ABSTRACT

Protein kinase D (PKD) is implicated in the regulation of a variety of important functions in small cell lung cancer (SCLC) cell lines, but the downstream signaling targets stimulated by PKCs in these cells remain poorly characterized. Here we report that treatment of the SCLC cell lines H 69, H 345, and H 510 with phorbol-12,13-dibutyrate (PDB) led to a rapid and striking activation of protein kinase D (PKD), a novel serine/threonine protein kinase distinct from all PKC isoforms. PKD activation induced by PDB in these SCLC cell lines was completely abrogated by treatment of the cells with the PKC inhibitor GF 109203X (GF I) at concentrations (0.5–2.5 μM) that did not inhibit PKD activity when added directly to the in vitro kinase assays. Treatment with the biologically active phorbol ester 12-O-tetradecanoylphorbol-13-acetate or with membrane-permeable diacylglycerols also stimulated PKD activation, which was also completely prevented by prior exposure of the cells to GF I. The PKC inhibitors Ro 31-8220 and Go 7874 also blocked PKD activation in response to PDB. Addition of the autocrine growth factor bombesin to cultures of H 345 cells induced significant PKD activation that also was prevented by GF I. Our results demonstrate, for the first time, the existence of a PKC/PKD pathway in SCLC cells and raise the possibility that PKD may be an important mediator of some of the biological responses elicited by PKC activation in SCLC cells.

INTRODUCTION

SCLC1 constitutes 25% of all pulmonary cancers and is characterized by a very low 5-year survival, despite initial sensitivity to radiotherapy and chemotherapy (1, 2). Thus, novel therapeutic strategies are needed, and these could arise from defining the factors and signaling pathways that stimulate the proliferation of SCLC.

PKC plays a central role in signal transduction pathways that mediate the action of growth factors, neuropeptides, tumor promoters, and cellular oncogenes (3, 4). In particular, PKC has been implicated in the regulation of a variety of important functions in SCLC cells including E-cadherin-mediated adhesion (5), drug resistance (6), protection against c-myc-induced apoptosis (7), colony formation in semisolid medium (8), and tumor growth in nude mice (9). Direct activation of PKCs by phorbol esters stimulates MAPK (8), transcription of gastrin-releasing peptide mRNA (10), and colony growth (8, 10) in SCLC cell lines. PKC also mediates MAPK activation and colony growth induced by a variety of neuropeptides (8) that function as autocrine/paracrine growth factors for SCLC cells (8, 11–13). It is known that SCLC cell lines express multiple diacylglycerol- and phorbol ester-sensitive PKCs, including α, β, δ, ε, and η (6, 14, 15).

However, the downstream signaling targets stimulated by PKCs in SCLC cells remain poorly characterized.

The newly identified PKD is a serine/threonine protein kinase with distinct structural features and enzymological properties (16). In particular, the catalytic domain of PKD shows little homology to the kinase domain of the PKC family and consistent with this, PKD does not phosphorylate a number of known PKC substrates in vitro, indicating that PKD has a distinct substrate specificity (16, 17). In contrast to all known PKCs, including mammalian, Drosophila, and yeast isoforms, the NH2-terminal region of PKD contains a pleckstrin homology domain that regulates enzyme activity (18) and lacks a sequence with homology to a typical PKC autoinhibitory pseudosubstrate motif (16). However, the NH2-terminal region of PKD contains a tandem repeat of cysteine-rich, zinc finger-like motifs that binds phorbol esters with high affinity (16). PKD, and its homologue PKCμ (19), can be activated in vitro by DAG/phorbol esters in the presence of PS (17, 20, 21), indicating that PKD/PKCμ are phorbol ester/DAG-stimulated protein kinases (22).

More recently, a second mechanism of PKD activation has been identified that involves PKD phosphorylation (21). Specifically, treatment of intact mouse Swiss 3T3 cells with biologically active phorbol esters (21), bryostatin (23), or growth factors (24) induces PKD activation that persists during cell disruption and immunoprecipitation. Several lines of evidence, including the use of selective PKC inhibitors and cotransfection of PKD with constitutively active mutants of PKC ε and η, indicate that PKD is activated by phosphorylation in living cells through a novel PKC-dependent signal transduction pathway (21–24). These results implied that PKD can function downstream of PKCs in signal transduction (22). Little is known, however, about the novel PKC/PKD pathway in human cancer cells. Because, as summarized above, PKC plays a central role in SCLC signal transduction, we examined whether PKD is regulated via PKC in these cancer cells.

