Oxidant-Induced *S*-Glutathiolation Inactivates Protein Kinase C-α (PKC-α): A Potential Mechanism of PKC Isozyme Regulation†

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ABSTRACT: Protein kinase C (PKC) isozymes are subject to inactivation by reactive oxygen species (ROS) through as yet undefined oxidative modifications of the isozyme structure. We previously reported that Cys-containing, Arg-rich peptide−substrate analogues spontaneously form disulfide-linked complexes with PKC isozymes, resulting in isozyme inactivation. This suggested that PKC might be inactivated by oxidant-induced *S*-glutathiolation, i.e., disulfide linkage of the endogenous molecule glutathione (GSH) to PKC. Protein *S*-glutathiolation is a reversible oxidative modification that has profound effects on the activity of certain enzymes and binding proteins. To directly examine whether PKC could be inactivated by *S*-glutathiolation, we used the thiol-specific oxidant diamide because its oxidant activity is restricted to induction of disulfide bridge formation. Diamide weakly inactivated purified recombinant cPKC-α, and this was markedly potentiated to nearly full inactivation by 100 μM GSH, which by itself was without effect on cPKC-α activity. Diamide inactivation of cPKC-α and its potentiation by GSH were both fully reversed by DTT. Likewise, GSH markedly potentiated diamide inactivation of a PKC isozyme mixture purified from rat brain (α, β, γ, ϵ, ζ) in a DTT-reversible manner. GSH potentiation of diamide-induced cPKC-α inactivation was associated with *S*-glutathiolation of the isozyme. cPKC-α *S*-glutathiolation was demonstrated by the DTT-reversible incorporation of [^{35}S]GSH into the isozyme structure and by an associated change in the migration position of cPKC-α in nonreducing SDS−PAGE. Diamide treatment of NIH3T3 cells likewise induced potent, DTT-reversible inactivation of cPKC-α in association with [^{35}S] *S*-thiolation of the isozyme. Taken together, the results indicate that PKC isozymes can be oxidatively inactivated by *S*-thiolation reactions involving endogenous thiols such as GSH.

Protein kinase C (PKC)[1] is an isozyme family comprising 10 mammalian members (*1*) that plays an important role in cell growth and differentiation (*2*). PKC isozymes also contribute to key events in the development and progression of cancer, e.g., the tumor promotion phase of multistage carcinogenesis (*3*, *4*), the acquisition of multidrug resistance by cancer cell populations (*5*), and the induction of cancer cell expression of cyclooxygenase-2 (cox-2) (*6*). PKC isozymes are activated by phospholipid-dependent mechanisms, and phosphatidylserine (PS) serves as a universal lipid cofactor for the isozymes (*1*). The isozymes are divided into three subfamilies on the basis of structural distinctions among their regulatory domains and corresponding differences in their cofactor requirements for phospholipid-dependent activation (*1*, *2*). cPKC isozymes (α, β_1, β_2, γ) are Ca^{2+}-dependent and phorbol ester/diacylglycerol-responsive, nPKC isozymes (δ, ϵ, θ, η) are Ca^{2+}-independent and phorbol ester/diacylglycerol-responsive, and aPKC isozymes (ι (λ), ζ) are Ca^{2+}- and phorbol ester/diacylglycerol-independent (*1*, *2*).

Phosphoinositide-dependent kinase 1 (PDK-1) phosphorylates PKC isozymes at the activation loop (*7*, *8*). PDK-1-catalyzed activation-loop phosphorylation enables cPKC isozymes to autophosphorylate two carboxyl-terminal sites and thereby become catalytically competent, i.e., responsive to Ca^{2+} and stimulatory lipid cofactors (*8*−*10*). In contrast, aPKC-ζ is directly activated by PDK-1-catalyzed phosphorylation (*11*). Thus, negative regulatory mechanisms for PKC isozymes may include the action of phosphatases on phospho-residues required for catalytic competence (*10*, *12*) and direct interference with lipid cofactor-mediated PKC activation by endogenous molecules such as sphingosine (*13*).

We have found that certain Cys-containing peptide−substrate analogues such as *N*-biotinyl-Arg−Arg−Arg−Cys−Leu−Arg−Arg−Leu *S*-thiolate the active-site region of cPKC, nPKC, and aPKC isozymes, i.e., the peptides form disulfide-linked complexes with the isozymes, resulting in PKC inactivation (*14*−*16*). These results suggested that under appropriate conditions PKC might be negatively regulated by oxidant-induced *S*-thiolation involving endogenous molecules such as glutathione (GSH). Protein *S*-thiolation is a reversible oxidative modification that involves the disulfide

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[1] Abbreviations: DMEM, Dulbecco's modified Eagle medium; DTT, dithiothreitol; GSH, glutathione; HBSS, Hanks balanced salt solution; LMW, low molecular weight; NEM, *N*-ethylmaleimide; PBS, phosphate-buffered saline; PDK-1, phosphoinositide-dependent kinase 1; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine; ROS, reactive oxygen species; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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linkage of GSH or related endogenous LMW thiols, e.g., cysteine, to select proteins in vivo (*17*, *18*). Protein *S*-thiolation is induced in cells in response to mild oxidative stress, and it is thought to offer a mechanism of protection for select proteins against irreversible oxidative damage (*17*, *18*). Protein *S*-thiolation serves as an oxidative regulatory mechanism for certain enzymes and binding proteins with reactive Cys residues. For example, proteins that have been shown to be negatively regulated by *S*-glutathiolation include the enzymes HIV protease (*19*) and aldose reductase (*20*) and the transcription-factor jun (*21*).

We recently reported that GSH has nonredox (oxidation/reduction-independent) inhibitory effects against PKC isozymes but only at the high end of the physiological concentration range (>3mM GSH) (*22*). In this report, we focus on the question of whether oxidizing conditions could support PKC *S*-glutathiolation with consequential effects on the catalytic activity of the kinase. We demonstrate that in the presence of 100 μM GSH, the thiol-specific oxidant diamide (*23*) induces *S*-glutathiolation of purified cPKC-α and that marked GSH-dependent inactivation of the isozyme occurs in association with the modification. We also provide evidence that *S*-thiolation of cPKC-α may oxidatively regulate the isozyme in vivo, by showing that diamide treatment inactivates cPKC-α in a DTT-reversible manner in fibroblasts in association with *S*-thiolation of the isozyme. These results offer the first evidence that a protein kinase can be negatively regulated by *S*-thiolation.

