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Cellular protein kinase C isozyme regulation by exogenously delivered physiological disulfides—implications of oxidative protein kinase C regulation to cancer prevention

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We reported previously that cystine produces regulatory responses in purified, recombinant human protein kinase C-δ (PKCδ) and PKCε via *S*-thiolation-triggered mechanisms that are consistent with a cancer preventive effect, i.e. stimulation of the pro-apoptotic, tumor-suppressive isozyme PKCδ and inactivation of the growth-stimulatory, oncogenic isozyme PKCε, at *S*-cysteinylation stoichiometries that correspond to modification of a single redox-regulatory cysteine (Cys) switch in each isozyme. In this report, we show that the oxidative regulatory responses of purified PKCδ and PKCε to cystine are recapitulated in disulfide-treated cells. We report that treatment of COS7-PKCε transfectants with the cystine precursor cystine dimethyl ester (CDME) produced concentration- and time-dependent PKCε inactivation that was associated with oxidative PKCε modification manifested as attenuated band intensity in PKCε immunoblot analyses, and that both PKCε inactivation and modification were reversed by dithiothreitol (DTT) as well as by thioredoxin. We also show that CDME induced biphasic PKCδ regulation in COS7-PKCδ transfectants, with DTT-irreversible PKCδ stimulation at low and DTT-reversible PKCδ inactivation at high CDME concentrations. The degrees of PKCδ versus PKCε inactivation by CDME treatment of COS7-PKC transfectants indicate substantial resistance of PKCδ to inactivation. The PKCδ stimulatory response in COS7-PKCδ cells was triggered only by the disulfide agent and not by its reduced thiol counterpart, providing evidence for an oxidative mechanism. Also paralleling the oxidative stimulation of purified PKCδ by cystine, the stimulation of PKCδ elicited by CDME treatment of cells involved a stable structural change, which was evident from the stability of the stimulated form of PKCδ to immunoprecipitation. Demonstration of oxidative regulation of cellular PKCδ and PKCε by disulfides in this report provides evidence that redox-regulatory sites in PKCδ and PKCε may offer novel targets for development of cancer preventive or therapeutic agents that selectively inactivate PKCε or stimulate PKCδ.

Introduction

Protein kinase C (PKC) is a Ser/Thr protein kinase family with 10 members (1). The importance of PKC signaling to phorbol ester (PE)-mediated tumor promotion/progression (TP/P) was originally suggested by the discovery that 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and other PE tumor promoters potently and selectively activate several PKC isozymes through mimicry of the endogenous activating cofactor sn-1,2-diacylglycerol (DAG) (2,3), and was later corroborated by investigations that characterized the roles of individual PKC isozymes in cell growth regulation and transformation by enforcing their expression in non-tumorigenic cells (4–14). Recent studies in transgenic mice with enforced PKC isozyme expression targeted to the skin have firmly established that PKCε is a driving force in PE-mediated TP/P that markedly enhances formation of malignant tumors (carcinomas) and also potentiates tumor metastasis in response to DMBA (7,12-dimethylbenz[*a*]anthracene) and TPA (15,16), and that PKCδ robustly opposes the formation of benign skin tumors (papillomas) as well as carcinomas in response to DMBA/TPA (17). In excellent agreement with these findings, studies in non-transformed epithelial cells and fibroblasts have defined PKCε as growth-stimulatory and oncogenic (6–11,18,19) and PKCδ as growth-suppressive and pro-apoptotic (6,12,20–27). The molecular basis of the oncogenic activity of PKCε has been pinpointed to Raf-1 activation (8,11), and the pro-apoptotic action of PKCδ is strongly linked to loss of mitochondrial membrane potential (20,22,23,26,27). These observations constitute a solid rationale for cancer preventive strategies that inactivate PKCε or stimulate PKCδ activity.

Human PKC isozymes contain 16–28 cysteine (Cys) residues, including one or two Zn^{2+} finger structures in the regulatory domain with six Cys per structure (28). Activation of PKC isozymes by phospholipid-dependent DAG binding to the Cys-rich Zn^{2+} finger structures plays a critical role in PKC regulation, and DAG surrogates such as TPA potently activate PKC by this mechanism with profound pathophysiological consequences (3). In addition, reversible oxidative modifications of Cys in PKC isozymes alter kinase activity, implicating thiol-directed redox mechanisms in PKC regulation (28). Protein *S*-thiolation is an oxidative post-translational modification that entails non-enzymatic disulfide linkage of small thiols such as glutathione (GSH) to select, redox-sensitive Cys in proteins (29–31). We found recently that disulfide forms of thiol antioxidants that antagonize PE-mediated TP/P, such as GSH and cysteine (32–34), induce marked changes in PKC isozyme activity via *S*-thiolation that are consonant with a cancer preventive outcome, e.g. PKCδ stimulation and PKCε inactivation (35). In addition to PKCε, other isozymes with oncogenic or pro-inflammatory activity, e.g. PKCγ and PKCα, respectively (5,36), were inactivated by the *S*-thiolation-triggered mechanism (35). Importantly, in contrast with our previous observations that several growth-stimulatory PKC isozymes were similarly sensitive to inactivation by

Abbreviations: βME, β-mercaptoethanol; CDME, cystine dimethyl ester; Cys, cysteine; DAG, diacylglycerol; DMBA, dimethylbenz[*a*]anthracene; DTT, dithiothreitol; FBS, fetal bovine serum; GSH, glutathione; IP, immunoprecipitate; NAC, *N*-acetylcysteine; PE, phorbol ester; PKC, protein kinase C; PS, phosphatidylserine; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TP/P, tumor promotion/progression.

diamide-induced *S*-glutathiolation (37), a given disulfide produced variable rather than uniform responses among the isozymes inactivated by *S*-thiolation. For example, cystine fully inactivated PKCε but did not affect PKCα activity (35). Furthermore, disulfide-induced regulatory responses were associated with *S*-thiolation stoichiometries that implicated a single modified Cys in each isozyme (35). These observations have led us to hypothesize that redox-regulatory Cys in PKC may offer novel targets for design of isozyme-selective cancer preventive agents, e.g. PKCε inactivators and PKCδ activators.

Our previous studies of PKC isozyme regulation by disulfides focused on biochemical analysis of the regulatory mechanism with purified human recombinant PKC isozymes. A question of critical importance to our hypothesis is whether the disulfide-induced PKC regulatory responses are elicited in cells. In this report, we show that exogenously delivered, physiological disulfides induce marked PKCε inactivation and substantial PKCδ stimulation in mammalian cells by an oxidative mechanism.

Materials and methods

Materials

Cells. Human hepatoblastoma HepG2 cells and SV40-transformed, monkey kidney COS-7 cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). The cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), non-essential amino-acids, L-glutamine, and penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO_2/95% air. Both cell lines were used within 5–12 passages of the passage supplied by ATCC. DMEM, Hanks' Balanced Salt Solution, phosphate-buffered saline (PBS), trypsin, FBS and dialyzed FBS, other cell culture reagents and LipofectAMINE PLUS were from Invitrogen Life Technologies (Carlsbad, CA).

Plasmids, antibodies, chemicals and other reagents. PKC-pcDNA3 expression plasmids were generously provided by Dr Jae-Won Soh (Columbia University, NY). The isozyme-selective PKC antibodies employed for western analysis were monoclonal anti-PKCα, anti-PKCδ, anti-PKCε and anti-PKCλ from BD Biosciences (San Diego, CA), rabbit polyclonal anti-PKCβ_1 (sc-209), anti-PKC-β_2 (sc-210), anti-PKCγ (sc-211), anti-PKCδ (sc-937), anti-PKCε (sc-214), anti-PKCη (sc-215) and anti-PKCθ (sc-212) from Santa Cruz Biotechnology (Santa Cruz, CA), and rabbit polyclonal anti-PKCζ from Upstate Biotech (Lake Placid, NY). Purified human recombinant PKC isozymes from Pan Vera (Madison, WI) served as positive controls in immunoblots and were also used to analyze purified PKC isozyme activity. For immunoprecipitation of PKC isozymes, Santa Cruz rabbit polyclonal anti-PKCα (sc-208) and BD Biosciences monoclonal anti-PKCε were utilized. In addition, FLAG-tagged PKCδ was immunoprecipitated with anti-FLAG M2 monoclonal antibody (mAb) (Sigma Chemical Co., St Louis, MO). Horseradish peroxidase (HRP)-linked sheep anti-mouse and donkey anti-rabbit immunoglobulins from Amersham Pharmacia Biotech were respectively used as secondary antibodies with monoclonal and polyclonal primary antibodies. Molecular weight markers, 2-mercaptoethanol, all other SDS–PAGE reagents and protein assay reagent were purchased from Bio-Rad (Hercules, CA); gels were run on the Bio-Rad Mini-Protean II System. [γ-^{32}P]ATP was purchased from NEN Life Science (Boston, MA) and Hyperfilm enhanced chemiluminescence (ECL) from Amersham Pharmacia Biotech. [Ser25]PKC(19-31), a synthetic peptide-substrate of PKC isozymes, was purchased from Bachem Bioscience (King of Prussia, PA), and DAG and bovine brain PS (>98% pure) from Avanti Polar Lipids (Alabaster, AL). Purified *Escherichia coli* thioredoxin-reductase was purchased from American Diagnostica (Greenwich, CT), and recombinant *E.coli* thioredoxin, NADPH and microcystin from Calbiochem-Novabiochem (La Jolla, CA). Dithiothreitol (DTT) and GF109203X were purchased from Alexis Biochemicals (San Diego, CA). Cystine dimethyl ester (CDME), cystamine, cysteamine, *N*-acetylcysteine, ATP, histone III-S, TPA, IGEPAL, protein A–Sepharose, sodium orthovanadate, protease inhibitors, buffers, chelators and all other reagents were purchased from Sigma-Aldrich (St Louis, MO).