In the present study, we demonstrate that PKD is rapidly activated in intact H 69, H 345, and H 510 SCLC cell lines in response to biologically active phorbol esters and cell-permeant DAGs. Furthermore, the neuropeptide bombesin, an autocrine growth factor of H 345 cells (25), also induced a significant activation of PKD in these cells. Our results indicate that PKD is activated in intact SCLC cells through a PKC-dependent pathway.

MATERIALS AND METHODS

Cell Culture. SCLC cell lines were generously donated by Dr. A. Gazdar (Southwestern Medical Center, University of Texas, Dallas, TX) or purchased from the American Type Culture Collection. Stock cultures of cell lines H 69, H 345, and H 510 were maintained in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (heat inactivated at 57°C for 1 h) in a humidified atmosphere of 5% CO2, 95% air at 37°C. They were passaged every 7 days. For experimental purposes, the cells were grown in HITESA, which consists of RPMI 1640 supplemented with 10 mM hydrocortisone, 5 μg/ml insulin, 10 μg/ml transferrin, 10 nM estradiol, 30 nM selenium, and 0.25% BSA.

Immunoprecipitation. SCLC cells cultured in HITESA for 3–4 days were washed three times in RPMI 1640 and lysed in 50 μg/ml TritonX-100 (pH 7.6). 2 mM EGTA, 2 mM EDTA, 1 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, and 1% Triton X-100 were added to the lysis buffer. Lysates were immunoprecipitated with the appropriate antibodies against PKCε, PKCη, PKD, or α-tubulin (30 μg/ml) and then incubated with 40 μl of protein A-Sepharose CL-4B (Sigma) overnight at 4°C. Immunoprecipitated proteins were resolved by SDS-PAGE and transferred to nitrocellulose. Membranes were blocked with 5% (w/v) nonfat dry milk in Tris-buffered saline and then incubated with antibodies against PKCε, PKCη, PKD, or α-tubulin (30 μg/ml) for 2 h at room temperature. Membranes were washed and then incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution) for 1 h. Blots were washed and then developed using the enhanced chemiluminescence system (Amersham).
The kinase activity of PKD was determined in an in vitro kinase assay by mixing 20 μl of either immunocomplexes or eluted PKD with 10 μl of a phosphorylation mixture containing (final concentration) 10 μM [γ-32P]ATP (specific activity, 400–600 cpm/pmol), 30 mM Tris/HCl (pH 7.4), 10 mM MgCl2, and 1 mM DTT. In vitro stimulation of eluted PKD was performed as described previously using PDB (200 nM) and PS (100 μg/ml) micelles (15). After 30 min of incubation at 30°C, the reaction was stopped by washing with 200 μl of kinase buffer and then adding an equal volume of 2× SDS-PAGE sample buffer [200 mM Tris/HCl (pH 6.8), 2 mM EDTA, 0.1 M NaCl, 6% SDS, 10% glycerol, and 4% 2-mercaptoethanol], followed by SDS-PAGE analysis. The gels were dried, and the 110-kDa radioactive band corresponding to autophosphorylated PKD was visualized by autoradiography. Autoradiographs were scanned in a ScanJet 6000C/T (Hewlett Packard), and the labeled band was quantified using the NIH image software program.

Exogenous substrate phosphorylation by immunoprecipitated PKD was carried out by mixing 20 μl of the washed immunocomplexes with 20 μl of the phosphorylation mixture containing 2.5 mg/ml syntide-2 (PLARTLSVA-GLPGKK), a peptide based on phosphorylation site two of glycogen synthase (22, 23). After 10 min of incubation at 30°C, the kinase reaction was stopped by adding 100 μl of 75 mM H3PO4 and then spotting 80 μl of supernatant onto P-81 phosphocellulose paper. Free [32P]ATP was separated from the labeled peptides by eluting the P-81 paper with six successive washes of 2 ml of either immunocomplexes or eluted PKD. The resulting immunocomplexes were analyzed by autoradiography. Autoradiographs were scanned to quantify the phosphoprotein in terms of peak area. Values correspond to the autophosphorylation of PKD expressed as fold increase over the unstimulated values. Inset, a representative autoradiogram of the 110-kDa autophosphorylated PKD. The results shown are the mean values of two independent experiments; bars, SE.