MATERIALS AND METHODS

A purified PKC isozyme mixture consisting of the isozymes α, β, γ, ϵ, and ζ was prepared from frozen rat brains (Harlan Sprague-Dawley, Indianapolis IN) as previously described and stored at −20 °C (*15*). The histone kinase activity of the PKC preparation was stimulated 10−15-fold by 0.2 mM Ca^{2+} and 30 μg/mL PS but was unaffected by either Ca^{2+} or PS alone, indicative of cPKC catalysis (*15*). Baculovirus-produced, purified human cPKC-α and the cPKC-selective inhibitor Go6976 were purchased from Calbiochem-Novabiochem Corp (San Diego, CA). The radiochemicals [γ-^{32}P]ATP and [^{35}S] L-cysteine were purchased from Amersham Pharmacia Biotech, and [^{35}S]GSH was purchased from New England Nuclear (NEN) (Boston, MA). Murine NIH3T3 fibroblasts were kindly provided by Dr. Jon Wiener (MD Anderson Cancer Center). Dulbecco's modified Eagle medium (DMEM), DMEM lacking Cys, dialyzed fetal bovine serum (FBS), phosphate-buffered saline (PBS), Hanks balanced salt solution (HBSS), and other tissue culture reagents were purchased from Life Technologies. SDS−PAGE reagents, including molecular weight markers, were purchased from BioRad. Recombinant human glutaredoxin (250 U/mg) was purchased from American Diagnostica (Greenwich, CT). Diamide, GSH, bovine brain PS (≥98% pure), ATP, histone III-S, *N*-ethylmaleimide, cycloheximide, DTT, protease inhibitors, buffers, and all other reagents were purchased from Sigma.

Measurement of Diamide-Induced Inactivation of Purified PKC Isozymes. Purified rat brain PKC and recombinant human cPKC-α were pretreated with 2 mM DTT for 20 min at 4 °C to refresh PKC thiols. The reducing agent was then removed as previously reported by gel filtration at 4 °C on a 2-mL G-25 Sephadex column equilibrated in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 20 μg/mL soybean trypsin inhibitor, 10 μg/mL leupeptin, and 250 μM PMSF (buffer A) (*15*). Diamide stock solutions (*23*) were freshly made from the solid in 20 mM Tris-HCl, pH 7.5, on the day of the experiment. Gel-filtered rat brain PKC (100 ng) or human cPKC-α (800 ng) was preincubated with varying concentrations of diamide, in the presence or absence of 100 μM GSH, in buffer A (final volume = 100 μL) for 5 min at 30 °C. In some cases, a second preincubation step (5 min, 30 °C) was employed in the presence or absence of DTT, to assess DTT reversibility of PKC inactivation. Preincubated mixtures were briefly kept on ice prior to the execution of PKC assays that measured the inactivation of PKC induced by preincubation with diamide and potentiated by co-incubation with 100 μM GSH. The cPKC-α preincubation mixtures were also used to measure cPKC-α *S*-glutathiolation, as described under the section *Detection of cPKC-α S-Glutathiolation.* PKC activity was measured as previously described (*15*, *22*). PKC assay mixtures (120 μL) utilized the pseudosubstrate-based synthetic peptide [Ser25]PKC(19−31) (BACHEM Bioscience, Inc, King of Prussia, PA) as substrate and contained 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.2 mM $CaCl_2$ (or 1 mM EGTA), 30 μg/mL PS (or none), 6 μM [γ-^{32}P] ATP (5000−8000 cpm/pmol), 10 μM [Ser25]PKC(19−31), and approximately 10 μL of preincubated rat brain PKC or cPKC-α. A 10-min reaction period at 30 °C was initiated by the addition of [γ-^{32}P]ATP, and the reaction was terminated on phosphocellulose paper to quantitate ^{32}P incorporation into the peptide substrate, as previously described (*14*, *15*). Assays were performed in triplicate and expressed as the mean value ± SD, and all experimental results shown were determined to be reproducible in independent experiments.

Detection of S-Glutathiolation of Purified cPKC-α. cPKC-α was preincubated in the presence of varying concentrations of diamide ± 100 μM [^{35}S]GSH, under the conditions employed in the analysis of diamide-induced cPKC-α inactivation. To measure [^{35}S] radiolabeling of cPKC-α, the preincubation period was terminated by the addition of 2× SDS−PAGE sample buffer, and the preincubated samples were subjected to nonreducing 10% SDS−PAGE (120 ng of cPKC-α per lane). The gels were stained with Coomassie to visualize molecular weight markers, rinsed 30 min in deionized water, soaked in Amplify (Amersham Pharmacia Biotech) for 30 min, and vacuum-dried onto filter paper prior to autoradiography.

cPKC-α *S*-glutathiolation was also detected based on a change in the migration position of the isozyme in denaturing gels. In these experiments, nonradioactive GSH was employed in the cPKC-α preincubation mixtures. Immunoblot analysis was done as previously described (*15*) using a nonreducing 7.5% SDS−PAGE system with 120 ng of preincubated cPKC-α per lane, cPKC-α mAb (Transduction Laboratories, Lexington KY), and peroxidase-linked sheep antimouse Ig (Amersham Pharmacia Biotech) as the primary and secondary antibodies, respectively, and enhanced chemiluminescence (ECL) to detect immunoreactive bands (Amersham Pharmacia Biotech).

Measurement of Diamide-Induced cPKC-α Inactivation in NIH3T3 Cells. NIH3T3 fibroblasts cultured in DMEM with 10% serum (approximately 75% confluent) were

exposed to 50 μg/mL cycloheximide for 5 h at 37 °C to match conditions employed in the analysis of cPKC-α [^{35}S] *S*-thiolation in the cells. The cells were rinsed with 10 mL of HBSS and treated with diamide for 10 min at 37 °C in serum-free media. Cells were then washed with ice-cold PBS, and PKC was extracted from cell lysates by DEAE chromatography as previously described (*22*). Briefly, the cells were lysed in buffer A containing 1% Triton X-100. Lysates were stirred for 15 min at 4 °C, spun at 14000*g*, and the supernatants were loaded onto 0.5-mL DEAE Sepharose columns equilibrated in buffer A. The columns were washed with 2 mL of buffer A, and the PKC was eluted with 1 mL of buffer A containing 0.3 M NaCl (*22*). The DEAE-extracted PKC samples were preincubated for 15 min with/without 30 mM DTT at 30 °C to allow ascertainment of the DTT reversibility of diamide effects on cPKC-α activity and then assayed for Ca^{2+}- and PS-dependent PKC (cPKC) activity. Because cPKC-α is the only cPKC isozyme expressed in the cells (see Results), the assay directly measures cPKC-α activity in this system. Samples were assayed for cPKC-α activity as described for the purified isozyme (5 μg of sample protein/assay mixture) except that the cPKC inhibitor Go6976 (100nM) was included in the assay mixtures that measured background kinase activity (*22*). Assays were performed in triplicate, and results shown were reproducible in independent experiments.

