ANTIOXIDANTS & REDOX SIGNALING
Volume 7, Numbers 7 & 8, 2005

Forum Original Research Communication

PKC Sulfhydryl Targeting by Disulfiram Produces Divergent Isozymic Regulatory Responses that Accord with the Cancer Preventive Activity of the Thiuram Disulfide

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ABSTRACT

The protein kinase C (PKC) isozyme family plays key roles in cell growth regulation and influences neoplastic disease development and progression. For example, PKCϵ is oncogenic, and PKCδ tumor-suppressive. PKC isozymes are characterized by distinct activation mechanisms entailing phosphatidylserine-dependent cofactor binding to the regulatory domain. Evidence is now emerging that redox signaling offers another platform of PKC regulation. We have established that PKC isozymes are regulated by *S*-thiolation, a posttranslational modification entailing disulfide linkage of low-molecular-weight species to select protein sulfhydryls. Our recent studies demonstrate that physiologically occurring disulfides with cysteinyl constituents, *e.g.,* cystine, regulate cellular PKC isozymes by *S*-thiolation-triggered mechanisms. This report shows that PKC isozymes are also molecular targets of a chemically distinct class of disulfides. Disulfiram is a thiuram disulfide with potent cancer preventive activity in *in vivo* models of chemical carcinogenesis. Our results indicate that PKC *S*-thiolation by disulfiram induces differential regulatory effects on PKC isozymes that correlate with the cancer preventive activity of the drug. The implication of these findings is that PKC-regulatory effects of thiuram disulfides may offer a useful pharmacological guide for development of disulfiram analogues with superior cancer preventive activity. *Antioxid. Redox Signal.* 7, 855–862.

INTRODUCTION

Protein kinase C (PKC) is an isozyme family that plays key roles in the regulation of cell growth and survival (23). Aberrations in PKC signaling contribute to neoplastic disease development and progression. Individual PKC isozymes influence tumor development and malignant progression in divergent ways in *in vivo* models, *e.g.,* PKCβ_2 induces hyperproliferation of epithelial cells (12), PKCϵ is oncogenic and prometastatic (4, 17, 27, 31), and PKCδ is tumor-suppressive (28, 30). This provides a strong rationale for isozyme-selective PKC targeting in cancer prevention and therapy (1, 25). One strategy of isozyme-selective PKC targeting is to exploit mechanisms of isozyme regulation. PKC isozymes are classified based on distinct phosphatidylserine-dependent activation mechanisms that involve binding of the stimulatory cofactors Ca^{2+} and *sn*-1,2-diacylglycerol (DAG) to the regulatory domain. Conventional PKCs (α, β_1, β_2, γ) are stimulated by Ca^{2+} and DAG, and novel PKCs (δ, ϵ, θ, η) by DAG; atypical PKCs (ι, ζ) are activated independently of Ca^{2+} and DAG (23). The DAG-binding site is under investigation as a pharmacophore for antineoplastic drug development (1).

Redox signaling offers another platform of PKC isozyme regulation. Oxidative stimuli regulate PKC by direct and indirect mechanisms. For example, exposure of cells to hydrogen peroxide has been reported to stabilize critical Tyr residues in PKC isozyme catalytic domains in a phosphorylated state (P-Y) that transduces lipid-independent PKC activation (19). Oxidative stimuli exert direct regulatory effects on PKC isozymes by reversible modification of PKC-sulfhydryls. Human PKC isozymes contain 16–28 Cys residues. Among these are Cys residues that invariably occur in tandem Zn^{2+}-finger structures that form the DAG-binding site, and several

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Cys residues conserved to various extents in the PKC catalytic domain (23). Nitric oxide inactivates PKC by *S*-nitrosylating critical Cys residues (13), whereas superoxide produces the opposite effect. Superoxide stimulates PKC activity by sulfhydryl oxidation-induced Zn^{2+} release, with Cys oxidation in the DAG-binding site hypothesized as the causative event (18).

PKC isozymes are also regulated by *S*-thiolation (41), which is an oxidative posttranslational modification entailing disulfide linkage of low-molecular-weight species, *e.g.,* glutathione, to reactive Cys residues in target proteins (11). Using *N*-biotinylcysteine as a probe, Eaton *et al.* have shown that PKCα is *S*-thiolated in the myocardium during ischemia and reperfusion of isolated rat hearts (10). In affirmation of PKCα regulation by *S*-thiolation in cells, we have determined that diamide induces reversible PKCα inactivation by *S*-thiolation in mouse fibroblasts (40), and that the *S*-thiolating agent cystamine triggers dithiothreitol (DTT)-reversible PKCα inactivation in a human hepatoma cell line (8).