**RESULTS**

Expression of PKD in H 69, H 345, and H 510 SCLC Cell Lines. To examine whether PKD is expressed in SCLC cell lines, lysates of H 69, H 345, and H 510 cells were subjected to Western blot analysis using a specific antibody that recognizes the COOH-terminal region of PKD. As shown in Fig. 1A (upper), a major immunoreactive band of 110 kDa, which corresponds to the molecular mass of PKD (16), was detected in each SCLC cell line. In some experiments, we noticed that PKD isolated from SCLC cells migrates in SDS-PAGE gels as a...
PROTEIN KINASE D IN SMALL CELL LUNG CANCER CELLS

Fig. 2. PDB induces PKD activation in intact SCLC cell lines. Cultures of H 69, H 345, and H 510 SCLC cell lines cultured in HITESA were incubated in the absence (−) or in the presence (+) of 200 nM PDB for 30 min and lysed. Lysates were immunoprecipitated with PA-1 antiserum in the absence (−) or in the presence (+) of the immunizing peptide (im.pep.). To determine PKD activity, the immunoprecipitates were incubated with [γ-32P]ATP in phosphorylation mixture, and the products of the reaction were further analyzed by SDS-PAGE and autoradiography. Quantification of the level of PKD auto-phosphorylation was performed by scanning densitometry. The results shown are expressed as fold increase over the unstimulated values. The values of PKD activity from lysates of unstimulated and PDB-stimulated cells that were immunoprecipitated without the immunizing peptide are the means of 20 independent experiments, and the corresponding values with the immunizing peptide are means of three independent experiments; bars, SE. The autoradiogram depicts a representative experiment.

doublet. The detection of this (doublet) band was completely blocked when the immunoblots were incubated with the antibody in the presence of the synthetic peptide EEREMKALSERVSIL that corresponds to the COOH-terminal region of the predicted amino acid sequence of PKD.

A prominent band migrating with an identical molecular mass (110 kDa) was also obtained when lysates from the same SCLC cells lines were immunoprecipitated with the PA-1 antiserum (see “Materials and Methods”), and the immunoprecipitates were analyzed by Western blotting using a different antibody directed against PKD (Fig. 1A, lower). Detection of the 110-kDa band was blocked by the inclusion of the immunizing peptide during the immunoprecipitation (Fig. 1A). The less striking band migrating with a lower molecular mass, the detection of which was also extinguished by the immunizing peptide, is likely to represent a proteolytic fragment of PKD. These results clearly demonstrate the expression of PKD in H 69, H 345, and H 510 SCLC cell lines.

To determine the catalytic activity of PKD isolated from SCLC cells, cultures of H 69, H 345 and H 510 cells were lysed, and PKD was immunoprecipitated with PA-1 antiserum, eluted from the immunocomplexes by exposure to the immunizing peptide, and incubated with [γ-32P]ATP in the absence or presence of PS and PDB. PKD was analyzed by SDS-PAGE and autoradiography to determine the level of autophosphorylation. As shown in Fig. 1B, PKD isolated from SCLC cells had low catalytic activity. However, addition of PS and PDB to the incubation mixture caused a marked stimulation of PKD kinase activity. These results demonstrate that PKD isolated from human tumor cells can be activated in vitro by phorbol esters and indicate that this enzyme is not constitutively activated in SCLC cell lines.

PDB induces PKD Activation in SCLC Cell Lines. Next, we examined whether phorbol esters can induce PKD activation in intact H 69, H 345, and H 510 SCLC cell lines. As shown in Fig. 2, stimulation of intact SCLC cell lines with 200 nM PDB for 30 min induced a striking increase in PKD activity (measured by PKD autophosphorylation), which was maintained during cell lysis and immunoprecipitation. We verified that the immunoprecipitation of the kinase activity in PKD immunocomplexes was also abolished by the inclusion of the immunizing peptide. These results demonstrate that PDB stimulation of intact SCLC cells induces the conversion of PKD into a state that persists during cell disruption and protein isolation.

PKD activation was a rapid consequence of the addition of PDB to SCLC cells (Fig. 3A). An increase in PKD activity was detectable within 1 min and reached a maximum after 10 min of PDB stimulation in the H 69, H 345, and H 510 cell lines. Treatment with PDB also induced a time-dependent electrophoretic mobility shift, resulting in the appearance of slowly migrating forms of PKD. The mobility shift induced by PDB is likely to reflect the increase in phosphorylated species of PKD (21, 24). Thus, PKD activation is one of the early events induced by phorbol esters in SCLC cells.

Stimulation of intact H 69, H 345, and H 510 SCLC cell lines with increasing concentrations of PDB for 30 min induced a striking dose-dependent increase in PKD activity. Half-maximal PKD activation by PDB was achieved at 10–25 nM in each cell line.

