Phorbol Esters Regulate Preprogastrin-releasing Peptide Messenger RNA in Small Cell Lung Cancer Cells

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Abstract
The expression of preprogastrin-releasing peptide (GRP) mRNA was studied using human small cell lung cancer (SCLC) cells. By Northern analysis, preproGRP mRNA was stimulated by 4β-phorbol 12-myristate 13α-acetate (PMA) in a concentration- and time-dependent manner in these cells. In cell line NCI-H209, the addition of 10^{-6} M PMA increased a 0.9-kb mRNA after 8 h. An inactive phorbol ester, 4α-PMA, had little effect on preproGRP mRNA. A nuclear run-on assay indicated that 10^{-6} M PMA increased preproGRP transcription 3-fold, whereas β-actin and glyceraldehyde 3-phosphate dehydrogenase transcription was unaltered. In contrast, PMA had little effect on β-actin mRNA expression. PMA (1 μM) in the presence of 100 μM 1-(5-isouquinolinesulfonyl)-2-methylpipеразине (H7), a protein kinase C inhibitor, had little effect on preproGRP mRNA. Addition of PMA after protein kinase C down-regulation did not alter preproGRP mRNA. PMA (1 μM) caused translocation of protein kinase C from the cytosol to the membrane of SCLC cells. Also, PMA (10^{-6} μM) stimulated and H7 (10^{-4} M) reduced SCLC growth in vitro. When new synthesis of preproGRP mRNA was blocked by the addition of actinomycin D, preproGRP mRNA remained stable for 15 h. These data suggest that PMA induces transcription of GRP mRNA in SCLC cells.

Introduction
The 27-amino acid GRP is an autocrine growth factor for some human SCLC cell lines (1). Some of the initial steps in the signal transduction pathway following binding to GRP receptors have recently been defined (2). After release, GRP binds to high affinity G-protein-coupled receptors on the cell surface (3). Activation of the G-proteins stimulates phospholipase C activity, which in turn cleaves phosphatidylinositol diphosphate to inositol 1,4,5-trisphosphate and diacylglycerol (4). These events result in a rapid elevation of intracellular calcium and activation of protein kinase C (5). These changes initiate a series of events that are as yet poorly defined which ultimately may stimulate cellular proliferation (6).

The gene for preproGRP, which has been cloned and localized to human chromosome 18q21, is comprised of three exons and two introns (7–9). GRP is synthesized as a preprohormone containing a 23-amino acid signal sequence followed by a 27-amino acid GRP, a glycine amidation don- nor, a dibasic cleavage site, and a COOH-terminal extension peptide. Three forms of preproGRP are found in SCLC cells due to alternative splicing. Each of the 3 forms of mRNA is identical over regions encoding the signal sequence, GRP, and the first 68 amino acids of the extension peptide. Heterogeneity results from alternative splicing of the primary transcripts at the second and third exon splice junction. After posttranslational processing, preproGRP is metabolized to GRP and GRP\textsubscript{1827}, which are biologically active. High levels of immunoreactive GRP are present in classic SCLC (10, 11). The secretion rate of GRP from SCLC cells is increased by muscarinic cholinergic agonists (12) and vasoactive intestinal peptide, which elevates the cAMP levels (13).

Stimulation of preproGRP gene expression provides an additional mechanism for maintaining autocrine growth stimulation by potentially increasing synthesis of a growth-promoting peptide. In this study, we investigated the effects of signal transduction events on preproGRP gene expression. PMA activates protein kinase C, mimicking one of the known biochemical events in the GRP signal transduction pathway. In this study, we examined the effects of PMA on preproGRP gene expression.

Results
PMA Increases preproGRP Gene Expression. Fig. 1 shows an overexposure of preproGRP mRNA. A major 0.9-kb band and minor 3.6- and 7.2-kb bands were detected by Northern blot analysis. An increase in the intensity of all three bands was observed after treating NCI-H209 with 10 nM PMA for 8 h. Minor bands at 3.6 and 7.2 kb, which may represent partially spliced precursor transcripts, were also increased by PMA. Similar results were observed using cell line NCI-H345 except that the overall intensity of the bands was lower. The data suggest that PMA increases the steady-state levels of preproGRP mRNA.

A dose-response curve was determined using PMA on preproGRP mRNA. Fig. 2 shows that the 0.9-kb band was increased approximately 3-fold by 1 μM PMA after 8 h. preproGRP gene expression was not affected by 1 nM PMA and increased approximately 2-fold by 10^{-7} M PMA after 8 h. After hybridization with the preproGRP probe, the nitrocellulose was rehybridized with a β-actin probe. PMA had little effect on β-actin gene expression. The resulting autoradiogram was then analyzed on a scanning densitometer (Fig. 3). In the absence of PMA, the relative density of the β-actin and preproGRP mRNA appeared similar. After treatment of NCI-
H209 cells with 1 μM PMA for 8 h, the density of β-actin mRNA was similar; however, the density of preproGRP mRNA increased approximately 3-fold. These results indicate that GRP but not β-actin gene expression of NCI-H209 is regulated by PMA in a dose-dependent manner.