We have found that redox-regulatory effects of physiologically occurring disulfides on PKCδ and PKCϵ are concordant with a cancer preventive outcome (7, 8). Studies in transgenic mice have established that PKCδ strongly suppresses and PKCϵ vigorously drives chemical carcinogenesis in mouse skin (17, 30, 31). Buttressing the implications of these findings to cancer prevention are reports that PKCϵ transforms immortalized fibroblasts and epithelial cells (4, 27), and PKCδ facilitates the intrinsic apoptosis pathway in epithelial cells (21). We have established that cystine and cystamine oxidatively stimulate PKCδ and inactivate PKCϵ by *S*-thiolation-triggered mechanisms, whether the disulfides are applied to purified isozymes or cells transiently expressing the isozymes (7, 8). These findings provide evidence for sulfhydryl pharmacophores in PKCδ and PKCϵ that may be exploited for cancer prevention and therapy.

Disulfiram (Fig. 1) is a thiuram disulfide that has proven effective as a cancer preventive agent in several *in vivo* models of chemical carcinogenesis (9, 16, 22). Disulfiram undergoes thiol–disulfide exchange with select protein sulfhydryls, *e.g.,* a critical Cys residue in aldehyde dehydrogenase, the key target in alcohol aversion therapy (15, 38). Use of disulfiram as a therapeutic to deter alcohol consumption has revealed that disulfiram can be taken with moderate risk of adverse side effects for months or years (6). It logically follows from the safety evident for disulfiram that thiuram disulfides may hold value as a novel class of cancer preventive agents for use in individuals at risk for neoplastic disease. In addition, interest in disulfiram as a potential cancer therapeutic has been sparked by recent observations that disulfiram alleviates P-glycoprotein (Pgp)-driven multidrug resistance, which is an important clinical problem in the treatment of hematological malignancies and some solid cancers (33), by impairing the drug transport activity of Pgp through interactions with its ATP- and drug-binding sites and by impeding Pgp maturation (20, 34). Also of interest, disulfiram synergizes with 5-fluorouracil, the standard cytotoxic drug for colorectal cancer therapy, in the induction of apoptosis of cultured human colorectal cancer cells. Disulfiram-induced inhibition of the nuclear factor-κB survival pathway was implicated in the 5-fluorouracil sensitization mechanism (39). In addition, disulfiram was recently shown to induce apoptosis of several human melanoma cell lines, but not normal melanocytes *in vitro*, suggestive of a favorable therapeutic index (5).

In this report, we identify PKC isozymes as molecular targets of disulfiram. Our results demonstrate that disulfiram induces differential regulatory effects on PKC isozymes through thiol–disulfide exchange reactions with critical PKC-sulfhydryls. PKC-regulatory effects of thiuram disulfides may help to guide the development of disulfiram analogues with superior cancer preventive activity.

MATERIALS AND METHODS

Materials

COS7 cells were obtained from ATCC (Manassas, VA, U.S.A.). Cell culture reagents, LipofectAMINE PLUS, and purified human recombinant PKC isozymes were purchased from Invitrogen Life Technologies (Carlsbad, CA, U.S.A.). PKC-pcDNA3 plasmids were provided by Dr. I.B. Weinstein. PKC assay reagents include [Ser25]PKC(19–31) (Bachem, King of Prussia, PA, U.S.A.) and *sn*-1,2-dioleoylglycerol (Avanti, Alabaster, AL, U.S.A.). GF109203X was from Alexis (San Diego, CA, U.S.A.) and microcystin from Calbiochem (La Jolla, CA, U.S.A.). PKCδ and PKCϵ monoclonal antibodies (mAbs) were from BD Biosciences (San Diego, CA, U.S.A.), FLAG M2 mAb from Sigma (St. Louis, MO, U.S.A.), and horseradish peroxidase (HRP)-linked sheep anti-mouse Ig and enhanced chemiluminescence (ECL) detection reagents from Amersham (Piscataway, NJ, U.S.A.). Nitrocellulose membranes and protein assay reagent were from Bio-Rad (Hercules, CA, U.S.A.). Disulfiram [*bis*(diethylthiocarbamoyl) disulfide], IGEPAL, and protein A-Sepharose were from Sigma. Disulfiram was prepared on the day of the experiment as a 100 m*M* stock in dimethyl sulfoxide.

Transient PKC expression

COS7 cells maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin at 37°C in 5% CO_2 were cultured for 48 h at 37°C and transfected with pcDNA3 (control plasmid), C-terminally FLAG-tagged mouse PKCδ-pcDNA3 (PKCδ-FLAG-pcDNA3), or mouse PKCϵ-pcDNA3 (35) in combination with LipofectAMINE PLUS (8).