Methods

COS-7 cell transfection. COS-7 cells were seeded at a density of 1×10^6 cells/10 cm dish and allowed to proliferate at 37°C in growth media until reaching 75–80% confluency ~48 h later. Growth media was removed and replaced with serum-free media for 30 min at 37°C. The cells were transfected by incubation for 5 h at 37°C with 20 μg pcDNA3 ('empty vector' control), mouse PKCδ-pcDNA3, mouse C-terminally FLAG-tagged PKCδ (PKCδ-FLAG)-pcDNA3, or mouse PKCε-pcDNA3 combined with LipofectAMINE PLUS, according to the manufacturer's instructions. Cells were then incubated for 20 h at 37°C with growth media lacking sulfur-containing amino acids and containing 10% dialyzed serum, and treated with disulfides as described below.

Disulfide treatment of cells and preparation of cell lysates. HepG2 and COS-7 cells were depleted of low molecular weight thiols by replacing growth media with DMEM lacking sulfur-containing amino acids (SAA-free media) and containing 10% dialyzed FBS for 20 h at 37°C (31,38). Next, the cells were treated with disulfide (cystamine or CDME) or with the corresponding reduced thiol at indicated concentrations in SAA-free media containing 5% dialyzed FBS, typically for 30 min at 37°C. At the end of the treatment period, the cells were washed with ice-cold PBS. PBS was then removed, and 0.65 ml lysis buffer was added per plate at 4°C; [lysis buffer = 20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 10 μg/ml leupeptin, 250 μM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, 20 mM sodium fluoride and 10 nM microcystin]. The cells were immediately scraped from the plate and transferred to a microcentrifuge tube that was kept on ice. The cells were lysed by sonication on ice (three 10-s pulses), and cellular debris removed by centrifugation at 4°C (5 min, 14 000 *g*). Lysates were assayed for protein content using a Coomassie dye-based reagent and then normalized to protein concentrations of 1.5 mg/ml (HepG2 cells) or 0.2 mg/ml (COS-7 transfectants) by adjusting sample volumes.

Western analysis of PKC isozymes. Western analysis of PKC isozymes in cell lysates was done under both non-reducing and reducing conditions. Cell-lysate samples were prepared for 10% SDS–PAGE by boiling for 5 min in SDS–PAGE sample buffer lacking or containing 5% 2-mercaptoethanol (final concentration) (31,38). Protein was transferred from gels to nitrocellulose membranes and analyzed for PKC content by standard immunoblot methods with isozyme-selective PKC antibodies and HRP-linked secondary antibodies (listed under Materials). Immunoreactive bands were detected by ECL (31,38).

Analysis of cell-lysate PKC activity. To detect DTT-reversible changes produced in cellular PKC activity by disulfide treatment of cells, each cell-lysate sample was divided into two fractions incubated with or without 30 mM DTT for 15 min at 30°C. At the end of the incubation period, the samples were placed on ice and assayed for PKC activity (35). Where indicated, thioredoxin-reversible changes in PKC activity were measured by incubating cell lysates with or without 25 μM thioredoxin–0.5 μM thioredoxin reductase–1 mM NADPH for 1 h at 30°C (35) followed by PKC activity analysis. In the COS7-PKC cell-lysate analysis, PKC assay mixtures contained 20 mM Tris–HCl, pH 7.5, 1 mM EGTA, 10 mM $MgCl_2$, 30 μg/ml PS, 30 μg/ml DAG, 50 μM [Ser25]PKC(19–31), 6 μM [γ-^{32}P]ATP (5000–8000 c.p.m./pmol) and 5 μg cell-lysate protein in a volume of 120 μl (35). For analysis of HepG2 cell lysates, the assay mixture composition was modified to include 40 μg cell-lysate protein and increased concentrations of PS (125 μg/ml), [Ser25]PKC(19–31) (100 μM), and [γ-^{32}P]ATP (100 μM). Background kinase activity was measured by including the pan PKC inhibitor GF109203X (1 μM) (39) in control assay mixtures. Assays (10 min, 30°C) were initiated by addition of [γ-^{32}P]ATP and terminated on phosphocellulose paper (35). This method was also utilized to assay purified PKC isozymes. PKC activity is defined as total minus background kinase activity. Each activity value is expressed as the mean of triplicate determinations, and results shown were reproduced in independent experiments.

Kinase assays of immunoprecipitated PKC isozymes. To immunoprecipitate (IP) mouse PKCδ-FLAG and PKCε from COS7-PKC cell lysates, 500 μg cell-lysate protein was incubated with 5 μg FLAG M2 mAb or 5 μg PKCε mAb overnight at 4°C in IP buffer in a total volume of 1 ml (IP buffer = 20 mM Tris–HCl, pH 7.5, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% IGEPAL, 10 μg/ml leupeptin, 0.2 mM PMSF). Next, 200 μl of a 50% protein A–Sepharose slurry was added to the mixture, which was further incubated for 2 h at 4°C. Beads were spun down and subjected to three cycles of washing/centrifugation with 1 ml IP buffer per cycle followed by suspension of the beads in 500 μl 20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA. To assay the kinase activity of immunoprecipitated PKCδ-FLAG or PKCε, PKC assays were carried out as described under 'Analysis of cell-lysate PKC activity' except that the beads were added to kinase assay mixtures (4–10 μl beads per assay tube) in lieu of cell-lysate protein. The same procedures were followed to assay PKCα immunoprecipitated from HepG2 cells, except that 1 mg cell-lysate protein was incubated with 5 mg anti-PKCα, and PKC assay mixtures contained increased concentrations of PS (125 μg/ml), [Ser25]PKC(19–31) (100 μM) and [γ-^{32}P]ATP (100 μM).