Analysis of Diamide-Induced cPKC-α [^{35}S] S-Thiolation in NIH3T3 Cells. Cellular protein *S*-thiolation was measured by an established method that entails [^{35}S] metabolic labeling of GSH and other cysteine-derived, low molecular weight (LMW) thiols under conditions in which protein backbone radiolabeling does not occur (*18*, *24*−*26*). This is achieved by depleting cellular LMW thiols and inhibiting protein synthesis, prior to labeling cells with [^{35}S]cysteine (*18*, *24*−*26*). Endogenous LMW thiols were depleted from NIH3T3 fibroblasts cultured in DMEM with 10% serum and at a confluency of approximately 75% by replacing the culture media with DMEM lacking sulfur-containing amino acids and containing 10% dialyzed serum for 16 h at 37 °C. Next, the protein synthesis inhibitor cycloheximide (50 μg/mL) was added to the treatment media for 1 h at 37 °C, and then [^{35}S]-cysteine (30 μCi/mL) was added to the media in the continued presence of cycloheximide for 4 h at 37 °C. Cells (10^7 cells/treatment group) were washed 3× with 10 mL of HBSS, followed by treatment with 5 mM diamide (or none) in serum-free media for 10 min at 37 °C. At the end of the treatment period, the cells were washed with ice-cold PBS and then lysed using 1 mL of buffer A with 1% Triton X-100 containing either 50 mM *N*-ethylmaleimide (NEM) or 25 mM DTT. NEM is employed to modify free sulfhydryls and thereby prevent scrambling of the label in the lysate by thiol-disulfide exchange, and DTT is utilized to reverse protein [^{35}S] *S*-thiolation to distinguish the DTT-reversible, post-translational modification from protein backbone labeling (*18*, *24*). Lysates were stirred for 15 min at 4 °C and then spun at 14000*g*. Prior to immunoprecipitation of cPKC-α, 50 mM NEM was added to DTT-containing lysates to quench the DTT.

cPKC-α was immunoprecipitated from the lysates based on methods described in (*15*, *27*, *28*) as follows. The cell lysates were first precleared with 100 μL of protein A Sepharose (50% slurry)/mL of lysate with end-over-end rotation for 30 min at 4 °C and then spun at 14000*g* for 10 min. The supernatant was retained, and the protein concentration was ascertained using the Bio-Rad protein assay. Equal volumes of lysate protein (300 μg) in buffer A and 5 μg of polyclonal cPKC-α antibody (Santa Cruz, CA) in 2× IP buffer (IP buffer is 10mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% NP-40, 10% glycerol, 10 μg/mL leupeptin, and 0.2 mM PMSF) were co-incubated for 2 h with end-over-end rotation at 4 °C. Protein A Sepharose (100 μL, 50% slurry) was added, followed by further incubation for 1 h. Sepharose beads were subjected to three cycles of washing with IP buffer followed by centrifugation. The washed beads were resuspended with 100 μL of 2× nonreducing SDS−PAGE sample buffer, boiled for 5 min and spun, and the supernatants were retained for gel analysis. 7.5% SDS−PAGE was performed on the samples, and Western analysis was done with a cPKC-α mAb (Transduction Labs) using standard procedures (*15*). Gels utilized to analyze [^{35}S] *S*-thiolation of cPKC-α were stained with Coomassie dye to visualize molecular weight markers, soaked in Amplify (Amersham) for 30 min, dried, and then exposed to Hyperfilm MP for autoradiography.

RESULTS

DTT-Reversible Inactivation of PKC by the Thiol-Specific Oxidant Diamide. The catalytic domains of cPKC isozymes contain several conserved Cys residues, and the regulatory domains contain two conserved Cys-rich Zn^{2+} finger structures that participate in phorbol ester/diacylglycerol binding (*29*). Previous investigations of oxidant effects on PKC catalysis have utilized reactive oxygen species (ROS) such as hydrogen peroxide (*30*−*32*) that can readily introduce irreversible oxidative modifications into diverse residues in proteins, e.g., Cys, Met, Trp, Phe, Tyr, and His, through free radical mechanisms (*33*, *34*). To focus on the question of whether PKC is subject to oxidant-induced *S*-glutathiolation and, if so, whether the modification could influence PKC activity, our approach was to use the thiol-specific oxidant diamide in conjunction with GSH. Diamide promotes disulfide bridge formation by a thiol-specific addition/displacement reaction (*23*). The use of diamide produces chemically defined, DTT-reversible oxidative Cys modifications in proteins, i.e., disulfide bridges, and avoids the generation of irreversible oxidative modifications that are encountered with nonspecific oxidants (*23*).

We first evaluated the influence of diamide on PKC catalysis in the absence of GSH. In these studies, we employed a purified rat brain PKC isozyme mixture composed of cPKC-α, cPKC-*β*, cPKC-*γ*, nPKC-*ϵ*, and aPKC-*ζ* (*15*) and the synthetic peptide-substrate [Ser25]PKC(19−31), which is based on the pseudosubstrate sequence in cPKC-α (*35*). The [Ser25]PKC(19−31) kinase activity of the PKC preparation was enhanced 3.0−3.5-fold by 0.2 mM Ca^{2+} plus 30 μg/mL PS but was not enhanced by either Ca^{2+} or PS alone, indicating that ≥67% of the activity observed reflected cPKC catalysis. To measure diamide effects on PKC catalysis, PKC was preincubated in the absence of reducing thiol with diamide (0.01−10mM) in buffer A for 5 min at 30 °C and then diluted 12-fold into assay mixtures. The Ca^{2+}- and PS-stimulated [Ser25]PKC(19−31) kinase activity of PKC was inactivated by preincubation with diamide in a concentration-dependent manner (●, Figure 1).

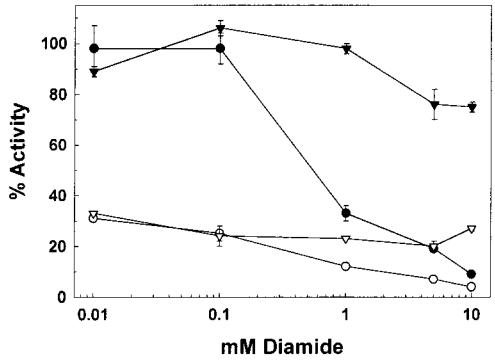

FIGURE 1: DTT-sensitive inactivation of rat brain PKC by the thiol-specific oxidant diamide. Purified rat brain PKC was preincubated with diamide at the concentrations shown for 5 min. at 30 °C in the absence (●, ○) or presence (▼, ▽) of 30 mM DTT, as described in Materials and Methods. The cofactor-stimulated cPKC activity of the preincubated enzyme was measured in the presence of 0.2 mM Ca^{2+} and 30 μg/mL PS (●, ▼), and the basal activity was measured in their absence (○, ▽). For other experimental details, see Materials and Methods. 100% activity = 19.0 ± 2.5 pmol of ^{32}P transferred/min. Each point is the average ± SD of triplicate assays, and the results shown were reproduced in separate experiments.