PKC isozyme regulation by disulfiram in COS7 cells

PKCϵ transfectants were incubated for 18–20 h posttransfection in DMEM lacking sulfur-containing amino acids (SAA-free DMEM) supplemented with 10% dialyzed FBS, and then treated with disulfiram in SAA-free DMEM (5% dialyzed FBS) at 37°C, washed with ice-cold phosphate-buffered saline, placed on ice, harvested with 0.65 ml of lysis buffer (8) per 10-cm dish, lysed by sonication, and centrifuged at 4°C to remove debris (5 min, 14,000 *g*). Effects of disulfiram on cellular PKCϵ activity were determined by analyzing the cell lysates and PKCϵ immunoprecipitated from

the cell lysates for PKC activity. In the cell-lysate analysis, 100% PKCϵ activity is defined as the difference between the PKC activity level of PKCϵ transfectants versus control-plasmid transfectants. PKCϵ regulation was analyzed by dividing each lysate into two portions, incubating one with and the other without 30 m*M* DTT (15 min, 30°C), and analyzing both for PKC activity and the DTT-free sample for PKCϵ expression (western analysis.)

PKCϵ was immunoprecipitated by incubating 400 μg of cell-lysate protein with 5 μg of PKCϵ mAb overnight at 4°C in 1 ml of immunoprecipitation buffer (8), followed by incubation with 100 μl of protein A-Sepharose (2 h, 4°C). Beads were spun down, washed 3× with 1 ml of immunoprecipitation buffer, and resuspended in 20 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 1 m*M* EGTA (500 μl). To analyze regulation by disulfiram, immunoprecipitated PKCϵ was incubated with and without DTT, as done with cell lysates, followed by PKC assays and western analysis. Disulfiram regulation of PKCδ activity was investigated in COS7 cells similarly; 5 μg of FLAG mAb was used to immunoprecipitate PKCδ.

Oxidative regulation of purified PKC isozymes by disulfiram

PKC-sulfhydryls were refreshed prior to disulfiram exposure by incubating the isozyme under analysis (5 μg) with 2 m*M* DTT (0.5 ml) (30 min, 4°C) followed by G25 chromatography (7). Isozymes were incubated with disulfiram (20 min, 30°C) and assayed. Incubation mixtures (100 μl) contained 2.0% dimethyl sulfoxide; this did not affect isozyme activity. In experiments where DTT reversal of disulfiram regulation was measured, PKC-disulfiram incubation mixtures were subjected to a second incubation with and without DTT (20 min, 30°C) that directly followed the first, and assayed.

PKC assays

PKC isozymes were assayed in reaction mixtures (120 μl) that contained 20 m*M* Tris-HCl, pH 7.5, 10 m*M* $MgCl_2$, 30 μg/ml phosphatidylserine, 30 μg/ml DAG, 0.2 m*M* $CaCl_2$ (or 1 m*M* EGTA), 50 μ*M* [Ser25]PKC(19–31), which is the synthetic peptide-substrate RFARKGSLRQKNV (41), 6 μ*M* [γ-^{32}P]ATP. The sample assayed was 5 μg of cell-lysate protein, 6–10 μl of resuspended immunoprecipitated isozyme, or 50 ng of purified isozyme. For cell lysates and immunoprecipitated isozymes, background activity was measured in control reaction mixtures that contained the PKC inhibitor GF109203X (1 μ*M*) and subtracted from total activity. Assays entailed a 10-min reaction at 30°C initiated with [^{32}P]ATP (8).

Western analysis

Western analysis was conducted under nonreducing and standard conditions [5% β-mercaptoethanol (β-ME) in samples]. Samples were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), blotted on nitrocellulose, and analyzed using the designated mAb, HRP-linked secondary antibody, and an ECL detection system.

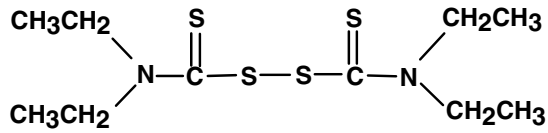

FIG. 1. Disulfiram.

RESULTS

We recently reported that disulfides containing cysteinyl constituents regulate PKC isozymes by *S*-thiolation-triggered mechanisms (7, 8). To explore whether PKC isozymes may be targets of a chemically distinct class of disulfides, thiuram disulfides, we investigated effects of disulfiram (Fig. 1) on the activity of purified human PKC isozymes. The analysis was conducted under nonreducing conditions, so that regulation stemming from thiol–disulfide exchange would be evident. We analyzed PKC isozymes that can transform immortalized fibroblasts (PKCβ_1, PKCγ, PKCϵ) (4, 14, 29), PKCδ, which is tumor-suppressive (30), and PKCα, which is implicated in breast cancer based on its elevated expression in tamoxifen-resistant human breast carcinomas and involvement in human breast cancer cell migration (26, 37).

Purified human PKC isozymes were preincubated with disulfiram for 20 min at 30°C and assayed. Figure 2 (upper panel) shows that disulfiram biphasically regulated PKCδ, with 10–50 μ*M* disulfiram stimulating PKCδ activity more than fourfold and higher disulfiram concentrations inactivating the isozyme. In contrast, disulfiram monophasically inactivated PKCα, PKCβ_1, PKCγ, and PKCϵ, eradicating isozyme activity at ≤25 μ*M* disulfiram (Fig. 2, lower panel); the IC_{50} values ranged from 1 to 8 μ*M* disulfiram (Table 1).