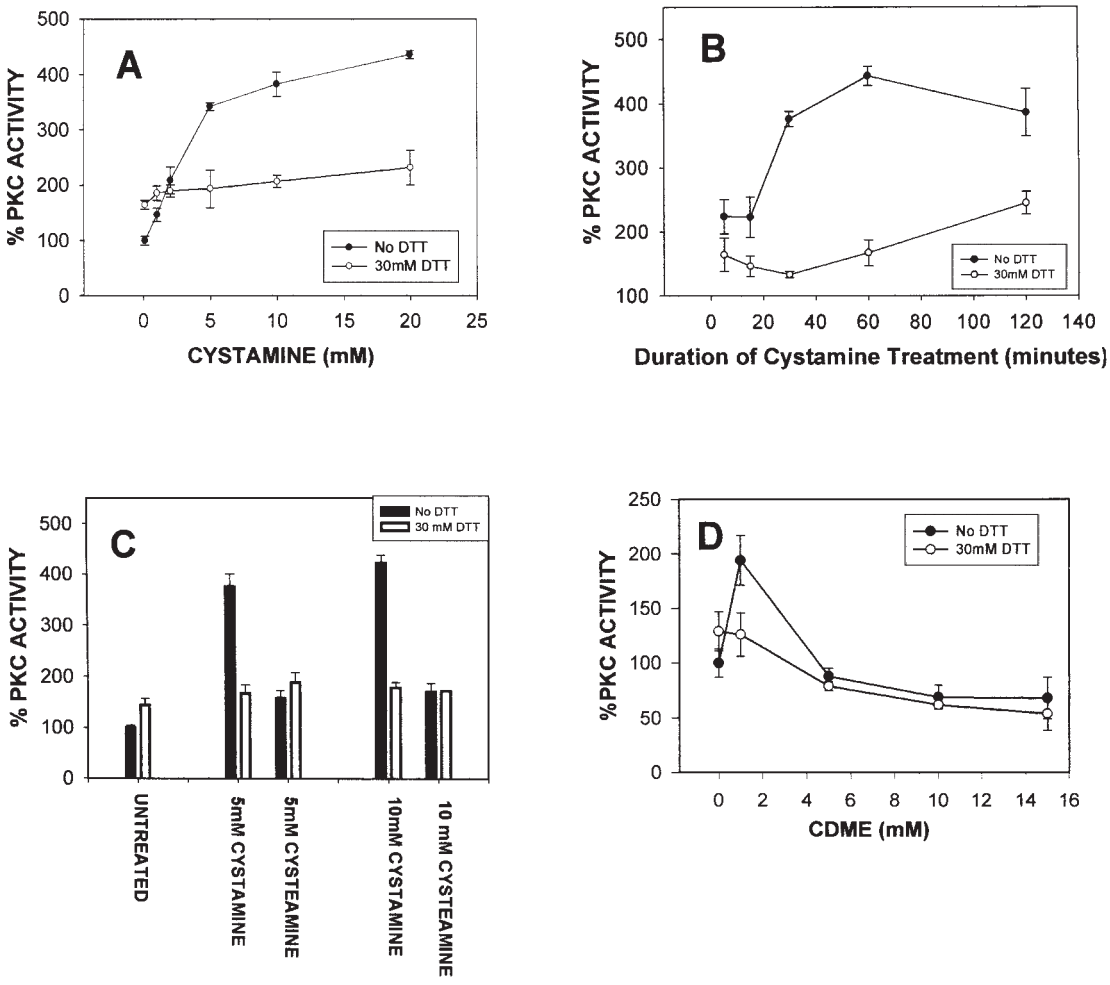


Fig. 1. Cystamine induces DTT-reversible PKC regulation in HepG2 cells. HepG2 cells, incubated in media lacking sulfur-containing amino acids (20 h, 37°C), were treated with cystamine or CDME, and lysed by sonication. Lysates were incubated with/without 30 mM DTT (15 min, 30°C) and then assayed for PKC activity (40 μg cell-lysate protein/assay). In each panel, 'No DTT' and '30 mM DTT' denote assays of the lysate fractions preincubated without or with DTT, respectively. For other experimental details, see Methods. Each PKC activity value is the average from triplicate determinations (±SD), and results shown in each panel (A–D) were reproducible in an independent analysis. (**A**) Concentration dependence of cystamine-induced, DTT-reversible PKC regulation in HepG2 cells. Cells were treated with a range of cystamine concentrations (30 min, 37°C), and cell-lysate PKC activity was assayed. (**B**) Time course of cystamine-induced, DTT-reversible PKC regulation in HepG2 cells. Cells were treated with 10 mM cystamine at 37°C for the time intervals specified, and cell-lysate PKC activity was assayed. (**C**) PKC-regulatory effects of HepG2 cell treatment with cystamine versus cysteamine. Cells were treated with cystamine or cysteamine (30 min, 37°C), and cell-lysate PKC activity was assayed. (**D**) Concentration dependence of CDME-induced, DTT-reversible effects on PKC activity in HepG2 cells. Cells were treated with a range of CDME concentrations (30 min, 37°C), and cell-lysate PKC activity was assayed.

Results

Cystamine treatment induces protein *S*-thiolation in isolated hepatocytes and perfused liver (40) and potently inhibits tissue transglutaminase in various cell models by a thiol-disulfide exchange mechanism (41). To investigate whether cystamine oxidatively regulates PKC activity in cells, we examined the effects of cystamine on PKC activity in human hepatoma HepG2 cells, a widely studied epithelial cell model (42–44). To mitigate cystamine induction of GSH synthesis in the cells (45,46), which would counteract production of oxidative protein modifications by the disulfide, we maintained HepG2 cells in growth media lacking sulfur-containing amino acids for 20 h prior to cystamine treatment.

HepG2 cells were treated at 75–80% confluency with graded concentrations of cystamine for 30 min at 37°C, and then lysed. The induction of DTT-reversible PKC regulation by cystamine was measured by PKC assays of the cell lysates, which were first incubated with/without 30 mM DTT (15 min, 30°C). The analysis of cell lysates that were not exposed to DTT (Figure 1A, closed circles) demonstrated concentration-dependent PKC stimulation by cystamine with a maximal effect of 4-fold at 10–20 mM cystamine and a half-maximal response at ~2 mM cystamine. In the lysates treated with DTT, the concentration dependence of the response was attenuated and flattened (Figure 1A, open circles), revealing a major DTT-reversible component in cystamine-induced PKC stimulation, which is indicative of oxidative regulation (28,31). The modest enhancement of PKC activity by DTT alone (Figure 1A, open circles, 0 mM cystamine) is consistent with non-specific thiol stabilization against air oxidation by the reducing agent. The time course analysis in Figure 1B shows that 4-fold DTT-reversible PKC stimulation was reached between 15 and 30 min of HepG2 cell exposure to 10 mM cystamine and was sustained at 2 h, although the DTT-reversible character of the response appeared to subside at the 2 h time point.

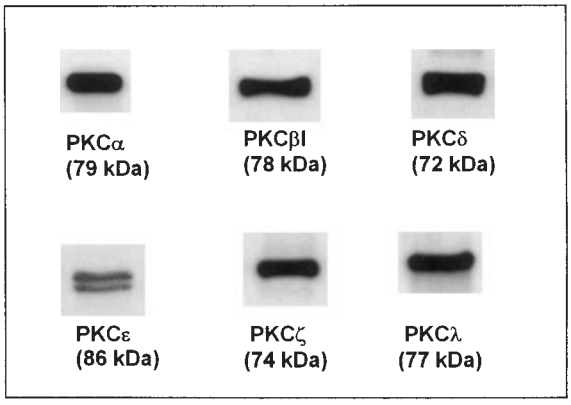


Fig. 2. PKC isozyme expression in HepG2 cells. PKC isozymes were detected by western analysis of a HepG2 cell lysate with a comprehensive panel of isozyme-selective, human-reactive PKC antibodies (40 μg cell-lysate protein/lane). For other details, see Methods. Isozymes detected are shown and the apparent molecular weights measured (10% SDS–PAGE) are specified; PKCβ_2, γ, η and θ were not detected.

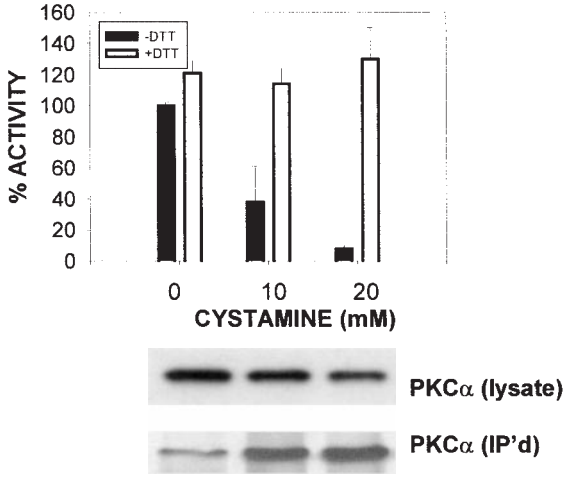


Fig. 3. Cystamine induces DTT-reversible PKCα inactivation in HepG2 cells. HepG2 cells were treated with cystamine (30 min, 37°C) under the conditions employed in Figure 1A followed by cell lysis, immunoprecipitation of PKCα, treatment of immunoprecipitated PKCα with/without 30 mM DTT (15 min, 30°C) and assay of the kinase activity. Black and white bars are PKC activity values measured for immunoprecipitated PKCα preincubated without and with DTT, respectively. Immunoblot analyses with PKCα antibodies were done under standard reducing conditions (SDS–PAGE) with 40 μg cell-lysate protein/lane and 60 μl immunoprecipitated PKCα/per lane. Other experimental details are provided in Methods. PKCα activity values are expressed as means ± SD of triplicate determinations, and the results shown were reproduced in an independent analysis.

To probe the importance of the disulfide bond in cystamine to induction of oxidative PKC regulation in HepG2 cells, we compared the effects of treating HepG2 cells with cystamine versus cysteamine, the reduced thiol counterpart of the disulfide. Figure 1C shows that under conditions where cystamine (5 and 10 mM) produced 4-fold DTT-reversible PKC stimulation, cysteamine produced only a 1.5–1.8-fold DTT-insensitive enhancement tantamount to the stimulation achieved by DTT, suggesting involvement of non-specific thiol-stabilizing effects. These results indicate an essential role for the disulfide bond in the oxidative stimulation of PKC activity provoked by cystamine treatment of HepG2 cells. Furthermore, in addition to cystamine, CDME, which is a metabolic precursor of cystine (47), induced oxidative PKC regulation in HepG2 cells, albeit modestly. CDME is much more efficient than cystine itself for loading cells with the physiological disulfide because of membrane permeant properties afforded by esterification and the ready intracellular conversion of CDME to cystine by esterases (47). CDME produced a biphasic response characterized by 2-fold DTT-reversible PKC stimulation at 1 mM CDME and a return to near-baseline PKC activity levels at higher CDME concentrations, with minor DTT-insensitive loss of PKC activity evident (Figure 1D).