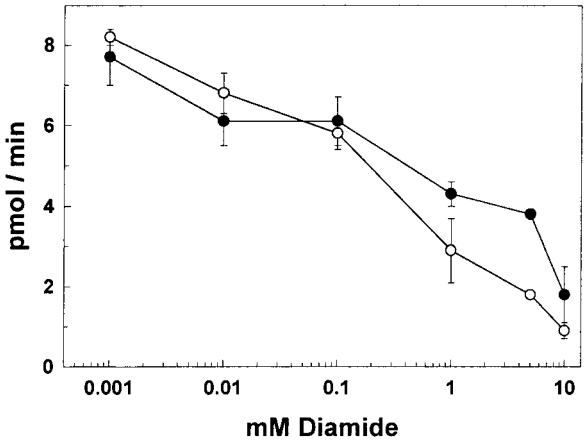

FIGURE 2: Inactivation of the basal activity of rat brain PKC by diamide. Purified rat brain PKC was preincubated with diamide as described in Materials and Methods and the legend to Figure 1. The basal activity of the preincubated enzyme was measured in the presence of either 0.2 mM Ca^{2+} (●) or 30 μg/mL PS (○). Each point is the average ± SD of triplicate assays, and the results shown were reproduced in separate experiments.

Substantial inactivation was achieved when PKC was preincubated with 1 mM diamide, and >75% inactivation was reached at 10 mM diamide (●, Figure 1). Thus, diamide was effective against cPKC catalysis, but robust inactivation was observed only in the millimolar concentration range. Diamide preincubation likewise inactivated the basal [Ser25]PKC-(19−31) kinase activity of PKC observed in the absence of Ca^{2+} and PS in a concentration-dependent manner (○, Figure 1). When 30 mM DTT was included in the preincubation mixtures, diamide did not achieve substantial inactivation of either the Ca^{2+}/PS- stimulated activity (▼) or the basal activity (▽) of PKC (Figure 1). Thus, the PKC inhibitory effects observed with diamide were lost when its thiol-oxidant activity was quenched by DTT.

The ROS sodium periodate has been reported to activate PKC up to 8-fold at low concentrations and to inactivate the enzyme at higher concentrations (*31*). In contrast, preincubation with diamide (0.01−10 mM) did not enhance PKC activity, whether the enzyme was assayed under basal or Ca^{2+}/PS-stimulated conditions (Figure 1). We next tested whether diamide could replace either the Ca^{2+} or the PS cofactor requirement in the activation of cPKC. Figure 2 shows that across a concentration range of 0.001−10 mM diamide, diamide preincubation in the absence of reducing thiol resulted in concentration-dependent inactivation of basal PKC activity when assay mixtures contained either 0.2 mM Ca^{2+} (●) or 30 μg/mL PS (○). Thus, inactivation of PKC catalysis was the predominant effect of diamide across a concentration range spanning three to four orders of magnitude, whether PKC was assayed in the presence of Ca^{2+}/PS, Ca^{2+} alone, or PS alone or in the absence of stimulatory cofactors.

To establish whether diamide-induced PKC inactivation entailed disulfide bridge formation, we investigated whether the reductant DTT could reverse the inactivation. In these experiments, PKC was preincubated with/without diamide in the absence of reducing thiol for 5 min at 30 °C followed by a second preincubation with/without 30 mM DTT (5 min, 30 °C) and then added to assay mixtures for measurement of Ca^{2+}/PS-stimulated PKC activity. Table 1 shows the results of two experiments done under identical conditions. In each case, about 30% of the Ca^{2+}/PS-stimulated PKC activity remained after PKC was preincubated with 5 mM diamide in the absence of DTT treatment, and 80−90% of the activity was recovered when the diamide preincubation step was followed by preincubation with 30 mM DTT (Table 1). The observed DTT reversibility indicates that diamide-induced PKC inactivation resulted from disulfide bridge formation (*18*, *23*). The observation that diamide had pronounced effects on PKC activity only at relatively high concentrations and solely through its ability to induce disulfide bridge formation indicates the suitability of this oxidant for investigations of oxidant-induced PKC *S*-glutathiolation.

Table 1: DTT Reverses Diamide-Induced Inactivation of Ca^{2+}/PS-stimulated Rat Brain PKC Activity

	percent (%) activity remaining	
condition[a]	experiment 1	experiment 2
no treatment	100 ± 3	100 ± 10
5 mM diamide	26 ± 1	31 ± 6
5 mM diamide + 30 mM DTT	89 ± 5	80 ± 2

[a] Purified rat brain PKC was preincubated in 20 mM Tris-HCl, pH 7.5, either alone or in the presence of 5 mM diamide at 30 °C for 5 min, followed by incubation with/without 30 mM DTT for 5 additional minutes at 30 °C, and then assayed for Ca^{2+}/PS-stimulated [Ser25]-PKC(19−31) kinase activity. Conditions and procedures are described in detail in Materials and Methods.

Glutathione Potentiates Diamide-Induced Inactivation of Purified Rat Brain PKC. In the presence of GSH, diamide can induce protein *S*-glutathiolation, e.g., carbonic anhydrase III and protein tyrosine phosphatase 1B have been reported to undergo *S*-glutathiolation upon exposure to diamide in the presence of 125 μM and 2 mM GSH, respectively (*36*, *37*). We previously reported that PKC isozymes are subject to nonredox inhibition by GSH but only at GSH concentrations exceeding 3 mM (*22*). To investigate whether PKC activity might also be influenced by *S*-glutathiolation, we next examined whether a subinhibitory concentration of GSH (100 μM) could potentiate diamide-induced inactivation of purified rat brain PKC.