To determine whether isozyme regulation in Fig. 2 entailed sulfhydryl oxidation, we examined the effects of coincubating the isozyme-disulfiram mixtures with DTT on the regulatory responses. Coincubation with 2 m*M* DTT (40 min, 30°C) eliminated disulfiram inactivation of PKCα, PKCβ_1, PKCγ, and PKCϵ (Fig. 3). This was due to quenching of the inactivation responses, because DTT alone stimulated PKCγ activity less than twofold and affected the other isozymes negligibly (Fig. 3). Similarly, DTT coincubation (40 min, 30°C) quenched PKCδ stimulation by disulfiram, whereas DTT alone affected PKCδ activity negligibly (Fig. 3). These results show that a reducing environment quenches PKC isozyme regulation by disulfiram, implicating thiol–disulfide exchange between the thiuram disulfide and PKC-sulfhydryls in the regulatory mechanism.

To investigate whether disulfiram monophasically inactivated PKC isozymes by a switch mechanism, where PKC-sulfhydryl oxidation by disulfiram would switch the isozyme to an inactive state and reversal by a reducing agent would restore isozyme activity, we preincubated the isozymes with disulfiram under conditions that induced inactivation in Fig. 2 (20 min, 30°C), and initiated a second incubation by adding 25 m*M* DTT (20 min, 30°C). Postincubation with DTT substantially increased the activity of disulfiram-treated PKCα, PKCβ_1, PKCγ, and PKCϵ over the activity remaining after treatment with disulfiram alone (Fig. 3). In the absence of disulfiram, DTT postincubation affected isozyme activity

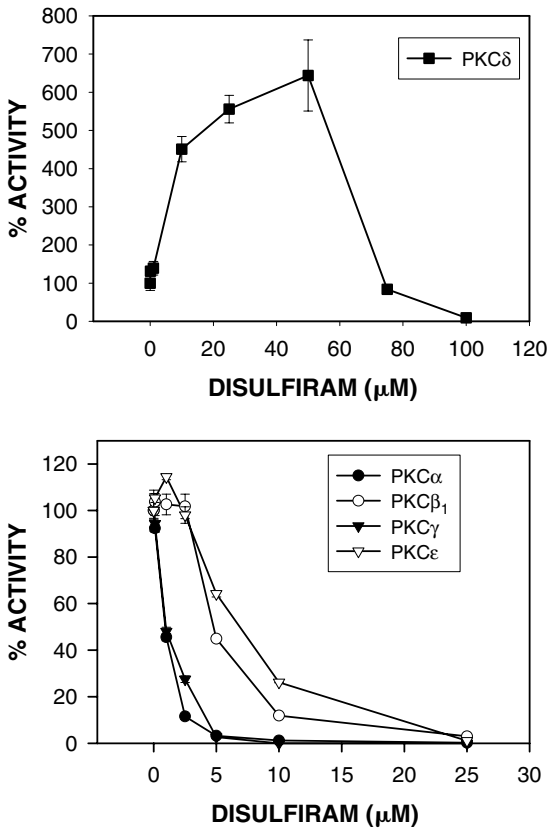

FIG. 2. Disulfiram regulation of purified PKC isozymes. PKC isozymes were incubated with disulfiram at the concentrations shown (20 min, 30°C) and assayed. Activity values are means ± SD from assays done in triplicate. "100% activity" corresponds to incubation without disulfiram. These results were reproduced in an independent analysis.

modestly or negligibly (Fig. 3). To illustrate, PKCβ_1 was inactivated by disulfiram to 20% of the original activity level, remained within 10% of the original activity level when treated with 25 m*M* DTT alone, and was restored to ~80% of the original activity level when treated with the disulfiram/DTT combination (Fig. 3). These results support a switch mechanism for disulfiram-induced PKC isozyme inactivation. In contrast, PKCδ stimulation by disulfiram was not reversed by DTT at all (Fig. 3). Thus, DTT prevented, but did not reverse, PKCδ stimulation by disulfiram, indicating a sulfhydryl oxidation-triggered, thiol-irreversible activation mechanism.