Purified PKC isozymes exhibit differential responses to disulfides (35). The results shown in Figure 1 pertain to the effects of cystamine and CDME treatment of HepG2 cells on the net activity of PKC isozymes present in the cell lysates. Western analysis of the HepG2 cell lysate with a complete battery of isozyme-selective PKC antibodies revealed expression of PKCδ and PKCε, which are respectively stimulated and inactivated by cystamine and cystine in their purified forms (35), as well as PKCα, PKCβ_1, PKCζ and PKCλ (Figure 2). We therefore focused on individual isozyme responses to characterize the robust DTT-reversible PKC regulatory response to cystamine in HepG2 cells. Analysis of DTT-reversible regulation of purified PKCα by cystamine established that pretreatment with the disulfide (20 min, 30°C) monophasically inactivates PKCα by an *S*-thiolation-triggered mechanism with an IC_{50} of 0.21 ± 0.04 mM. To ascertain the effects of cystamine treatment on PKCα activity in HepG2 cells, we immunoprecipitated PKCα from lysates of HepG2 cells treated with cystamine. For purposes of measuring DTT-reversible regulation, immunoprecipitated PKCα was pretreated with/without 30 mM DTT (15 min, 30°C) and then assayed for kinase activity. Cystamine treatment of the HepG2 cells produced concentration-dependent inactivation of immunoprecipitated PKCα that was fully reversed by DTT treatment of the isozyme (Figure 3). Only modest changes were evident in PKCα expression in response to cystamine treatment by western analysis of the cell lysates (Figure 3), and western analysis of immunoprecipitated PKCα indicated that cystamine treatment of the cells improved recovery of PKCα by immunoprecipitation (Figure 3). Thus, DTT-reversible inactivation of purified PKCα by cystamine is recapitulated in cystamine-treated cells, suggestive of a common inactivation mechanism entailing PKCα *S*-cysteaminylation. Furthermore, the results provide evidence that PKCα regulation by cystamine does not contribute to, and may detract from, the DTT-reversible stimulation of PKC activity observed in lysates of cystamine-treated HepG2 cells (Figure 1).

We next analyzed the effects of cystamine on purified forms of other PKC isozymes expressed in the HepG2 cells. Preincubation with cystamine (20 min, 30°C) inactivated PKCβ_1 (IC_{50} = 0.34 ± 0.05 mM) and PKCζ (IC_{50} = 0.12 ± 0.04 mM). Likewise, we found previously that cystamine inactivates purified PKCε (IC_{50} = 95 ± 5 μM) (35). In contrast, cystamine stimulated PKCδ (35), with 3.5–4.0-fold PKCδ stimulation sustained by 0.5–2.5 mM cystamine. PKCλ (also designated PKCι) is not available in purified form but its close structural relatedness to PKCζ suggests that it too is probably inactivated by cystamine. The purified isozyme responses to cystamine suggest PKCδ involvement in the stimulation of HepG2 cell-lysate PKC activity shown in Figure 1. However, with the exception of PKCα, the kinase activity recovered when PKC isozymes were immunoprecipitated from the HepG2 cells was inadequate for analysis, probably due to factors that include

isozyme expression levels, specific activities and attenuated catalytic efficiency of the kinases in immunoprecipitated complexes. We therefore turned to transient expression of PKC isozymes in COS-7 cells as an alternative system producing robust PKC isozyme expression (48,49) to define cystamine and CDME effects on the activity of individual cellular PKC isozymes. In these studies, we focused on PKCδ and PKCε because their prominent roles in TP/P (15–17) implicate the cystine- and cystamine-induced regulatory responses of the isozymes in purified form, i.e. PKCδ stimulation and PKCε inactivation, as attractive cancer preventive strategies (35).

COS7-PKCε transfectants, produced as described in Methods, typically exhibited a 5-fold enhancement in cell-lysate PKC activity compared with COS-7 cells transfected with empty vector (pcDNA3), and this was accompanied by a sharp increase in PKCε expression (compare V versus 0, Figure 4A). To measure the effects of CDME on PKCε activity in COS7-PKCε cells, the cells were treated with CDME under the conditions employed in Figure 1, and cell lysates were recovered and preincubated with/without 30 mM DTT (15 min, 30°C) prior to analysis of PKC activity. Figure 4A (black bars) shows that treatment of COS7-PKCε cells with graded CDME concentrations (30 min, 30°C) produced a concentration-dependent loss of up to 80% of the PKC activity measured in cell lysates lacking DTT, while having no effect on PKCε expression, measured by western analysis under standard reducing conditions (βmercaptoethanol; +βME). Because ectopically expressed PKCε contributed most of the PKC activity measured in COS7-PKCε cell lysates in Figure 4A, the results are indicative of marked PKCε inactivation. Furthermore, inactivation was strongly reversed by DTT (Figure 4A, white bars), indicating an oxidative inactivation mechanism. Also supportive of CDME-induced oxidative PKCε regulation in the cells, PKCε inactivation was associated with the introduction of thiol-reversible, covalent modifications in PKCε. This is indicated by the loss of PKCε band intensity in the non-reducing western analysis in response to CDME treatment (−βME, Figure 4A), when compared with the uniform PKCε band intensity in the corresponding reducing western analysis (+βME). Loss of PKCε band intensity in the non-reducing western analysis could reflect a diffuse migration pattern of the oxidatively modified isozyme in SDS–PAGE or direct interference with antibody recognition of the PKCε epitope by the redox modification. To distinguish between these possibilities, we treated the nitrocellulose membrane used in the non-reducing PKCε immunoblot analysis with 0.14 M 2-mercaptoethanol (30 min, 25°C) and then reprobed the membrane with the PKCε antibody. The loss of band intensity associated with CDME-induced PKCε inactivation persisted, indicating that the redox modification caused PKCε to migrate diffusely in SDS–PAGE (data not shown).

The results shown in Figure 4A were corroborated by analyzing PKCε immunoprecipitated from lysates of CDME-treated COS7-PKCε cells. Figure 5A shows that 5 and 10 mM CDME applied to COS7-PKCε cells produced >95% inactivation of PKCε immunoprecipitated from cell lysates with 60–70% of the control activity level recovered by incubation of the immunoprecipitated isozyme with 30 mM DTT (1 h, 30°C). In the western analysis of the immunoprecipitated PKCε complexes, PKCε band intensities were uniform (Figure 5A). Thus, pronounced DTT-reversible PKCε inactivation is evident in COS7-PKCε cells in response to CDME treatment, when PKCε is recovered and assayed in an immunoprecipitated complex, where the baseline activity contributed by endogenous kinases is excluded, and when PKCε is assayed in its more physiologically relevant form, free in solution in the cell lysate.

Time course analysis indicated that 10 mM CDME elicited marked PKCε inactivation in the COS7-PKCε cells within 15 min, which was evident from the sharp loss of cell-lysate PKC activity, and pronounced PKCε inactivation was sustained as a DTT-reversible response for the duration of the 2 h time course, with no change in PKCε expression (Figure 4B). Comparing the reducing and non-reducing western analyses in Figure 4B, it is evident that CDME exposure produced a progressive, thiol-reversible loss of PKCε band intensity over time, reinforcing the association between thiol-reversible PKCε inactivation and reversible oxidative PKCε modification. To test the role of the disulfide bond of CDME in the oxidative inactivation of cellular PKCε, we compared the effects of treating COS7-PKCε cells with CDME versus *N*-acetylcysteine (NAC) on cellular PKCε activity. Figure 4C shows that NAC had no discernible effect on cellular PKCε activity, measured by assays of cell lysates, at concentrations where CDME profoundly inactivated PKCε. Consistent with this, the accompanying non-reducing western analysis in Figure 4C shows that, in contrast with CDME, NAC failed to affect PKCε band intensity. These results indicate a role for the disulfide bond of CDME in the oxidative inactivation of cellular PKCε.