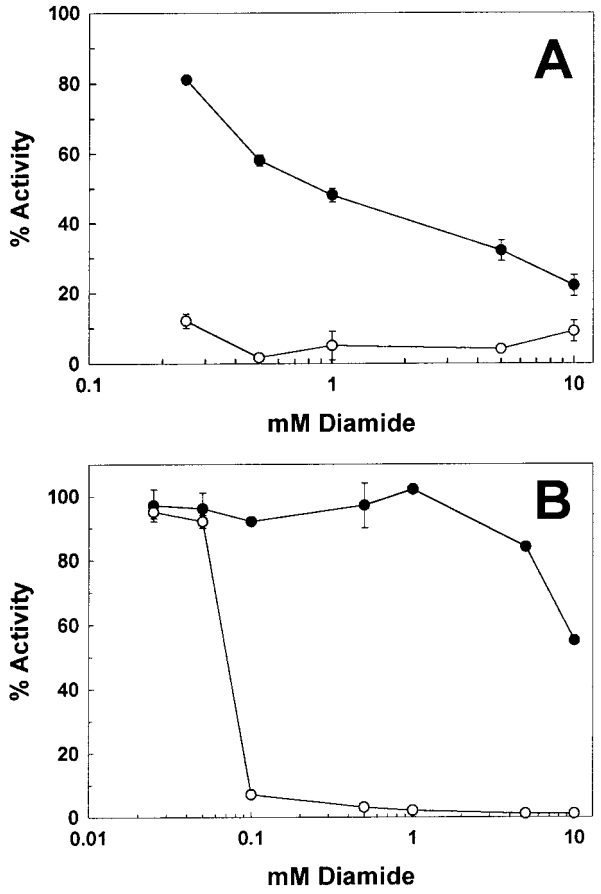

FIGURE 3: GSH potentiates diamide inactivation of PKC isozymes. GSH potentiation of diamide-induced inactivation of (A**)** rat brain PKC and (B**)** cPKC-α was measured as follows. (A**)** Purified rat brain PKC was preincubated with diamide as described in the legend to Figure 1, in the presence (○) or absence (●) of 100 μM GSH. The activity of the preincubated enzyme was measured in the presence of Ca^{2+} and PS. Preincubation with 100 μM GSH alone was without effect on PKC activity (see Results). 100% activity = 12.4 ± 0.7 pmol of ^{32}P transferred/min. Each point is the average ± SD of triplicate assays, and the results shown were reproduced in separate experiments. (B) Purified recombinant human cPKC-α was preincubated with diamide as described in Materials and Methods and the legend to Figure 1, in the presence (○) or absence (●) of 100 μM GSH. The catalytic activity of cPKC-α was measured in the presence of 0.2 mM Ca^{2+} and 30 μg/mL PS. Preincubation with 100 μM GSH alone was without effect on cPKC-α activity (see Results). 100% activity = 140 ± 10 pmol ^{32}P transferred/min. Each point is the average ± SD of triplicate assays, and the results shown were reproduced in separate experiments.

In these experiments, purified rat brain PKC was preincubated with 0.25−10 mM diamide in the presence or absence of 100 μM GSH for 5 min at 30 °C and then added to assay mixtures for measurement of Ca^{2+}/PS-stimulated [Ser25]PKC(19−31) kinase activity. Figure 3A shows representative results obtained in a single experiment that compare the effects of diamide alone (●) versus diamide plus 100 μM GSH (○) on Ca^{2+}/PS-stimulated rat brain PKC activity. The extent of diamide-induced PKC inactivation was markedly increased in the presence of GSH (○) across a broad range of diamide concentrations (Figure 3A). Most striking was the effect of GSH at 250 μM diamide, in which diamide alone (●) versus diamide + 100 μM GSH (○) resulted in a loss of 19 ± 1 versus 88 ± 2% of the PKC activity (Figure 3A). Because at least 67% of the Ca^{2+}/PS-stimulated [Ser25]PKC(19−31) kinase activity of the purified rat brain PKC isozyme mixture reflects cPKC catalysis, these results indicate that GSH markedly enhanced diamide-induced inactivation of cPKC catalysis. The enhancement of inactivation was not due to additive inhibitory effects introduced by GSH but was instead due to GSH potentiation of diamide-induced PKC inactivation, because when PKC was preincubated with 100 μM GSH in the absence of diamide, 99 ± 3% of the activity was recovered. (The dependence of GSH potentiation of inactivation on the concentration of diamide is demonstrated for recombinant cPKC-α in Figure 3B.) Thus, GSH potentiated diamide-induced cPKC inactivation with remarkable efficacy, providing evidence that cPKC isozymes may be subject to inactivation by *S*-glutathiolation.

Table 2: DTT Reverses GSH Potentiation of Diamide-Induced PKC Inactivation

first preincubation[a,b]	second preincubation[a,b]	percent (%) activity remaining[c]
rat brain PKC alone	no addition	100 ± 1
rat brain PKC + diamide	no addition	60 ± 1
rat brain PKC + diamide	DTT	108 ± 2
rat brain PKC + diamide & GSH	no addition	0 ± 1
rat brain PKC + diamide & GSH	DTT	107 ± 3
cPKCα alone	no addition (20 min)	100 ± 2
cPKCα + diamide	no addition (20 min)	70 ± 3
cPKCα + diamide and GSH	no addition (20 min)	5 ± 1
cPKCα + diamide and GSH	DTT (20 min)	102 ± 1
cPKCα + diamide and GSH	DTT (5 min)	45 ± 2
cPKCα + diamide and GSH	DTT (10 min)	88 ± 3

[a] Purified rat brain PKC was preincubated in 20 mM Tris-HCl, pH 7.5, for two successive 5-min intervals at 30 °C. Where indicated, 0.5 mM diamide and/or 100 μM GSH were included in the first preincubation step, and 30 mM DTT was added at the initiation of the second preincubation period. [b] cPKC-α was analyzed analogously. Purified cPKC-α was preincubated for 5 min at 30 °C in 20 mM Tris-HCl, pH 7.5, with/without 0.1 mM diamide plus 100 μM GSH. This was followed by a second preincubation at 30 °C with/without 30 mM DTT for the time interval specified in the Table under the column second preincubation. [c] The Ca^{2+}/PS-stimulated [Ser25]PKC(19−31) kinase activity of preincubated rat brain PKC/cPKC-α was measured. Other experimental details are provided under Materials and Methods.

Protein *S*-glutathiolation is readily reversed by DTT (*18*). As a test of whether the potentiation of diamide-induced PKC inactivation by GSH entailed *S*-glutathiolation of PKC isozymes, we investigated whether PKC inactivation by diamide plus 100 μM GSH could be reversed by DTT. This was accomplished by preincubation of rat brain PKC for 5 min at 30 °C either alone, with 0.5 mM diamide, or with 0.5 mM diamide plus 100 μM GSH followed by a second preincubation (5 min, 30 °C) with/without 30 mM DTT. When the second preincubation step was complete, Ca^{2+}/PS-stimulated PKC activity was assayed. Table 2 shows the results of a single experiment that was reproduced in a second analysis. In the case of preincubation mixtures that were not treated with DTT, the activity remaining after PKC was preincubated with diamide alone was 60 ± 1%, and this value fell to 0 ± 1% when PKC was preincubated with diamide in the presence of GSH (Table 2). This indicates that, under the experimental conditions employed, approximately 60% of the PKC inactivation produced by diamide plus GSH was due to GSH potentiation of diamide-induced inactivation. Inclusion of DTT in the second preincubation step fully

recovered PKC activity that had been lost due to incubation with diamide plus GSH in the first preincubation step, i.e., 107 ± 3% of the initial activity was recovered (Table 2). Taken together, these results show that DTT fully reverses GSH potentiation of diamide-induced PKC inactivation. These results are consistent with an oxidative inactivation mechanism involving PKC *S*-glutathiolation.