To explore whether disulfiram could inactivate PKC isozymes in cells, where other sulfhydryl-containing proteins and peptides may compete as disulfiram targets, we focused on PKCε in consideration of its attractiveness as a cancer prevention target (4, 27, 31), and conducted the analysis by transiently expressing the isozyme in COS7 cells. Transfection of COS7 cells with PKCε-pcDNA3 produced a sixfold higher level of cell-lysate PKC activity compared with pcDNA3 transfection (Fig. 4A, black bars, v = pcDNA3; 0 μ*M* disulfiram = PKCε). Western analysis of the cell lysates (upper blot) confirmed abundant PKCε transgene-product expression. Treatment of the PKCε transfectants with graded disulfiram concentrations (25–200 μ*M*) (1 h, 37°C) produced a concentration-dependent loss of PKC activity measured in the cell lysates without affecting PKCε expression (Fig. 4A, black bars; upper blot). The results are indicative of disulfiram-induced cellular PKCε inactivation, because >80% of the cell-lysate PKC activity derived from the transgene product. Inactivation was only moderately DTT-reversible, according to PKC activity measurements in lysates treated with 30 m*M* DTT (Fig. 4A, gray bars). However, analysis of PKCε immunoprecipitated from the cell lysates revealed a more pronounced inactivation response to disulfiram (Fig. 4B, black bars) with stronger DTT-reversible character (gray bars). These results provide evidence that disulfiram inactivates cellular PKCε through reversible PKCε-sulfhydryl oxidation. In further support of inactivation by sulfhydryl oxidation, western analysis of the cell lysates with nonreducing SDS-PAGE revealed a concentration-dependent decline in PKCε band intensity in response to disulfiram treatment of the cells (Fig. 4A, lower blot). Because this loss did not occur in the reducing western analysis, it is clearly reflective of sulfhydryl-reversible, oxidative modification of PKCε. The uniform intensity of the PKCε bands in the reducing western analysis (Fig. 4A) also provides evidence that the cells were not apoptotic after disulfiram treatment, because several caspases, including caspase-3, proteolyze PKCε (90 kDa) into 30–50-kDa fragments (2).

Stimulation of tumor-suppressive PKCδ is a potential avenue for cancer prevention or therapy (28, 30). We investigated whether disulfiram could stimulate PKCδ in COS7 transfectants by applying the approach taken to analyze PKCε. FLAG-tagged PKCδ was used, because the isozyme exhibits meager activity in immunoprecipitated complexes with commercial PKCδ antibodies. Similar to the PKCε analysis, COS7 cells transfected with PKCδ had a sevenfold higher cell-lysate PKC activity level than control transfectants (Fig. 5A, black bars). Western analysis with PKCδ Ab (upper blot) confirmed abundant transgene-product expression. Disulfiram treatment of the PKCδ transfectants (30 min, 37°C) produced biphasic changes in the cell-lysate PKC activity; the PKC activity level was increased twofold at 50 μ*M* disulfiram, and descended to slightly below the original activity level at 200 μ*M* disulfiram (Fig. 5A, black bars); PKCδ expression was unaffected by disulfiram (upper blot). Concordant biphasic PKCδ activity changes were observed in

TABLE 1. DISULFIRAM REGULATION OF PURIFIED PKC ISOZYMES

Isozyme	*IC_{50} (μM)*	*Maximal fold stimulation*
PKCα	2.5 ± 1.6	None
PKCβ_1	5.7 ± 0.9	None
PKCγ	1.3 ± 0.3	None
PKCδ	N/A	6.4 ± 0.1
PKCε	7.9 ± 1.0	None

IC_{50} is the disulfiram concentration achieving 50% PKC isozyme inactivation. Maximal fold stimulation is across 0.1–100 μ*M* disulfiram. Values are means ± SEM of the results in Fig. 2 and a separate analysis. N/A, not applicable.

