorbol ester-stimulated KRTLRR peptide kinase activity (Fig. 2, panel C), the pseudosubstrate peptide inhibitor of protein kinase C (protein kinase C19-36 peptide) also inhibited the KRTLRR peptide kinase activity transiently stimulated by growth factors (Table III). This result demonstrated that the growth factor-regulated KRTLRR peptide kinase activity was indeed protein kinase C. When the protein kinase C19-36 peptide-inhibitable KRTLRR peptide kinase activity was compared in control and growth factor-treated PC12 cells, the apparent-fold stimulation observed in NGF- and EGF-treated cells was 2.6- and 2.0-fold, respectively, which was again similar in magnitude to the change in diacylglycerol levels observed over this early time of NGF treatment of PC12 cells.

### Footnotes

1 C. N. S. Sperber and G. L. Johnson, unpublished observation.
TABLE III
Inhibition of NGF- and EGF-stimulated KRTLRR peptide kinase activity by protein kinase C peptide

<table>
<thead>
<tr>
<th>Treatment</th>
<th>KRTLRR peptide kinase activity (pmol/min/mg protein)</th>
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<tr>
<td></td>
<td>-PKC19-36 peptide</td>
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<tr>
<td>Control</td>
<td>15.9 ± 0.6</td>
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<tr>
<td>NGF</td>
<td>25.2 ± 3.1</td>
</tr>
<tr>
<td>EGF</td>
<td>21.2 ± 1.6</td>
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<tr>
<td>TPA</td>
<td>54.1 ± 0.6</td>
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TABLE IV
Regulation of KRTLRR peptide and S6 peptide kinases in control and protein kinase C-deficient PC12 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Peptide kinase activity (pmol/min/mg protein)</th>
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<tr>
<td></td>
<td>KRTLRR peptide</td>
</tr>
<tr>
<td>Control cells</td>
<td>9.7 ± 0.4</td>
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<tr>
<td>Buffer</td>
<td>12.3 ± 0.6</td>
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<tr>
<td>EGF</td>
<td>13.2 ± 0.0</td>
</tr>
<tr>
<td>NGF</td>
<td>24.5 ± 1.1</td>
</tr>
<tr>
<td>TPA</td>
<td></td>
</tr>
<tr>
<td>Protein kinase C-deficient cells</td>
<td>9.3 ± 0.4</td>
</tr>
<tr>
<td>Buffer</td>
<td>9.0 ± 0.6</td>
</tr>
<tr>
<td>EGF</td>
<td>8.1 ± 0.7</td>
</tr>
<tr>
<td>NGF</td>
<td>8.1 ± 0.7</td>
</tr>
<tr>
<td>TPA</td>
<td></td>
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</table>

Long term treatment of cells with TPA has been shown to markedly down-regulate protein kinase C levels (28). In contrast to the marked stimulation of KRTLRR peptide kinase activity observed following brief exposures to TPA (Fig. 3), we observed that PC12 cells grown in the presence of 1 μM TPA for 50 h exhibited KRTLRR peptide kinase activity that was not different from basal activity in untreated cells (Table IV). Furthermore, re-exposure of the cells to TPA no longer stimulated KRTLRR peptide kinase activity. Thus, PC12 cells treated for extended periods of time with TPA appeared to be functionally protein kinase C-deficient. As one would predict if the KRTLRR peptide kinase activity represents protein kinase C activity, no growth factor-stimulated KRTLRR peptide kinase activity was observed in PC12 cells that had been depleted of protein kinase C by long term TPA exposure (Table IV), although NGF and EGF elicited a characteristic 1.4- and 1.3-fold stimulation, respectively, of KRTLRR peptide kinase activity in untreated control cells. Thus, the KRTLRR peptide kinase activity regulated by growth factors directly correlates with the presence of protein kinase C. In contrast, treatment with NGF and EGF stimulated S6 peptide kinase in both control (1.7-2-fold) and protein kinase C-deficient cells (2-2.7-fold). This observation supports a previous finding by Blenis and Erikson (10) that NGF and EGF appear to regulate ribosomal S6 kinase by a mechanism independent of protein kinase C.

DISCUSSION

For the first time, our studies directly measure protein kinase C activation in response to NGF and EGF and define the temporal involvement of the kinase in growth factor action in PC12 cells. Unlike phorbol esters which markedly increased protein kinase C activity for durations of at least several hours, NGF and EGF caused modest stimulations of protein kinase C that persisted for only 15-30 min. Temporally, NGF and EGF regulate protein kinase C with a time course similar to that observed in a variety of cell lines upon treatment with hormones that stimulate phosphatidylinositol hydrolysis or diacylglycerol generation (21, 29-31). Thus, our results indicate that protein kinase C serves as an early and transient signal in NGF and EGF action in PC12 cells, not as a continuously generated signal like ribosomal S6 kinase.