Diamide Induces cPKC-α S-Glutathiolation in Concert with Isozyme Inactivation. Having established that GSH markedly potentiated diamide-induced inactivation of cPKC catalysis by a PKC isozyme mixture purified from mammalian tissue, our next objective was to determine whether GSH potentiation of inactivation occurred in association with covalent linkage of GSH to cPKC by disulfide bridges. To accomplish this, we shifted the focus of the study to purified, baculovirus-produced recombinant cPKC-α. We first established that GSH potentiated diamide-induced inactivation of the recombinant isozyme. Preincubation of cPKC-α with diamide alone (0.025−10 mM) for 5 min at 30 °C weakly inhibited the isozyme (●, Figure 3B). Inclusion of 100 μM GSH in the preincubation mixtures markedly potentiated cPKC-α inactivation by the thiol-specific oxidant (○, Figure 3B). As much as 80−100% of the loss of cPKC-α activity achieved when GSH was included with diamide in the cPKC-α preincubation mixtures was due to GSH potentiation of cPKC-α inactivation, across a broad range of diamide concentrations (0.1−5.0 mM diamide) (○, Figure 3B). No loss of cPKC-α activity was observed when preincubation mixtures contained 100 μM GSH plus 25−50μM diamide (○, Figure 3B) or 100 μM GSH alone (experimental error, ± 10%), indicating the dependence of the observed potentiation on the concentration of diamide.

We also assessed the ability of DTT to reverse GSH potentiation of cPKC-α inactivation. In these experiments, cPKC-α was first preincubated with 0.1 mM diamide and 100 μM GSH for 5 min at 30 °C and then subjected to a second preincubation period with/without 30 mM DTT (20 min, 30 °C), followed by an assay of cPKC-α activity. In the absence of DTT treatment, diamide plus GSH achieved >90% inactivation of cPKC-α (Table 2). When DTT was present during the second preincubation period, the inactivation was fully reversed (Table 2). Shorter incubation periods with DTT (5 and 10 min) resulted in partial recovery of cPKC-α activity (Table 2), indicating the time-dependence of the recovery (Table 2). The DTT-reversible effects of GSH/diamide on the catalytic activity of cPKC-α provided evidence that diamide induced cPKC-α *S*-glutathiolation, resulting in isozyme inactivation.

The enzyme thioltransferase (also known as glutaredoxin) selectively dethiolates *S*-glutathiolated proteins by a GSH-dependent mechanism (*37*, *38*). To investigate whether thioltransferase catalysis could reverse GSH-potentiated cPKC-α inactivation, we modified the DTT reversal analysis (Table 2) by substituting recombinant human thioltransferase and 500 μM GSH (*37*) for DTT in the second preincubation step (10 min, 30 °C). We found that the thioltransferase cosubstrate 500 μM GSH was by itself very effective in nonenzymatically restoring the activity of cPKC-α that had been inactivated in the first preincubation step by 0.1 mM diamide plus 100 μM GSH; 500 μM GSH restored the inactivated isozyme to 80 ± 1% of its original activity. This result is consistent with an inactivation mechanism involving

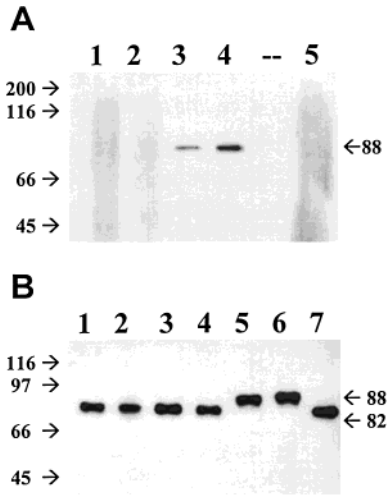

FIGURE 4: Diamide induces cPKC-α *S*-glutathiolation. (A) To measure cPKC-α *S*-glutathiolation using radiolabeled GSH, purified human cPKC-α was preincubated with 100 μM [^{35}S]GSH in the presence/absence of diamide under the conditions employed in Figure 3B. cPKC-α preincubation mixtures contained 100 μM [^{35}S]-GSH either alone (lane 1) or in the presence of 25 μM diamide (lane 2), 100 μM diamide (lane 3), 500 μM diamide (lane 4), or 500 μM diamide with subsequent addition of 10 mM DTT (lane 5). For experimental details, see Materials and Methods. The results shown were reproduced in separate experiments. (B) cPKC-α *S*-glutathiolation was detected by immunoblot analysis, based on the change in the migration position of the isozyme in nonreducing SDS−PAGE associated with the modification (82 kDa → 88 kDa). cPKC-α was preincubated under the conditions employed in Figure 3B and described in Materials and Methods, either alone (lane 1) or with 500 μM diamide (lane 2), 100 μM GSH (lane 3), 100 μM GSH plus 25 μM diamide (lane 4), 100 μM GSH plus 100 μM diamide (lane 5), 100 μM GSH plus 500 μM diamide (lane 6), or 100 μM GSH plus 500 μM diamide with subsequent addition of 10 mM DTT (lane 7). Preincubated samples were subjected to immunoblot analysis with cPKC-α mAb, and immunoreactive bands were detected by enhanced chemiluminescence. The results shown were reproducibly obtained in additional experiments.

S-glutathiolation, based on previous observations that excess GSH nonenzymatically dethiolates *S*-glutathiolated creatine kinase (*17*). In the same experiment, thioltransferase produced a substantial further recovery of cPKC-α activity. In the presence of 0.015 U/mL thioltransferase plus 500 μM GSH, cPKC-α activity was restored to 93 ± 3% of the initial activity, and increasing the thioltransferase concentration to 0.15 U/mL produced a recovery of 100 ± 2%. The relative abilities of GSH versus GSH plus thioltransferase to reverse the inactivation were reproduced in an independent analysis. We next sought to directly demonstrate the predicted covalent modification of cPKC-α.