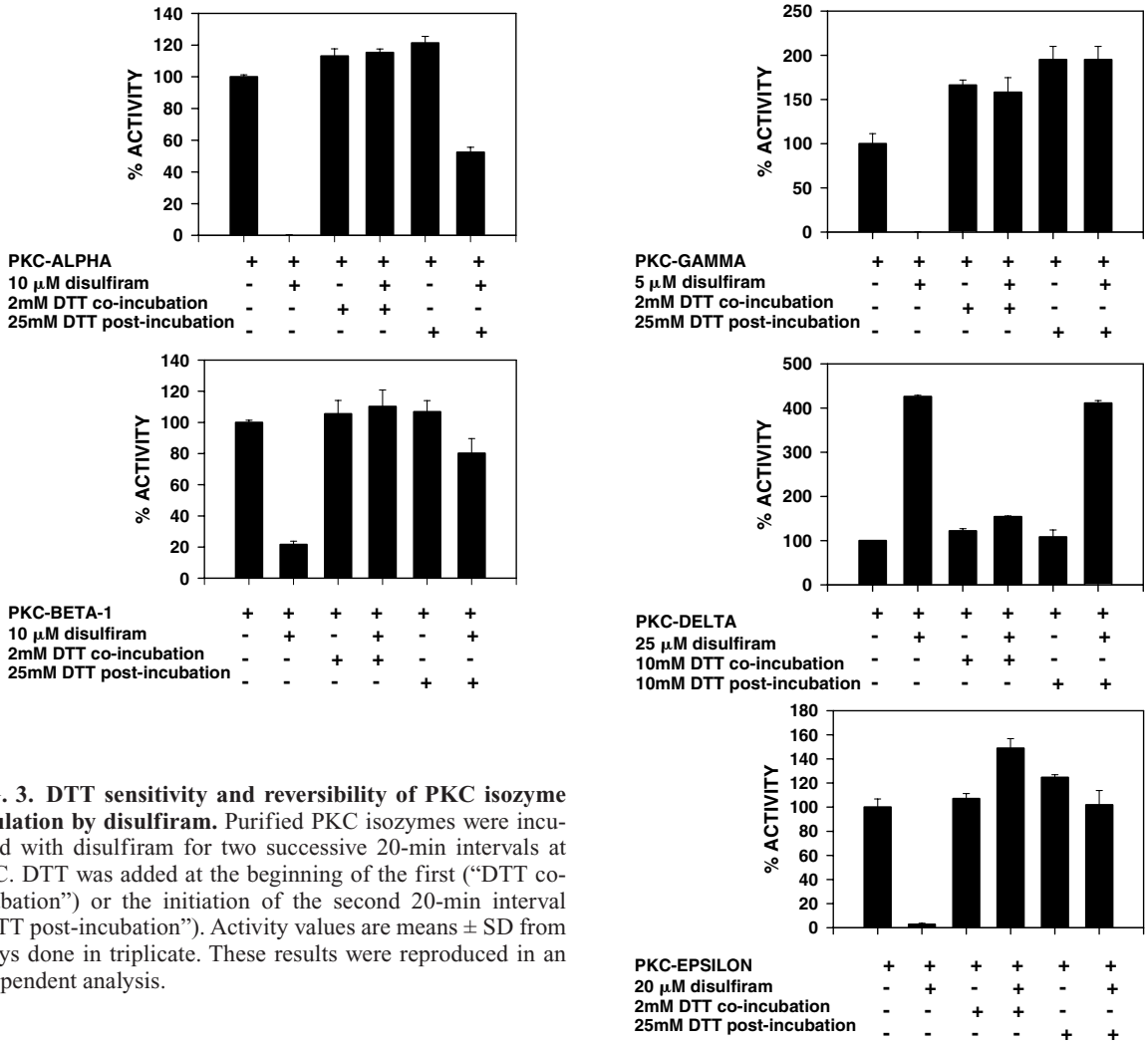

FIG. 3. DTT sensitivity and reversibility of PKC isozyme regulation by disulfiram. Purified PKC isozymes were incubated with disulfiram for two successive 20-min intervals at 30°C. DTT was added at the beginning of the first ("DTT co-incubation") or the initiation of the second 20-min interval ("DTT post-incubation"). Activity values are means ± SD from assays done in triplicate. These results were reproduced in an independent analysis.

a parallel analysis of PKCδ immunoprecipitated from the cell lysates with FLAG mAb (Fig. 5B), establishing the observed biphasic cellular PKCδ regulation by disulfiram as authentic.

Recapitulating the DTT-irreversible, oxidative stimulation of purified PKCδ by disulfiram in Fig. 3, disulfiram produced DTT-irreversible stimulation of cellular PKCδ in association with sulfhydryl-reversible, oxidative PKCδ modification, manifest as attenuated PKCδ band intensity in the nonreducing western analysis of the lysates (Fig. 5A, lower blot). Contrasting with this, cellular PKCδ inactivation at 200 μ*M* disulfiram was DTT-reversible (Fig. 5), like the inactivation responses of other isozymes in Fig. 3. Uniform PKCδ band intensities in the reducing western analysis across the disulfiram concentrations investigated (Fig. 5A, upper blot) provide evidence that the cells were not apoptotic after disulfiram treatment, because caspase-3 proteolyzes PKCδ (72 kDa) into ~40-kDa fragments (32).

DISCUSSION

In this report, we demonstrate that disulfiram oxidatively inactivates PKC isozymes that transform immortalized cells ($PKC\beta_1$, PKCγ, PKCϵ) (4, 14, 29) and PKCα, which is implicated in breast cancer (26, 37). The isozymes were inactivated by a DTT-reversible mechanism that was recapitulated in disulfiram-treated COS7 cells transfected with PKCϵ. In contrast, the tumor-suppressive isozyme PKCδ was stimulated by disulfiram by a DTT-irreversible mechanism operative in COS7 transfectants. Studies under way indicate that the PKCϵ-inactivating sulfhydryl switch is in the catalytic domain and, interestingly, conserved in PKCδ, suggesting that the sulfhydryl may resist disulfiram modification in PKCδ (Chu and O'Brian, unpublished observations).