Having established that CDME treatment of COS7-PKCε cells recapitulates the oxidative inactivation of purified PKCε by cystine, we next examined the effects of cystamine in the COS-7 system. Figure 4D shows that cystamine induced marked, concentration-dependent PKCε inactivation in COS7-PKCε cells, measured by analysis of the cell-lysate activity that was robustly reversed by DTT. [It is worth noting that non-reducing western analysis was not a reliable approach to detect cystamine-induced oxidative modification of cellular PKCε (compare Figures 4D and 5B), suggesting lability of the modification during the analysis.] Analysis of PKCε immunoprecipitated from COS7-PKCε cell lysates (Figure 5A) revealed 80–90% PKCε inactivation in cells treated with 5 and 10 mM cystamine. Strikingly, the control PKCε activity level (100% activity) was fully restored by treatment of inactivated immunoprecipitated PKCε with DTT. These results indicate a purely oxidative mechanism of cystamine-induced inactivation of cellular PKCε.

The profound degree of cellular PKCε inactivation attained by cystamine and CDME is suggestive of a mechanism that directly targets the active site. Theoretically, an active site-directed inactivation mechanism could be potentiated by agents that expose the active site by inducing the activated conformation of PKCε. To examine this question, we co-treated COS7-PKCε cells with the PKC activator TPA (20 nM) and either CDME or cystamine (30 min, 37°C). To facilitate detection of potentiating effects of TPA co-treatment, cystamine and CDME were each administered at 1 mM, which achieved ~50% inactivation of cell-lysate COS7-PKCε activity (Figure 6). Treatment of COS7-PKCε cells with TPA alone produced a decline of ~25% in cell-lysate PKC activity, consistent with modest PKCε down-regulation but too subtle to detect by western analysis (Figure 6). The effect of TPA co-treatment with either disulfide was to produce a further decline of ~20–25% in the cell-lysate PKC activity level, indicating a simple additive effect and ruling out potentiation of disulfide-induced cellular PKCε inactivation by the PE.

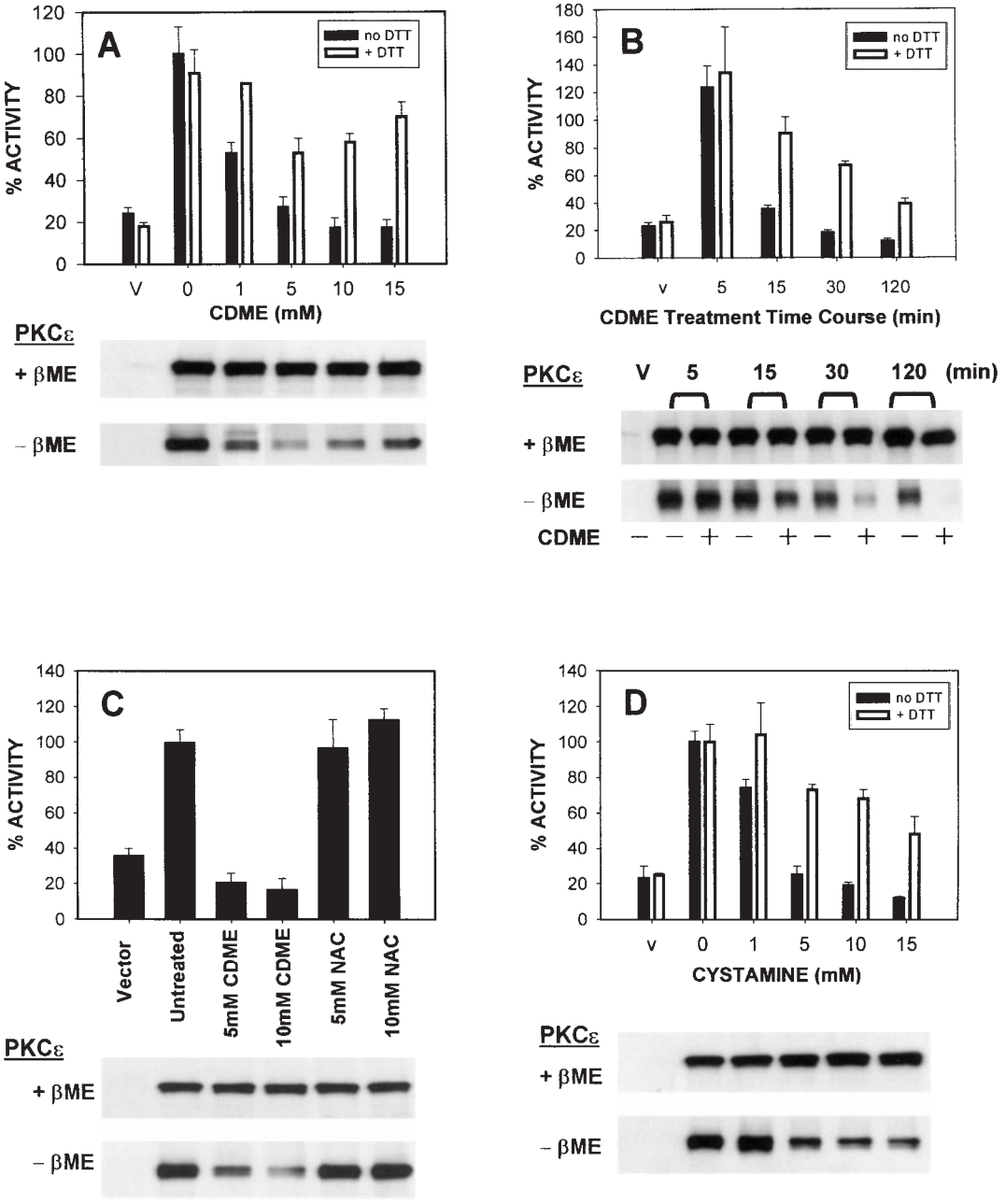
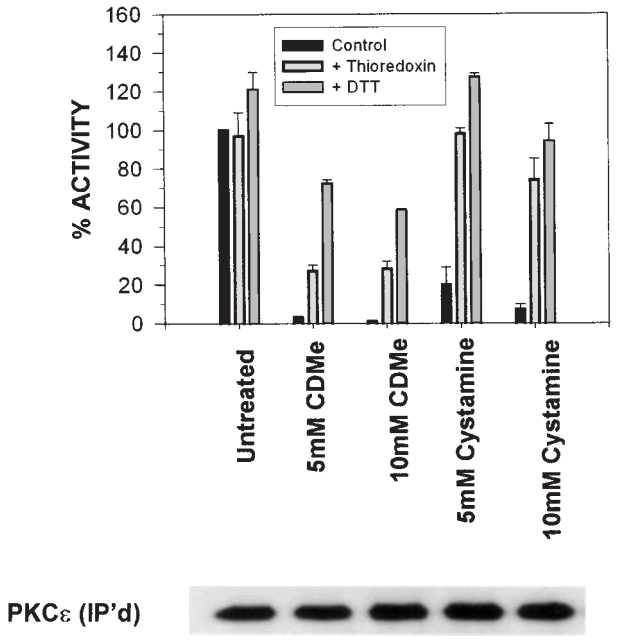


Fig. 4. CDME and cystamine induce DTT-reversible PKCε inactivation in COS7-PKCε transfectants. COS7-PKCε transfectants, incubated in media lacking sulfur-containing amino acids (20 h, 37°C), were treated with CDME or cystamine as specified. Cell lysates were prepared, incubated with/without 30 mM DTT (15 min, 30°C), and assayed for PKC activity (5 μg protein/assay). Black and white bars are PKC activity values measured by assays of the lysates preincubated without and with DTT, respectively. The PKC activity in the lysate of COS-7 empty-vector transfectants (V) was measured in parallel. Each PKC activity value is the average from triplicate determinations (±SD) western analyses corresponding to the cell lysates analyzed in the bar graphs were done with PKCε antibodies under reducing (+βME) and non-reducing conditions (−βME) and are shown in each panel (A–D). The results presented in each panel were reproducible in a separate analysis. (**A**) Concentration dependence of CDME-induced, DTT-reversible PKCε inactivation in COS7-PKCε cells. Cells were treated with a range of CDME concentrations (30 min, 37°C); western analysis was done with 25 μg cell-lysate protein/lane. (**B**) Time course of CDME-induced, DTT-reversible PKCε inactivation in COS7-PKCε cells. Cells were treated with 10 mM CDME for the indicated time interval at 37°C; western analysis was done with 12 μg cell-lysate protein/lane. (**C**) PKC-regulatory effects of COS7-PKCε cell treatment with CDME versus NAC. Cells were treated with CDME or NAC (30 min, 37°C); western analysis was done with 30 μg cell-lysate protein/lane. (**D**) Concentration dependence of cystamine-induced, DTT-reversible PKCε inactivation in COS7-PKCε cells. Cells were treated with a range of cystamine concentrations (30 min, 37°C); Western analysis was done with 20 μg cell-lysate protein/lane.