The time course of PMA on preproGRP gene expression was investigated. Fig. 4 shows that 1 μM PMA increased preproGRP gene expression, and the increase was maximal after 8 h (3-fold increase). In contrast, preproGRP gene expression was minimally affected after 0.5 h. As a control, the inactive phorbol ester 4α-PMA had little effect on preproGRP gene expression (Table 1). Therefore, preproGRP but not β-actin gene expression is regulated by active phorbol esters in a time-dependent manner.

The ability of PMA to interact with PKC was investigated. Fig. 5 (top) shows by Western blot analysis that a major 80 kilodalton band is present in the cytosol of NCI-H345 cells (Lane d) but not the membrane fraction (Lane c). After addition of PMA, the intensity of the band in the cytosol was greatly reduced (Lane b), and most of the PKC after addition of PMA was found in the membrane fraction (Lane a). PKC was not translocated by 4α-PMA (data not shown). These data suggest that PMA activates PKC, resulting in translocation of the enzyme from the cytosol to the membrane.

Protein kinase C can be down-regulated by phorbol esters such as PMA (14). After treatment with PMA for 36 h, PKC was not detected in either the cytosolic or membrane fractions by Western blot (data not shown). If SCLC cells were pretreated with PMA for 36 h and then treated with PMA for an additional 8 h, this had little effect on preproGRP gene.
expression (Fig. 6). In the absence of pretreatment with PMA, 1 μM PMA increased preproGRP but not β-actin mRNA levels (Fig. 5).

The effect of protein kinase C inhibitors was investigated (Fig. 7). H7 had little effect on basal or PMA-stimulated preproGRP mRNA levels in SCLC cells at a 10 μM dose but significantly decreased both basal and PMA-stimulated preproGRP mRNA levels at a 100 μM dose. Also, H7 (100 μM) blocked the increase in protein kinase C translocation caused by PMA (data not shown).

**Effects of PKC Agents on SCLC Growth.** The effects of PMA and H7 on SCLC growth were investigated. Table 2 shows that 10 nM PMA had little effect on SCLC colony formation, whereas 1 μM PMA significantly stimulated growth using NCI-H209 and H345 cell lines. In contrast, H7 (100 μM) decreased the number of NCI-H209 and H345 colonies in the absence of exogenous PMA. Also, H7 decreased the number of SCLC colonies in the presence of 10 nM PMA from 348 to 168 and 63 to 36 using NCI-H209 and H345, respectively. Also, H7 decreased the number of SCLC colonies in the presence of 1000 nM PMA by 63% and 45% using NCI-H209 and H345, respectively. These data suggest that protein kinase C may regulate SCLC growth in vitro.

**Effects of PMA on preproGRP Synthesis and Degradation.** The effect of PMA on preproGRP gene transcription was investigated using a nuclear run-on assay. Fig. 8 shows that there is a 3.4-fold increase in GRP transcription in NCI-H209 cells 8 h after treatment with 10^{-6} M PMA. In contrast, glyceraldehyde 6-phosphate dehydrogenase and β-actin were unaffected by PMA. The pSp64 vector served as a control for hybridization background and was undetectable in this assay.

The stability of the NCI-H209 preproGRP mRNA was investigated by Northern analysis. Fig. 9 shows that 8 h after treatment with 10^{-6} M PMA, actinomycin D was added, and the preproGRP mRNA was determined as a function of time. For the first 15 h, the intensity of the GRP mRNA band was unaltered; in all cases, the amount of preproGRP mRNA was approximately 3-fold greater in the presence relative to the absence of PMA. Similarly, for the first 15 h, the β-actin mRNA did not change; PMA had little effect on the β-actin mRNA expression. In contrast, the histone 2B mRNA was high initially but decreased 2 h after the addition of actinomycin D and was absent after 6 h. These data suggest that the half-life of the preproGRP and β-actin mRNA is greater than 15 h, whereas the histone 2B mRNA half-life is approximately 2 h.