The highly transient nature of protein kinase C activation relative to other NGF- and EGF-regulated protein kinases we have characterized begins to restrict the roles that protein kinase C may serve in the growth factor responses of PC12 cells. NGF, EGF, and phorbol ester treatment of PC12 cells clearly increases the phosphorylation state of tyrosine hydroxylase (5, 24, 25, 32). Based on observations that growth factors and phorbol esters stimulate the phosphorylation of a common tryptic phosphopeptide, workers have suggested that protein kinase C mediates, at least in part, the growth factor-stimulated phosphorylation of tyrosine hydroxylase (24, 25). However, the slower and persistent signal over a period of hours with which NGF and EGF stimulate tyrosine hydroxylase phosphorylation (5, 32) contrasts with the time course of protein kinase C activation (Fig. 4, panel A), suggesting that other kinases besides protein kinase C mediate growth factor-stimulated tyrosine hydroxylase phosphorylation in PC12 cells.

Another proposed role for protein kinase C is as the mediator by which NGF and EGF inhibit a protein kinase/substrate system referred to as Nsp 100 kinase and Nsp 100 (7). Similar to growth factor-stimulated phosphorylation of tyrosine hydroxylase, phorbol esters also mimicked the ability of NGF and EGF to inhibit Nsp 100 phosphorylation. However, maximal reduction of Nsp 100 phosphorylation by NGF and EGF occurred after 4-6 h of treatment, a time at which our data indicate that protein kinase C activity would have returned to control levels. Additionally, other workers observed that down-regulation of protein kinase C by chronic phorbol ester treatment had no effect on the ability of NGF or EGF to reduce this kinase activity (8).

Finally, protein kinase C has been implicated in such distal events as neurite outgrowth in PC12 cells which occurs following days of NGF treatment. In these studies, workers observed that extension of neurites by PC12 cells, which requires continuous exposure to NGF, was suppressed by sphingosine, a pharmacologic inhibitor of protein kinase C (26). Furthermore, treatment of PC12 cells with TPA alone did not elicit neurite outgrowth, but continuous exposure of cells to phorbol esters appeared to potentiate the mitogenic action of NGF (26). However, these findings must be cautiously interpreted since sphingosine can modulate cellular function by mechanisms other than inhibition of protein kinase C (33). Also, cellular actions resulting from chronic exposure to TPA may arise as a consequence of depressed levels of protein kinase C due to down-regulation rather than persistent activation of the kinase.

While NGF treatment of PC12 cells promotes neuronal differentiation and cessation of cell division, evidence indicates that an early mitogenic action may precede differentiation (4). Interestingly, we have observed that PC12 cells transfected with the c-myc or E1A oncogenes no longer differentiate in response to NGF (34). Instead, NGF now exerts only a mitogenic action on these transfected cells similar to...
that observed for EGF. In view of these findings, we consider it likely that the early and transient activation of protein kinase C serves as a "switch" for stimulating the metabolic pathways regulated by growth factors in PC12 cells. These metabolic responses, observed in many cell types following treatment with mitogenic agents, include the rapid and transient transcription of c-fos and β-actin and the more delayed transcription of c-myc and ornithine decarboxylase (3), increased Na+/H+ exchange (4) and increased nutrient transport (1, 2). Clearly, these early metabolic events and protein kinase C activation by peptide growth factors appear to be a general response of cells to multiple stimuli and are not specific differentiation responses of PC12 cells to NGF.

In summary, we have described the use of the synthetic peptide, KRTLRR, to monitor protein kinase C activity in permeabilized PC12 cells following treatment with phorbol esters, NGF and EGF. The combination of the permeabilized cell assay and the synthetic peptide substrate has many advantages over translocation protocols. Among the advantages are the ease and specificity of the assay, the ability to perform rapid and accurate time courses, and the direct measure of protein kinase C activity independent of cell homogenization and fractionation of membranes and cytosol. Using this assay, we observed marked differences in the kinetics of protein kinase C activation by phorbol esters such as TPA and the growth factors, NGF and EGF. TPA activated the kinase for several hours, whereas even in the continued presence of NGF or EGF, protein kinase C activation was transient. Thus, TPA and other phorbol esters should be used with reservation when attempting to understand the role of protein kinase C in signaling events associated with NGF and EGF action, at least in PC12 cells. The synthetic diacylglycerol analogs such as diC8 are probably much more suitable for studies of the role of protein kinase C in NGF and EGF action and the control of specific cell responses.

REFERENCES