PKC isozymes contain Zn^{2+} in Cys-rich Zn^{2+}-fingers constituting the DAG-binding site. Superoxide activates PKC by sulfhydryl oxidation-induced Zn^{2+} release (18), and cystine stimulates PKCδ by the same mechanism (7). Observations here that PKCδ stimulation by disulfiram was DTT-sensitive, but not reversed by DTT, are consistent with the Zn^{2+} release mechanism.

A feature of the PKCδ/PKCϵ immunoblots, which was exclusive to nonreducing analysis of lysates from disulfiram-treated cells, was a high-molecular-weight smear at the top of the gels, reflective of isozyme aggregation with self or other proteins by intermolecular disulfide bonds. The high-molecu-

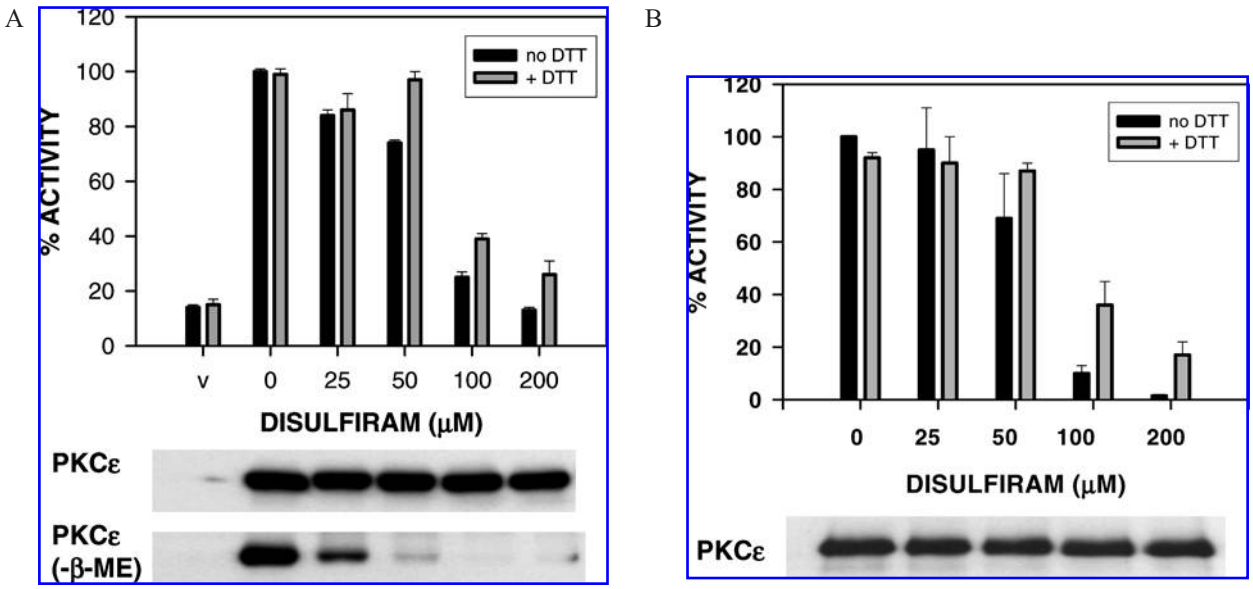

FIG. 4. Inactivation of PKCε by disulfiram in PKCε transfectants. COS7 cells transfected with PKCε were treated with disulfiram at the concentrations shown (1 h, 37°C) and lysed. **(A)** Cell lysates (with or without DTT) were assayed for PKC activity (5 μg of protein/assay); activity values are means ± SD from assays done in triplicate. v, untreated COS7 cells transfected with empty vector. Reducing and nonreducing (−β-ME) immunoblot analyses of the cell lysates with PKCε mAb (20 μg of protein/lane) are shown. The results were reproduced in a separate analysis. **(B)** PKCε was immunoprecipitated from the cell lysates, incubated with or without DTT, and assayed. The PKCε activity analysis is the mean ± SEM from two experiments with assays done in triplicate. Western analysis of immunoprecipitated PKCε is shown.

lar-weight species increased in abundance with increased disulfiram concentrations; this phenomenon was more pronounced for PKCδ than PKCε (data not shown). Disulfiram induction of disulfide cross-linked protein aggregates containing PKCδ and PKCε suggests that the isozymes may exist in cells as noncovalently associated multimeric species or as monomers associated with sulfhydryl-containing protein-binding partners. An important precedent for these observations is that the diazene-carbonyl diamide induces formation of disulfide-linked complexes containing PKCδ, PKCε, and