Our first approach to detect cPKC-α *S*-glutathiolation was to preincubate purified cPKC-α with diamide in the presence of 100 μM [^{35}S]GSH (specific activity = 125 mCi/mmol) under conditions employed in Figure 3B. The preincubated samples were then subjected to nonreducing 10% SDS−PAGE followed by autoradiography, to measure the incorporation of radiolabel into cPKC-α. Figure 4A shows results that are representative of four independent analyses. No radiolabeled band was evident when cPKC-α was preincubated with 100 μM [^{35}S]GSH alone (lane 1) or 100 μM [^{35}S]-GSH plus 25 μM diamide (lane 2) (Figure 4A), i.e., preincubation conditions that did not result in cPKC-α inactivation (Figure 3B). A single, prominent radiolabeled band was evident at 88 kDa in lanes 3 and 4, which

corresponded respectively to cPKC-α preincubation mixtures containing 100 μM [^{35}S]GSH plus either 100 μM or 500 μM diamide (Figure 4A), i.e., preincubation conditions that fully inactivated cPKC-α primarily as a result of the potentiating effects of GSH (Figure 3B). These results provided evidence that purified cPKC-α was covalently modified by [^{35}S]GSH with a consequential minor retardation of its normal migration position of 82 kDa (*15*), in association with GSH potentiation of cPKC-α inactivation by diamide. The increased radiolabeling of the 88 kDa band in lane 4 as compared with lane 3 (Figure 4A) also provided evidence that the extent of [^{35}S]GSH labeling of cPKC-α continued to increase as the diamide concentration was increased, even after conditions supporting full inactivation were reached. Consistent with the DTT reversibility of GSH potentiation of diamide-induced cPKC-α inactivation, the 88-kDa radiolabeled band disappeared when the sample preincubated with 100 μM [^{35}S]GSH and 500 μM diamide (lane 4) was further incubated with 10 mM DTT (5 min, 30 °C) (lane 5) (Figure 4A). These results constitute evidence that [^{35}S]GSH covalently modifies cPKC-α by forming intermolecular disulfide bridges with the isozyme, in association with GSH potentiation of diamide-induced cPKC-α inactivation.

To establish that the 88 kDa band labeled by [^{35}S]GSH in Figure 4A was indeed cPKC-α, we employed Western analysis to directly examine whether the migration position of cPKC-α was changed to 88-kDa, under conditions in which GSH potentiated diamide-induced cPKC-α inactivation. cPKC-α was preincubated with/without diamide ± GSH under the conditions employed in Figures 3B and 4A. The preincubated samples were subjected to nonreducing 7.5% SDS−PAGE followed by immunoblot analysis with cPKC-α mAb. The results shown in Figure 4B are representative of four independent analyses. Figure 4B shows that cPKC-α migrated at 82 kDa when the isozyme was preincubated alone (lane 1) or in the presence of 0.5 mM diamide (lane 2), 100 μM GSH (lane 3), or 100 μM GSH plus 25 μM diamide (lane 4), i.e., under conditions in which GSH did not potentiate cPKC-α inactivation (Figure 3B) and [^{35}S]GSH labeling of cPKC-α did not occur (Figure 4A). The migration position of cPKC-α shifted to 88 kDa when the isozyme was preincubated with 100 μM GSH plus either 100 μM diamide (lane 5, Figure 4B) or 500 μM diamide (lane 6, Figure 4B), i.e., conditions that also resulted in GSH-dependent PKC inactivation (Figure 3B) and [^{35}S]GSH labeling of an 88 kDa band (Figure 4A). These results clearly identify the [^{35}S]GSH-labeled band in Figure 4A as cPKC-α. Furthermore, when the GSH-modified cPKC-α sample (lane 6, Figure 4B) was treated with 10 mM DTT for 5 min at 30 °C prior to immunoblot analysis, the cPKC-α migration position was restored to 82 kDa (lane 7, Figure 4B). Taken together, the results shown in Figures 3B and 4 demonstrate that diamide induces cPKC-α *S*-glutathiolation in association with GSH-dependent cPKC-α inactivation. Furthermore, because the oxidant activity of diamide is limited to the induction of disulfide bridge formation (*23*), cPKC-α *S*-glutathiolation is the sole mechanism that can account for the GSH-potentiated inactivation of the isozyme observed in these studies.

To estimate the stoichiometry of cPKC-α *S*-glutathiolation under conditions of inactivation, we quantitated the [^{35}S]-GSH incorporated into cPKC-α when the isozyme was incubated with diamide plus [^{35}S] GSH under the conditions employed in Figure 4A. This was done by precipitating the [^{35}S] *S*-glutathiolated cPKC-α sample with an equal volume (120 μL) of 20% trichloroacetic acid (TCA), 1% PP_I at 4 °C, followed by recovery of the precipitated isozyme on glass fiber filters and quantitation of the cpm incorporated into the isozyme (460 cpm/pmol of [^{35}S]GSH). At diamide concentrations of 25, 100, and 500 μM, the respective stoichiometries of *S*-glutathiolation were 0.18 ± 0.07, 0.95 ± 0.03, and 2.32 ± 0.07 pmol of [^{35}S] GSH/pmol of cPKC-α. These results are representative of two independent analyses done in duplicate, and they are consistent with the concentration dependence of labeling shown in Figure 4A. Furthermore, the stoichiometry of 0.95 [^{35}S]GSH/cPKC-α observed at 100 μM diamide, a concentration that is sufficient to support cPKC-α inactivation by *S*-glutathiolation (Figure 3B), shows that, out of the 20 Cys residues in cPKC-α, at most very few and potentially only one is involved in the inactivation mechanism.

Diamide Treatment Induces DTT-Reversible Inactivation of cPKC-α in NIH3T3 Cells in Association with cPKC-α [^{35}S] S-Thiolation. To determine whether inactivation of cPKC-α by *S*-thiolation could serve as a point of control for the isozyme in vivo, we next investigated whether diamide treatment could likewise induce cPKC-α inactivation in mammalian cells. NIH3T3 cells were chosen for analysis of oxidative cPKC-α inactivation, because immunoblot analysis revealed that they express abundant cPKC-α but do not express other cPKCs (data not shown). Thus, the Ca^{2+}/PS-dependent activity of cPKC-α could be assayed directly in DEAE-extracted cell lysates. NIH3T3 cells were first cultured in the presence of 50 μg/mL cycloheximide for 5 h at 37 °C, because these conditions of protein synthesis inhibition were necessary for the analysis of cellular cPKC-α [^{35}S] *S*-thiolation in subsequent experiments. Next, the cells were treated with 0.25−5.0 mM diamide for 10 min at 37 °C and then lysed at 4 °C. cPKC-α was DEAE-extracted from the cell lysates, preincubated for 15 min with/without 30 mM DTT at 30 °C, and assayed.

Western analysis of the DEAE-extracted cPKC-α samples demonstrated that diamide treatment had no effect on cPKC-α expression in this system (Figure 5, lower panel). Inactivation of cellular cPKC-α activity in response to diamide treatment of the cells was measured by assays of the cPKC-α samples that were not preincubated with DTT. The upper panel of Figure 5 shows that diamide treatment of the NIH3T3 cells induced substantial cPKC-α inactivation (>50%) in a concentration-dependent manner (filled bars). The diamide-induced inactivation of cellular cPKC-α that persisted after preincubation with DTT (DTT-irreversible inactivation) is represented by open bars (Figure 5, upper panel). At 0.25−2.5 mM diamide, the inactivation of cellular cPKC-α was fully reversed by DTT, and DTT reversed the inactivation observed at 5.0 mM diamide (66 ± 5% inactivation) (filled bar) to 16 ± 3% inactivation (open bar, Figure 5). Thus, diamide treatment of NIH3T3 cells caused robust, DTT-reversible cPKC-α inactivation.