**preproGRP Promoter Sequence.** Previously, the preproGRP gene was demonstrated to have two introns. Here, the sequence derived from the 5'-flanking region and the first exon was determined (Fig. 10). A consensus CAAT box is 150 bases upstream from the TATA box. Sixty bases upstream
8 hr PMA Incubation - + - + 36 hr PMA Preincubation - - + +

**Fig. 6.** Effect of PKC down-regulation on preproGRP gene expression. NCI-H209 cells were treated with and without 1 μM PMA for 36 h. Cells were washed, incubated in low serum with and without 1 μM PMA for 8 h. RNA was isolated and analyzed for preproGRP mRNA. The β-actin mRNA was similar in each sample.

**Fig. 7.** Effect of H7 on GRP gene expression. NCI-H209 cells were treated with and without 10 μM H7, 100 μM H7, and/or 1 μM PMA for 8 h. RNA was isolated and analyzed for preproGRP mRNA. The f-actin mRNA was similar in each sample.

<table>
<thead>
<tr>
<th>PMA, 1μM</th>
<th>H-7</th>
<th>10μM</th>
<th>100μM</th>
<th>10μM</th>
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**Table 2.** Effect of PKC agents on SCLC growth in vitro

<table>
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<th>Addition</th>
<th>NCI-H209</th>
<th>NCI-H345</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>266 ± 27</td>
<td>45 ± 5</td>
</tr>
<tr>
<td>10 μM PMA</td>
<td>348 ± 52</td>
<td>63 ± 10</td>
</tr>
<tr>
<td>1 μM PMA</td>
<td>680 ± 61*</td>
<td>93 ± 16*</td>
</tr>
<tr>
<td>100 μM H7</td>
<td>133 ± 38</td>
<td>41 ± 4</td>
</tr>
<tr>
<td>10 μM PMA + 100 μM H7</td>
<td>168 ± 91</td>
<td>36 ± 9</td>
</tr>
<tr>
<td>1 μM PMA + 100 μM H7</td>
<td>253 ± 30</td>
<td>51 ± 7</td>
</tr>
</tbody>
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* P < 0.01 relative to control.

from the TATA box is a stretch of seven nucleotides homologous to the cAMP-regulatory elements previously observed for SRIF and proenkephalin genes (15, 16). Surprisingly, no phorbol ester-responsive element is in the vicinity of the cAMP-responsive element. Approximately 1345 nucleotides upstream from the TATA box is a potential phorbol ester-responsive element.

**Discussion**

The present study demonstrates that SCLC GRP mRNA is regulated by PMA, a known enhancer of peptide genes (17). For the GRP gene, the TATA box is approximately 18 base pairs upstream, and this may represent the start site of transcription. The upstream regulatory elements for the GRP gene include the CAAT box and the GC-rich region, which are approximately 169 and 144 base pairs upstream. GRP gene enhancers may include a PMA- and cAMP-regulatory sequence, 1364 and 79 base pairs upstream, respectively.

Here, the effects of PKC on GRP gene expression were investigated using SCLC cells. PMA-stimulated expression of a 0.9-kb GRP mRNA species in a concentration- and time-dependent manner. GRP mRNA was increased approximately 3-fold in SCLC cell lines NCI-H209 and H345 by treatment with PMA (10^{-6} M) for 8 h, whereas 4a-PMA was less potent. Also, pretreatment of NCI-H345 with PMA for 36 h caused down-regulation of PKC. In these pretreated cells, addition of 10^{-6} M PMA for 8 h had no effect on GRP gene expression. These data suggest that GRP gene expression may be regulated by PKC in SCLC cells. Previously, it was observed that expression of other peptides such as VIP was enhanced 4-fold by PMA (18, 19). In the VIP gene, PMA interacted with a cist-acting element that was approximately 4000 base pairs upstream of the promoter (20). Also, a PMA-responsive sequence (TGACTCA) was identified for the calcitonin gene (21).

The increase in GRP gene expression appears to result from increased transcription and not increased mRNA stability. Using a nuclear run-on assay, the density of the GRP band increased 3-fold after treating NCI-H209 with 1 μM PMA for 8 h. Also, the GRP mRNA was stable for 15 h in SCLC cells. β-Actin gene transcription was not increased by PMA treatment, and β-actin mRNA also had a long half-life. In contrast, histone 2B mRNA was increased by PMA, but the histone 2B mRNA was substantially degraded after 6 h.

Multiple isoforms of protein kinase C have been identified (22, 23). The ε isofom of PKC is present in variant SCLC cells (24, 25). Here, using an antibody which recognizes multiple PKC isoforms, an 80 kilodalton band was detected in classic SCLC cells. Within 5 min, 10^{-4} M PMA caused translocation of protein kinase C from the cytosol to the membrane using...
Also, cAMP-regulatory sequences may alter GRP gene expression. Forskolin (50 μM) stimulates GRP gene expression 2-fold after 8 h (29). For the VIP gene, a cAMP-responsive element was identified 70 base pairs upstream from the transcription initiation site, and cAMP increased VIP mRNA levels 4-fold (19). The sequence of the cAMP-responsive element for the GRP gene (GACGTCA) is similar to that seen for other peptides such as VIP and somatostatin (15, 16).