Thioredoxin is a dithiol-disulfide oxidoreductase that catalyzes disulfide reduction by an NADPH-dependent mechanism when coupled with thioredoxin reductase (50). A direct test of whether oxidative PKCε inactivation entails the introduction of disulfide bonds in PKCε is reversibility by thioredoxin. We demonstrated previously that cystine and cystamine inactivate purified PKCε by a thiol-disulfide exchange mechanism that is reversed by thioredoxin (35). Figure 5A shows that PKCε immunoprecipitated from lysates of cystamine-treated cells was inactivated 80–90% by the disulfide, and that the kinase activity of the immunoprecipitated isozyme was restored to 70–90% of the control activity level by treatment with



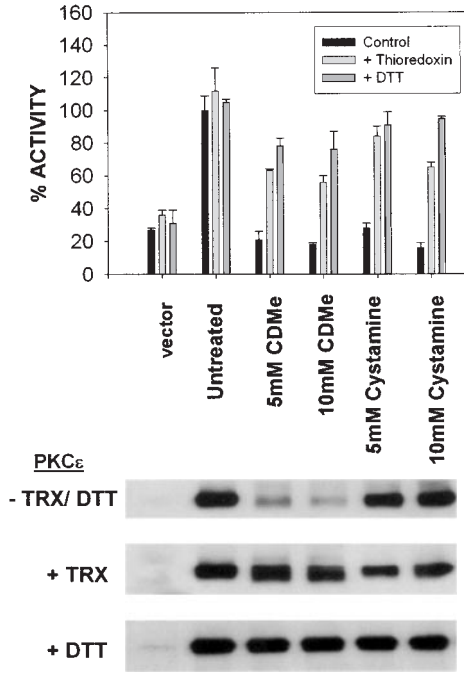


Fig. 5. Thioredoxin-reversible inactivation of PKCε by CDME and cystamine treatment of COS7-PKCε cells. COS7-PKCε cells were treated with CDME and cystamine (30 min, 37°C), and PKCε was immunoprecipitated from the cell lysates as described in Methods. The PKC activity of immunoprecipitated PKCε (**A**) and the corresponding cell lysate (**B**) was analyzed after a 1 h preincubation at 30°C alone, with thioredoxin (25 μM thioredoxin–0.5 μM thioredoxin reductase–1 mM NADPH), or with 30 mM DTT. Western analyses were done with PKCε antibodies. For other experimental details, see Methods. (**A**) The PKC activity analysis of immunoprecipitated PKCε and the corresponding western analysis of the DTT-treated immunoprecipitated PKCε are shown. The bar graph shown is the averaged result from two independently conducted experiments, and the western analysis corresponds to one of those experiments and is representative of the other. (**B**) The PKC activity analysis of the cell lysates is shown with corresponding western analysis done with the lysates under non-reducing conditions (–TRX/DTT) and with the thioredoxin-treated (+TRX) and the DTT-treated lysates (+DTT) (8 μg cell-lysate protein/lane). The results shown were reproducible in an independent analysis.

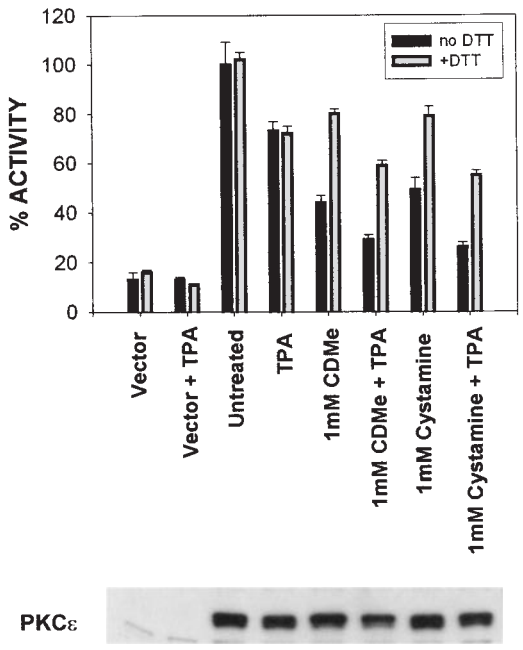


Fig. 6. TPA does not potentiate DTT-reversible PKCε inactivation induced by CDME or cystamine in COS7-PKCε transfectants. COS7-PKCε transfectants, incubated in media lacking sulfur-containing amino acids (20 h, 37°C), were treated with CDME or cystamine with/without 20 nM TPA co-treatment (30 min, 37°C). Cell lysates were prepared, incubated with/without 30 mM DTT (15 min, 30°C), and assayed for PKC activity (5 μg cell-lysate protein/assay). The PKC activity in the lysates of COS-7 empty-vector transfectants (vector and vector + TPA) was measured in parallel. Each PKC activity value is the average from triplicate determinations (±SD). The results of the PKC activity analysis and western analysis of the cell lysates, which was done under standard reducing conditions with PKCε antibodies and 20 μg cell-lysate protein/lane, are shown. These results were reproduced in an independent analysis.

thioredoxin/thioredoxin reductase. Similarly, CDME-induced inactivation of cellular PKCε was substantially reversed by thioredoxin/thioredoxin reductase treatment of the immunoprecipitated isozyme (Figure 5A). In addition to effects on immunoprecipitated PKCε, thioredoxin/thioredoxin reductase treatment of the lysates of cystamine- and CDME-treated COS7-PKCε cells also substantially reversed PKCε inactivation (Figure 5B). Furthermore, the structural modification of PKCε induced by CDME and detected by non-reducing western analysis as attenuated PKCε band intensity was fully restored by thioredoxin (Figure 5B), implicating disulfide bridge formation as the oxidative modification.

COS-7 cells transfected with mouse PKCδ-FLAG (COS7-PKCδ) exhibited a robust increase in PKCδ expression detected by western analysis with anti-PKCδ and an increase of ~5-fold in cell-lysate PKC activity (Figure 7A, vector versus 0). Treatment of COS7-PKCδ cells with CDME produced a concentration-dependent increase in cell-lysate PKC activity of up to 2-fold that peaked at ~0.25–0.50 mM CDME (Figure 7A) and at treatment periods of ~15–30 min (Figure 7B). Higher CDME concentrations produced a concentration-dependent decline in the cell-lysate PKC activity, with 70% of the original PKC activity remaining at the highest CDME concentration (10 mM) (Figure 7A). A similar biphasic response was observed in the analysis of the cell-lysate PKC activity of cystamine-treated COS7-PKCδ cells, which peaked at 1.6-fold PKC stimulation with 0.75 mM cystamine and declined to the original activity level (100%) at 10 mM cystamine