![Graph](image)

**Fig. 3.** PDB induces PKD activation in SCLC cell lines in a time- and dose-dependent manner. A, the H 69, H 345, and H 510 SCLC cell lines cultured in HITESA for 3–6 days were washed and incubated with 200 nM PDB for various times as indicated. They were then lysed; immunoprecipitated with PA-1 antiserum; and subjected to in vitro kinase assay. SDS-PAGE, and autoradiography. B, cultures of H 69, H 345, and H 510 SCLC cell lines were washed and treated with various concentrations of PDB for 30 min and then lysed. The cell extracts were immunoprecipitated with the PA-1 antiserum and subjected to in vitro kinase assays, SDS-PAGE, and autoradiography. In all of the cases, the autoradiograms are representative of two independent experiments.
PROTEIN KINASE D IN SMALL CELL LUNG CANCER CELLS

The PKC Inhibitor GF I Prevents PKD Activation by PDB in SCLC Cell Lines. To determine the role of phorbol ester-sensitive PKCs in PKD activation in SCLC cells, we used inhibitors that discriminated between PKCs and PKD. SCLC cell lines were treated with various concentrations of GF I (also known as GF 109203X or bisindolylmaleimide I), a potent inhibitor of phorbol ester-sensitive isoforms of PKC (26) but not PKD (21, 24), before PDB stimulation. As shown in Fig. 4A, GF I potently blocked PKD activation induced by PDB in a concentration-dependent fashion. Treatment with GF I at a concentration as low as 0.5 μM prevented PKD activation in all of the three cell lines. In striking contrast, GF I added directly to the in vitro kinase assay, even at concentrations (0.5–2.5 μM) higher than those required to block PKD activation, did not inhibit PKD activity directly but interferes with PDB-mediated PKD activation in intact cells by blocking PKC.

Subsequently, we examined whether the effects of PDB and GF I on PKD activation in SCLC cell lines could also be demonstrated when PKD activity was measured using an exogenous substrate instead of autophosphorylation. The synthetic peptide syntide-2 (27, 28) has been identified as an efficient substrate for the catalytic domain of PKD (16, 17). As shown in Fig. 5, PDB stimulated a marked increase in syntide-2 phosphorylation by PKD immunoprecipitates from H 69, H 345, and H 510 cells. Treatment of these SCLC cell lines with GF I completely blocked the increase in syntide-2 kinase activity induced by subsequent exposure of these cells to PDB.

To substantiate the results obtained with GF I, we examined whether other inhibitors of PKC including Ro 31-8220 and Go 7874 also prevent PKD activation in response to PDB in the H 345 SCLC cell line. As illustrated by Fig. 6, A and C, treatment of intact H 345 cells with various concentrations of Ro 31-8220 and Go 7874 for 1 h before stimulating with 200 nM PDB profoundly inhibited PKD activation. Importantly, Ro 31-8220 and Go 7874 did not reduce PKD activity when added directly to the in vitro kinase assay at identical concentrations to those required to block PKD activation in vivo (Fig. 6, B and D). In contrast, inhibition of a variety of kinases, including phosphoinositide 3-kinase with wortmannin (29, 30), p70 ribosomal S6 kinase (p70S6K) with rapamycin (31, 32), p42/44MAPK with the selective MEK-1 inhibitor PD 98059 (8, 33), and protein tyrosine kinases with genistein, did not affect PKD activation in response to PDB in intact H 345 cells (Fig. 6E). These results demonstrate the specificity of the PKC inhibitors and indicate that these kinases are not upstream regulators of PKD.

Biologically Active Phorbol Esters, Diacylglycerol Analogues, and Bombesin Induce PKD Activation in H 345 Cells: Inhibition by GF I. To examine further the specificity of the phorbol ester-induced activation of PKD in SCLC, we carried out in vitro kinase assays using immunoprecipitates from H 345 cells exposed to PDB, TPA, and the membrane-permeable DAG analogues OAG and diC8 in the absence or presence of GF I. Fig. 7A shows that treatment with TPA stimulated PKD activity to the same extent as PDB. The stimulatory effect of TPA was prevented by prior treatment with GF I. The exogenously added DAG analogues OAG and diC8 also stimulated PKD activation, which was completely abolished by pretreatment of the cells with 2.5 μM GF I. In contrast, neither 4-α-phorbol nor the biologically inactive Me-TPA added to intact SCLC cells induced PKD activation (results not shown).