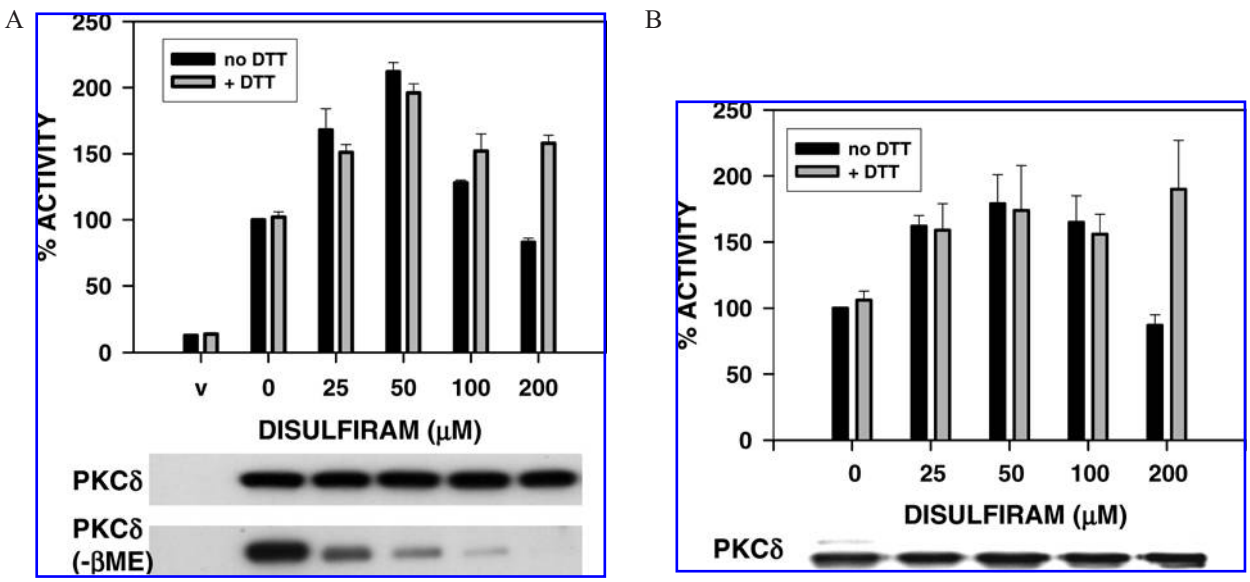

FIG. 5. Biphasic PKCδ regulation by disulfiram in PKCδ transfectants. COS7 cells transfected with FLAG-tagged PKCδ were treated with disulfiram at the concentrations shown (30 min, 37°C) and lysed. **(A)** Cell lysates (with or without DTT) were assayed for PKC activity (5 μg of protein/assay). Activity values are means ± SD of assays done in triplicate. v, untreated empty-vector transfectants. Reducing and nonreducing immunoblot analyses were done with PKCδ mAb (20 μg of protein/lane). The results were reproduced in a separate analysis. **(B)** PKCδ was immunoprecipitated from the cell lysates with FLAG mAb, incubated with or without DTT, and assayed. The PKCδ activity analysis is the mean ± SEM from two experiments with assays done in triplicate. Western analysis of immunoprecipitated PKCδ detected with PKCδ mAb is shown.

other proteins in cardiac myocytes (3). The disulfide-linked protein complexes previously observed in cardiac myocytes and detected in COS7 transfectants in this study support the notion that inducers of sulfhydryl oxidation may oligomerize PKC isozymes or cross-link them with protein-binding partners through intermolecular disulfides, with potential consequence to PKC-dependent signal transmission.

Disulfiram inactivates aldehyde dehydrogenase via *S*-thiolation of a critical sulfhydryl with rearrangement to an intraprotein disulfide (38). Disulfiram is transformed *in vivo* to *S*-methyl-*N*,*N*-diethylthiocarbamoyl sulfoxide and sulfone. These metabolites inactivate aldehyde dehydrogenase by sulfhydryl modification (15), suggesting that disulfiram and some metabolites react with related sets of targets. Thus, some metabolites may reinforce the pharmacological action of disulfiram.

Several disulfiram targets that may contribute to its cancer preventive activity have been identified. Disulfiram induction of glutathione *S*-transferase activity may protect against carcinogens, and nuclear factor-κB pathway antagonism may favor apoptosis of damaged cells (36, 39). Reports of apoptosis suppression by disulfiram would seem to conflict with other reports of apoptosis induction; this may reflect the complication that disulfiram also inhibits caspase-3 (24). Elimination of caspase targeting will be important in designing disulfiram analogues as antineoplastic agents. This report reveals that disulfiram targets implicated in its cancer preventive activity include oncogenic and tumor-suppressive PKC isozymes.

ACKNOWLEDGMENTS

This work was supported by the Robert A. Welch Foundation and Tobacco Settlement Funds (M.D.A.C.C.).

ABBREVIATIONS

DAG, *sn*-1,2-diacylglycerol; DMEM, Dulbecco's modified Eagle medium; DTT, dithiothreitol; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; HRP, horseradish peroxidase; mAb, monoclonal antibody; β-ME, β-mercaptoethanol; Pgp, P-glycoprotein; PKC, protein kinase C; SAA-free, sulfur-containing amino acid-free; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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Received for publication October 11, 2004; accepted January 19, 2005.

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