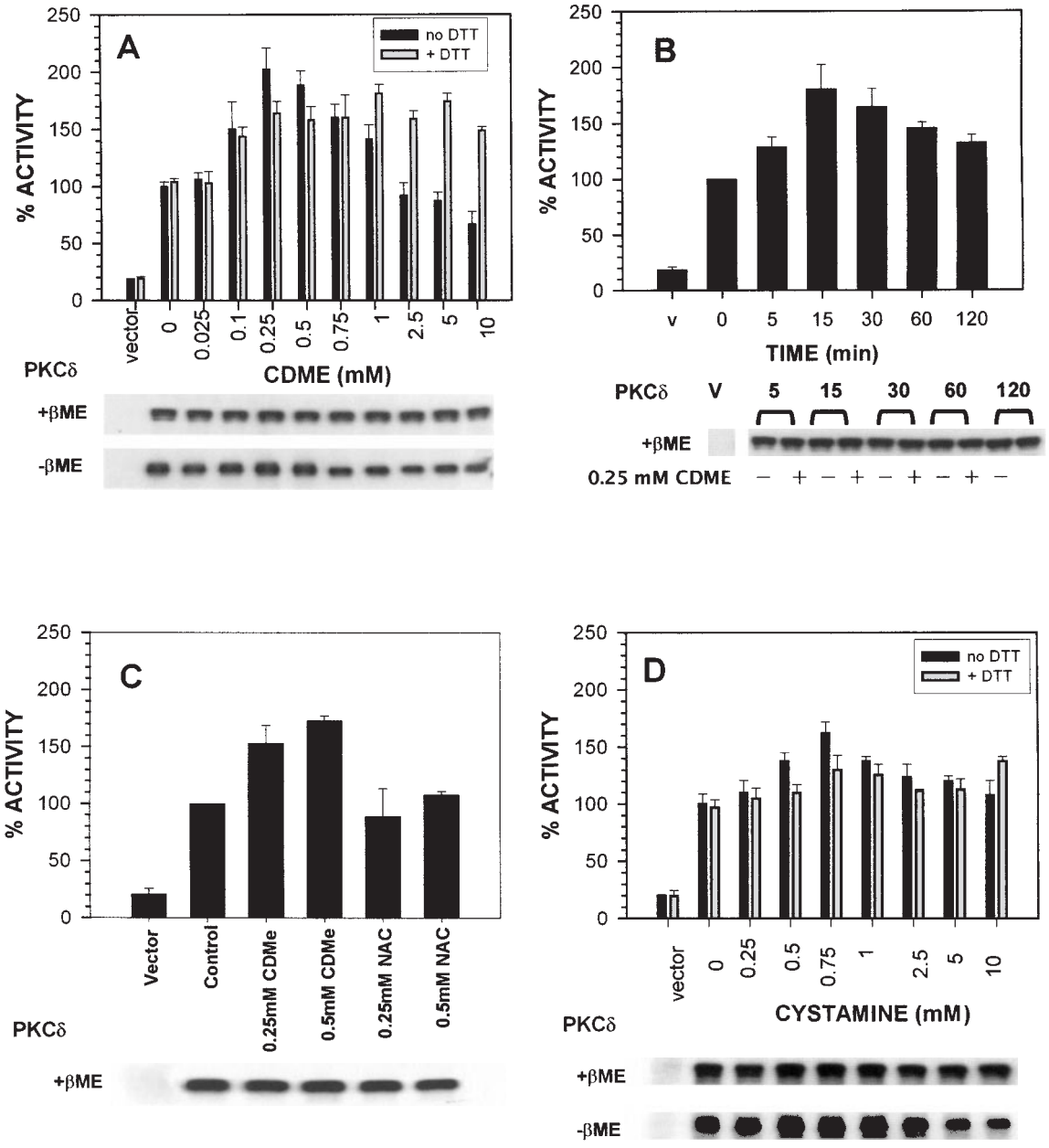


Fig. 7. CDME induces biphasic PKCδ regulatory responses in COS7-PKCδ transfectants. COS7-PKCδ transfectants, incubated in media lacking sulfur-containing amino acids (20 h, 37°C), were treated with CDME or cystamine as specified. Cell lysates were prepared, incubated with/without 30 mM DTT (15 min, 30°C), and assayed for PKC activity (5 μg protein/assay). Black and gray bars are PKC activity values measured by assays of the lysates preincubated without and with DTT, respectively. The PKC activity in the lysate of COS-7 empty-vector transfectants (V) was measured in parallel. Each PKC activity value is the average from triplicate determinations (±SD). Western analyses corresponding to the cell lysates analyzed in the bar graphs were done with PKCδ antibodies (12 μg cell-lysate protein/lane) under reducing (+βME) and non-reducing conditions (−βME) as shown. The results presented in each panel were reproducible in a separate analysis. (**A**) Concentration dependence of CDME-induced PKCδ regulation in COS7-PKCδ cells. Cells were treated with a range of CDME concentrations (30 min, 37°C). (**B**) Time course of CDME-induced PKCδ regulation in COS7-PKCδ cells. Cells were treated with 0.25 mM CDME for the indicated time interval at 37°C. For each time point, the PKC activity of the lysate from the CDME-treated cells is expressed as a percentage of the PKC activity of mock-treated cells corresponding to the same time point; the zero time point is shown for reference only (100%) and is not an experimental value. (**C**) PKC-regulatory effects of COS7-PKCδ cell treatment with CDME versus NAC. Cells were treated with CDME or NAC (30 min, 37°C). (**D**) Concentration dependence of cystamine-induced PKCδ regulation in COS7-PKCδ cells. Cells were treated with a range of cystamine concentrations (30 min, 37°C).

(Figure 7D). In both cases, PKCδ expression was unchanged across the disulfide concentration gradient (Figure 7A and D). To test whether the increase in the PKC activity level in COS7-PKCδ cells in response to CDME and cystamine could involve endogenous kinases in COS-7 cells, we examined the effects of CDME and cystamine treatment of vector-control cells on cell-lysate PKC activity. We found that the effects of both disulfides (0.25–0.75 mM) were negligible, with no change in the vector-control PKC activity level evident (data not shown).

CDME treatment of COS7-PKCδ cells stimulated the kinase activity of PKCδ-FLAG immunoprecipitated from the cell lysates up to 1.7-fold (Figure 8) (PKCδ was immunoprecipitated with anti-FLAG antibodies). As in the analysis of

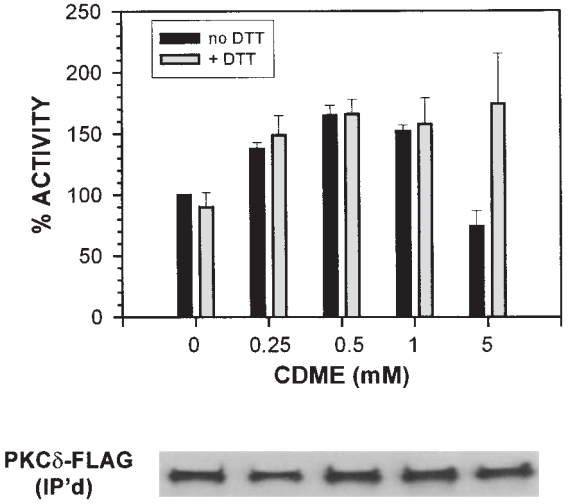


Fig. 8. CDME treatment of COS7-PKCδ cells induces biphasic regulation of PKCδ-FLAG immunoprecipitated from cell lysates. COS7-PKCδ cells were treated with CDME (30 min, 37°C), and PKCδ-FLAG was immunoprecipitated from the cell lysates with anti-FLAG mAb as described in Methods. Immunoprecipitated PKCδ-FLAG was incubated with/without 30 mM DTT (15 min, 30°C) and assayed for PKC activity. The results of the PKC activity analysis of immunoprecipitated PKCδ-FLAG and the corresponding western analysis of the immunoprecipitated isozyme with PKCδ antibodies under standard reducing conditions are shown. Black and gray bars are PKCδ activity values measured by assays of the immunoprecipitated isozyme preincubated without and with DTT, respectively. The bar graph shown is the averaged result from two independently conducted experiments, and the western analysis corresponds to one of those experiments and is representative of the other.

cell-lysate PKC activity (Figure 7A), CDME treatment produced biphasic effects on the activity of PKCδ-FLAG immunoprecipitated from COS7-PKCδ cells, with 70% PKCδ activity remaining at 5 mM CDME. Western analysis with PKCδ antibodies showed that the amount of PKCδ-FLAG in the immunoprecipitated samples was similar and could not account for observed activity changes (Figure 8). The CDME concentration dependencies in regulating PKC activity in the COS7-PKCδ cells were in general agreement for the cell-lysate PKC activity (Figure 7A) and the activity of PKCδ-FLAG immunoprecipitated from the lysates (compare Figures 7A and 8). Thus, CDME produces opposing effects on PKCδ and PKCε in COS7-PKC transfectants that recapitulate the opposing responses provoked by cystine treatment of the purified isozymes. While PKCε is potently inactivated by CDME treatment of COS7-PKCε cells (Figure 5A), PKCδ exhibits resistance to inactivation and is actually stimulated ~1.5-fold by CDME treatment of COS7-PKCδ cells (Figure 8).

The inactivation of PKCδ immunoprecipitated from COS7-PKCδ cells treated with 5 mM CDME was strongly DTT-reversible (Figure 8), as was the inactivation observed for the cell-lysate PKC activity when COS7-PKCδ cells were treated with 2.5–10 mM CDME (Figure 7A). This is indicative of an oxidative inactivation mechanism. In this regard, it is worth noting that the PKCδ band intensity in the non-reducing western analysis in Figure 7A was modestly attenuated at CDME concentrations that provoked inactivation of cell-lysate PKC activity. In contrast, the enhancement of cell-lysate PKC activity in COS7-PKCδ cells by low CDME concentrations was only marginally DTT-reversible (Figure 7A) and the ~1.5-fold stimulation of PKCδ immunoprecipitated from the cells was not DTT-reversible at all. Consistent with this, DTT not only reversed the inactivation of PKCδ immunoprecipitated from cells treated with 5 mM CDME, but actually restored PKCδ to the fully stimulated form (Figure 8). This indicates that the structural change corresponding to PKCδ stimulation persists at high CDME concentrations, where the net activity change under non-reducing conditions is inactivation. Likewise, the analysis of cell-lysate PKC activity in Figure 7A revealed that treating the lysates with DTT not only reversed inactivation produced at high CDME concentrations but restored the PKC activity to the enhanced levels produced by low CDME concentrations (Figure 7A).