Binding of bombesin to its receptor in H 345 cells is known to induce phospholipase C-mediated formation of the second messengers DAG and inositol 1,4,5-trisphosphate, which activate classic and novel isoforms of PKC and mobilize Ca2+, respectively (8, 34, 35). We assessed whether bombesin can stimulate PKD activation in H 345
cells. As shown in Fig. 7B, addition of bombesin to these cells induced a detectable increase in PKD activation, as measured by either auto-
phosphorylation or syntide-2 phosphorylation assays. The increase in
syntide-2 kinase activity induced by bombesin was abrogated by prior
treatment with GF I, indicating that bombesin stimulates PKD acti-
vation via PKC (Fig. 7B, right).

DISCUSSION

As a first step to elucidate the role of PKD in tumor cells, we exam-
ined the regulation of this enzyme in SCLC cells, a clinically
aggressive tumor that constitutes 25% of all pulmonary cancers. Our
results demonstrate that treatment of intact SCLC cell lines H 69, H
345, and H 510 with tumor-promoting phorbol esters or membrane-
permeant DAGs induces rapid PKD activation. PKD recovered from
stimulated SCLC cells is active in the absence of lipid effectors (e.g.,
PS and PDB). The conversion of PKD into this activated state occurs
within minutes of phorbol ester stimulation of intact SCLC cells and
thus is one of the early events induced by phorbol esters in SCLC
cells.

PKD activation in SCLC cells is associated with a time-dependent
retardation of its electrophoretic mobility, indicating that the enzyme
undergoes phosphorylation. It is likely that covalent modification of
PKD in living SCLC cells treated with phorbol esters is responsible
for the stable activated state of this enzyme. At least two different
mechanisms involving phosphorylation could be responsible for
maintaining the activated state of PKD. Direct stimulation of PKD by
phorbol esters or DAGs could induce an activating autoprophospho-
ylation of the enzyme. Alternatively, PKD activation could be induced by trans-phosphorylation involving a different proximal kinase.

We found that treatment of SCLC cells with the PKC inhibitors GF
I, Ro31-8220, and Go 7874 before stimulation with phorbol esters
strikingly prevents PKD activation. Importantly, these PKC inhibitors
do not reduce PKD activity when added directly to the in vitro kinase
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naling pathways, including the p70S6K which is constitutively active
in SCLC cells (32) or the p42 mapk /p44 mapk pathway which is mark-
edly stimulated by phorbol esters in SCLC cells via PKC (8), did not
affect PKD activation by PDB. Furthermore, the addition of bombesin
to cultures of H345, which are known to express receptors for this
neuropeptide (36), induced significant PKD activation that also was
prevented by inhibitors of PKC. These findings indicate that PKD is
activated in intact SCLC cells by biologically active phorbol esters,
DAGs, and the neuropeptide bombesin through a PKC-dependent
signal transduction pathway.

PKC consists of multiple isoforms with possibly different biologi-
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ical (ζ, λ, and ι) are not regulated by these effectors. Recent studies
demonstrated that cotransfection of PKD with constitutively activated mutants of PKCε and ε strongly induced PKD activation in COS-7 cells (21). SCLC cell lines are known to express both PKCε and ε (6, 14, 15), and a variant SCLC cell line (N417) has been reported to express a constitutively active fragment of PKCε (15), which could lead to constitutive activation of PKD. Our results with H 69, H 345, and H 510 SCLC cell lines indicate that PKD is not in a constitutively active state but can be strikingly activated within these cells via PKC.

A salient feature of the results presented here is that PDB-induced PKD activation in SCLC cells is blocked by PKC inhibitors at concentrations substantially (5-fold) lower than those required to attenuate PKD activation in other cell types including Swiss 3T3 cells (21, 23). It is likely that this difference is due to expression of PKC isoforms in SCLC (e.g., PKC ε), which are not found in Swiss 3T3 cells (37). Regardless of the precise explanation, our results demonstrate that PKD activation can be blocked by low doses of PKC inhibitors in SCLC cells.

PKD has been implicated as a major signal transduction pathway in the regulation of a variety of functions in SCLC cells including adhesion, apoptosis, colony formation, and tumor growth in nude mice (see the “Introduction” for references). PKC also mediates MAPK activation and colony growth induced by neuroepitopes, which function as autocrine/paracrine growth factors for SCLC cells. The precise signal transduction pathways by which PKC induces these effects in SCLC cells remain unknown. Our results, showing that PKD can function downstream of PKC in these cells, raise the possibility that PKD mediates some of the biological responses elicited by PKC in SCLC cells.

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Protein Kinase D in Small Cell Lung Cancer Cells: Rapid Activation through Protein Kinase C

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