In summary, SCLC GRP gene expression is increased by PMA, which activates protein kinase C. The increase in GRP gene expression caused by PMA is inhibited by H7. PMA (1 μM) stimulates SCLC growth, and H7 (100 μM) inhibits SCLC colony formation. These data suggest that protein kinase C may be an important regulatory enzyme in SCLC.

Materials and Methods

Cell Culture. SCLC cell lines NCI-H345 and NCI-H209, which have 4 and 18 pmol immunoreactive GRP/mg protein, respectively, were cultured in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (30). The cells, which grow as floating colonies, were split 1:1 weekly. The cells were placed in RPMI 1640 containing 0.5% fetal bovine serum prior to conducting an experiment. Stimuli such as 1 μM PMA were added for the indicated time.

Northern Analysis of preproGRP mRNA. Total RNA was isolated using the guanidinium isothiocyanate method (31). Ten μg of denatured RNA were separated in a 0.66% formaldehyde-1% agarose gel as described previously (32). The RNA was blotted onto a nitrocellulose membrane overnight, and the membrane was hybridized with a preproGRP probe (a 1-kb cDNA fragment cloned in a pSP64 vector). The 1-kb fragment was electroeluted from Seakem agarose gel and labeled with [32P]dCTP using a Bethesda Research Laboratories random priming kit. The blot was rehybridized with a β-actin probe, and films were analyzed on a densitometer. The relative ratio of the preproGRP/β-actin gene expression was calculated.

The preproGRP mRNA stability was assessed (33). SCLC cells were treated with actinomycin D (40 μg/ml) for up to 15 h after an 8-h incubation with PMA (10⁻⁶ M), preproGRP, β-actin, and histone 2B mRNA was determined by Northern analysis.

Nuclear Run-on. The effect of PMA on preproGRP gene transcription was determined using a nuclear run-on assay as described previously (34). Hybridization membranes were exposed for 10 days using a Dupont Quanta III intensifying screen. The bands were quantitated by densitometry analysis.

Western Blot Analysis of Protein Kinase C. SCLC cells (10⁷) were harvested and washed twice in SIT medium (RPMI 1640 containing 3 × 10⁻⁶ M Na₂SeO₃, 5 μg/ml insulin, and 10 μg/ml transferrin) with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-NaOH (pH 7.4). PMA (10⁻⁶ M) was added, and after 5 min at 37°C, the cells were centrifuged, washed once in phosphate-buffered saline, and lysed in ice-cold 20 mM Tris, 2 mM EDTA, 0.5 M ethyleneglycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 5 mM dithiothreitol, 100 μM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin (pH 7.4). The cells were centrifuged at 12,000 × g for 20 min at 4°C, and the supernatant was boiled for 5 min. The pellet was solubilized in 0.2% sodium dodecyl sulfate, 20 mM Tris-HCl, and 10 μg/ml leupeptin (pH 7.4) at 4°C for 30 min, and after a 5-min

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4 M. Draoui, unpublished observations.
centrifugation at 12,000 × g for 30 min, the membrane extract was boiled for 5 min. Protein (150 µg) was separated on an 8% sodium dodecyl sulfate-polyacrylamide gel (35), transferred to nitrocellulose, and treated with protein kinase C antiserum (monoclonal antibody clone MC5; Amersham Corp.) using Western blot techniques (36).

**Sequencing.** Bacteriophage clones from a human genomic library were plaque purified, and cloned inserts were subcloned into plasmid and M13 vectors (32). Sequence analysis was performed using a modification of the Sanger dideoxynucleotide chain termination method (37). Both strands of the preproGRP promoter region were sequenced.

**Growth.** The effects of PMA and H7 on colony formation were investigated. NCI-H209 cells (5 × 10⁴) were suspended in SIT medium (RPMI 1640 containing 3 × 10⁻⁶ M Se₂O₃, 5 µg/ml insulin, and 10 µg/ml transferrin) containing 0.2% bovine serum albumin, 0.3% agarose, and 1 µM PMA and/or 100 µM H7 over a base layer of 0.5% agarose in SIT medium containing 5% fetal bovine serum. The 6-well plates were incubated for 2 weeks in a humidified incubator at 37°C. Viable colonies containing more than 50 cells were counted after staining overnight with 1 mg/ml p-nitroiodotetrazolium violet (27).

**Acknowledgments**

The authors wish to thank Drs. J. Baumgold, M. Birrer, S. Jakowlew, and F. Zia for helpful comments.

**References**