The inability of DTT to reverse the CDME-induced stimulation of immunoprecipitated PKCδ in Figure 8 mirrors the lack of DTT reversibility that we have observed for cystine-induced stimulation of purified PKCδ (35). In the latter case, prevention of the PKCδ stimulatory response by DTT co-treatment was used to demonstrate an oxidative regulatory mechanism (35). To measure the contribution of oxidative regulation to CDME-induced stimulation of cellular PKCδ, we compared the effects of treating COS7-PKCδ cells with CDME versus NAC on PKC activity in the COS7-PKCδ cell lysate. At concentrations where CDME stimulated the cell-lysate PKC activity 1.5–1.6-fold, NAC was without effect (Figure 7C). These findings provide evidence that CDME-induced PKCδ stimulation in COS7-PKCδ cells reflects an oxidative regulatory response.

Discussion

We reported previously that cystine produces regulatory responses in purified PKCδ and PKCε via *S*-thiolation-triggered mechanisms that are consistent with a cancer preventive effect, i.e. stimulation of the pro-apoptotic, tumor-suppressive isozyme PKCδ and inactivation of the growth-stimulatory, oncogenic isozyme PKCε, at *S*-cysteinylation stoichiometries that correspond to modification of a single redox-regulatory Cys switch in each isozyme (35). In this report, we show that the regulatory responses of purified PKCδ and PKCε to cystine are recapitulated in disulfide-treated cells. We report that treatment of COS7-PKCε cells with the cystine precursor CDME produced concentration- and time-dependent PKCε inactivation that was associated with oxidative PKCε modification manifested as attenuated band intensity in PKCε immunoblot analyses, and that both PKCε inactivation and modification were reversed by DTT as well as by thioredoxin. These results provide strong evidence for an inactivation mechanism mediated by disulfide formation in PKCε but do not rule out potential involvement of another thioredoxin-reversible oxidative modification, sulfenic acid formation (51), in the isozyme structure.

We further show that CDME induced biphasic PKCδ regulation in COS7-PKCδ cells, with DTT-irreversible PKCδ stimulation at low and DTT-reversible PKCδ inactivation at high CDME concentrations, according to the analysis of the immunoprecipitated isozyme. The degrees of PKCδ versus PKCε inactivation by CDME treatment of COS7-PKC transfectants indicate substantial resistance of PKCδ to the inactivation mechanism. As in our analysis of purified PKCδ (35), the PKCδ stimulatory response in COS7-PKCδ cells was triggered only by the disulfide agent and not by its reduced thiol counterpart, providing evidence for an oxidative mechanism. Also paralleling the response of purified PKCδ to cystine (35), the stimulation of PKCδ elicited by disulfide treatment of cells

involved a stable structural change, as evident from the stability of the stimulated form of PKCδ in the cell lysate to IP, which has the effect of diluting reversibly bound ligands.

We also report that treatment of HepG2 cells with cystamine induced a robust increase in the PKC activity measured in the cell lysate. Because PKCδ is the only PKC isozyme expressed by HepG2 cells that was stimulated by cystamine in its purified form, and because purified, recombinant forms of other PKC isozymes expressed by HepG2 cells were actually inactivated by cystamine, our results would appear to implicate PKCδ as the mediator of the cystamine-induced PKC stimulatory response in HepG2 cells. However, the extent of cystamine-induced PKCδ stimulation in COS7-PKCδ cells was modest by comparison with HepG2 cells and followed a distinct concentration dependence. If PKCδ were responsible for the PKC stimulatory response in HepG2 cells, it would be logical to expect an even greater stimulatory response in COS7-PKCδ cells, where the relative contribution of PKC isozymes inactivated by cystamine to the net PKC activity level would presumably be diminished by enforced PKCδ expression. However, this simplified view ignores considerations such as the influence of other types of PKCδ post-translational modifications, e.g. Tyr phosphorylation (52).

One way to determine directly the identity of the cystamine-stimulated PKC isozyme(s) in HepG2 cells is to employ antisense or siRNA gene silencing strategies to measure the effect of quashing expression of each PKC isozyme in the cells on the cystamine-induced response; these studies are underway.

We established previously that cystamine was more potent than cystine in both the oxidative inactivation of purified PKCε and the oxidative stimulation of purified PKCδ (35). In this study, the cystine precursor CDME proved more effective than cystamine in inducing PKCε inactivation (Figure 5A) and PKCδ stimulation (Figure 7) in COS7-PKC transfectants. The superior induction of oxidative PKC regulation by CDME in the cells may reflect differences in cellular uptake rates of the lipophilic CDME versus cystamine or in the partitioning of the disulfides among cellular microenvironments. Another factor may be the extent to which CDME is hydrolyzed to cystine by cellular esterases. Incomplete hydrolysis might foster interactions of the lipophilic CDME with hydrophobic sites in the catalytic or regulatory domain of PKC, e.g. CDME interactions with cysteine-rich phospholipid binding pockets in the C1 domain could promote oxidative stimulation of cellular PKCδ. In the case of PKCε, the limited DTT and thioredoxin reversibility of inactivation by CDME compared with cystamine (Figure 5A) suggests that the metabolic stability of the redox-regulatory PKCε modification may be a factor in the superior potency of CDME against cellular PKCε.

Our findings in the present report that disulfides induce PKCε inactivation in cells by oxidative modification of the isozyme support our hypothesis that a redox-regulatory site present in PKCε offers a new molecular target for design of cancer preventive or therapeutic agents that inactivate this oncogenic isozyme (35). Based on the capacity of the disulfide-triggered mechanism to almost fully switch off PKCε in cells, we hypothesize that the redox-regulatory Cys mediating inactivation resides in or near the active site. However, this would suggest that treating cells with TPA to enhance PKCε active-site accessibility would potentiate inactivation by the disulfide (53), and we found that TPA did not potentiate PKCε inactivation by either cystamine or CDME in COS7-PKCε cells. Studies underway to identify the redox-regulatory Cys in PKCε may help to reconcile these observations. Furthermore, identification of the redox-regulatory Cys will allow assessment of whether heterogeneous regulatory responses of different PKC isozymes to a given disulfide (35) reflect structural distinctions among redox-regulatory sites that can be exploited for development of PKCε-selective inactivators. Finally, structure–activity studies focused on the modified regulatory Cys could resolve the critical mechanistic question of whether PKCε inactivation is mediated by a stable *S*-thiolation modification or by rapid conversion of the initial modification to an inactivating intraprotein disulfide bond.

Studies of PKCδ regulation by disulfide treatment of COS7-PKCδ cells in this report show that the cellular isozyme, like purified PKCδ (35), undergoes a stable structural change in response to disulfides that enhances PKCδ activity. As in the case of cystine-stimulated purified PKCδ (35), PKCδ immunoprecipitated from CDME-treated cells exhibited DTT-irreversible stimulation. This suggests that the modest DTT reversibility evident in cell lysates of CDME-treated COS7-PKCδ cells may not be reflective of direct effects on PKCδ. While the mechanism of cystine-induced stimulation of purified PKCδ is not yet known, the reversal of stimulation by millimolar $ZnCl_2$ suggests involvement of *S*-thiolation-triggered Zn^{2+} release from Zn^{2+} finger structures in the PKC regulatory domain (35); consistent with this, millimolar $ZnCl_2$ partially reversed CDME stimulation of cellular PKCδ measured in COS7-PKCδ cell lysates (Chu and O'Brian, unpublished observations). Studies are underway to identify the redox-regulatory Cys in PKCδ mediating isozyme stimulation by disulfides. Based on the tumor-suppressive and pro-apoptotic action of PKCδ (17,20–27), we hypothesize that the redox-regulatory Cys may offer a new target for development of isozyme-selective PKCδ activators. In this regard, we note that although the maximal degree of stimulation of PKCδ immunoprecipitated from CDME-treated cells was modest (1.7-fold), this is not necessarily reflective of the full potential of the oxidative PKCδ-stimulatory mechanism, because, in studies reported here and elsewhere (35), cystine and cystamine stimulated purified PKCδ up to 2.5- and 4.0-fold, respectively. Furthermore, the potential of the site to yield an isozyme-selective approach is evident from the absence of a stimulatory response to disulfides or to GSH in combination with the disulfide-inducing agent diamide for any of the eight PKC isozymes that we have examined other than PKCδ (35,37).

Pro-oxidant conditions are intrinsic to TP/P pathophysiology and thus characteristic of tissues at risk for tumor development and malignant conversion, e.g. DMBA/TPA-treated mouse skin (28,54). Protein *S*-thiolation is fostered by pro-oxidant environments (29,30). Thus, observations of oxidative regulation of cellular PKC isozymes by cystine and cystamine in this report strengthen the correlative evidence that TP/P antagonism by physiological thiols (32,33) may stem not only from simple quenching of reactive oxygen species but may also entail *S*-thiolation reactions between PKC isozymes and disulfide or other oxidized forms of the thiols that result in PKCδ stimulation and PKCε inactivation.

Acknowledgements

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