To establish whether diamide-induced cPKC-α inactivation in NIH3T3 cells was associated with *S*-thiolation of the isozyme, we employed a standard approach for the detection of protein *S*-thiolation in cells that entails selective [^{35}S] labeling of cellular LMW thiols (*18*, *24*−*26*). Cells were

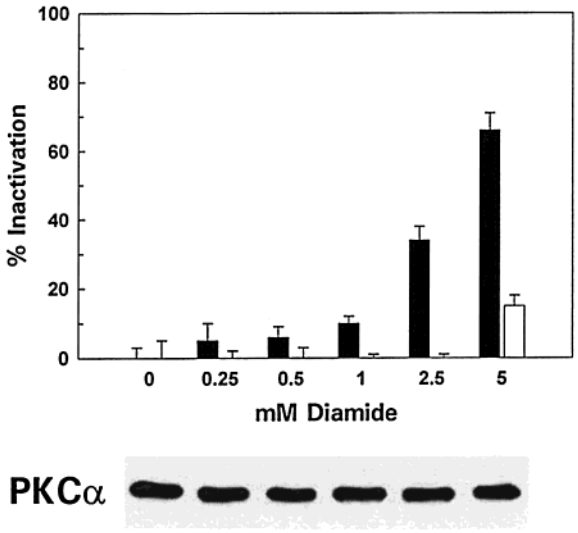

FIGURE 5: Diamide treatment induces DTT-reversible inactivation of cPKC-α in NIH3T3 cells. Subconfluent NIH3T3 cells were treated with diamide at the indicated concentrations for 10 min at 37 °C as described in Materials and Methods, and cPKC-α was DEAE-extracted from the cell lysates. Upper panel: The DEAE-extracted cPKC-α samples were assayed after preincubation for 15 min at 30 °C in the absence (filled bars) or presence of 30 mM DTT (open bars) (5 μg protein/assay). Open bars represent DTT-irreversible inactivation. At a given diamide concentration, DTT-reversible inactivation is the percent inactivation observed in the absence of DTT (filled bars) minus the percent inactivation observed in the presence of DTT (open bars). The cPKC-α activity recovered from the untreated cells (0 mM diamide) served as the 100% activity value; 100% activity = 3.18 ± 0.11 pmol ^{32}P transferred min^{-1} (5 μg of protein)$^{-1}$. Results are expressed as the mean of triplicate assays ± SD. Lower panel: Western analysis of the DEAE-extracted cPKC-α samples analyzed for activity in the upper panel (10 μg of protein/lane). Results shown were reproduced in independent experiments.

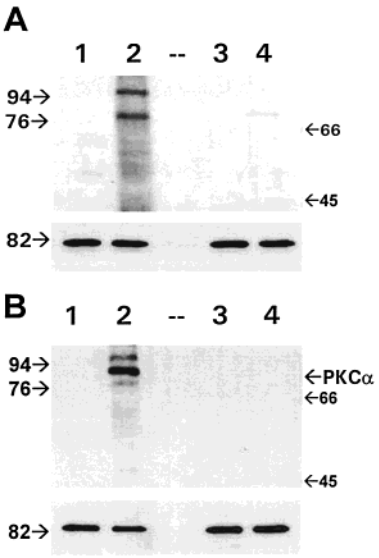

FIGURE 6: Diamide treatment induces cPKC-α [^{35}S] *S*-thiolation in NIH3T3 cells. [^{35}S]-labeled NIH3T3 cells were analyzed for induction of [^{35}S] *S*-thiolation of total cellular proteins (A) and cPKC-α (B) by 5 mM diamide, under the conditions employed to measure diamide-induced inactivation of cellular cPKC-α in Figure 5. Experimental procedures and conditions are described in detail in Methods and Results. (A) Upper panel: Cell lysates were analyzed by nonreducing SDS−PAGE/ autoradiography for [^{35}S] *S*-thiolated proteins (DTT-sensitive protein radiolabeling). Cells were lysed either in the presence of NEM to detect protein [^{35}S] radiolabeling, or in the presence of DTT to establish the DTT-sensitivity of the labeling. Lane 1 = untreated cells lysed with NEM; lane 2 = 5 mM diamide-treated cells lysed with NEM; lane 3 = untreated cells lysed with DTT; lane 4 = diamide-treated cells lysed with DTT. Protein radiolabeling was observed only in the NEM-lysate from diamide-treated cells, and prominent radiolabeled species are indicated by arrows at 76 and 94 kDa (lane 2). The disappearance of the labeled bands when the diamide-treated cells were lysed with DTT (lane 4) is indicative of DTT-sensitive labeling. Lower panel. cPKC-α Western analysis of cell lysates analyzed in the upper panel (20 μg of cell lysate protein/lane). (B) Analysis of cPKC-α [^{35}S] *S*-thiolation was done by immunoprecipitating cPKC-α from the cell lysates shown in (A) (lanes 1−4) and then subjecting the immunoprecipitated samples to nonreducing SDS−PAGE/autoradiography (upper panel) and Western analysis (lower panel). [^{35}S] *S*-thiolated cPKC-α is indicated by an arrow at 82 kDa (upper panel). Results shown were reproduced in independent experiments.

metabolically labeled with [^{35}S] cysteine under conditions of protein synthesis inhibition and then treated for 10 min at 37 °C with 5 mM diamide, a concentration that achieved robust, DTT-reversible cPKC-α inactivation in the cells (Figure 5). To detect protein [^{35}S] *S*-thiolation, the cells were lysed under two sets of conditions. Lysis in the presence of *N*-ethylmaleimide (NEM) was done to detect protein [^{35}S] *S*-thiolation produced in the cells by preventing scrambling of the label in the lysate by thiol−disulfide exchange reactions (*18*, *24*); lysis in the presence of DTT was done to establish that the observed labeling of cellular proteins was indeed due to [^{35}S] *S*-thiolation, based on the DTT reversibility of the posttranslational modification (*18*, *24*).

We first measured [^{35}S] *S*-thiolation of cellular proteins in response to diamide treatment of the NIH3T3 cells by analyzing aliquots of the cell lysates by nonreducing SDS−PAGE/autoradiography. A representative autoradiogram corresponding to this analysis is shown in Figure 6A (upper panel). [^{35}S] *S*-thiolation of cellular proteins was not detected in the lysate of untreated cells (lane 1, Figure 6A). In contrast, [^{35}S]-labeling of cellular proteins was readily detected in the lysate from the diamide-treated cells (lane 2, Figure 6A), and the labeling was lost when the cells were lysed in the presence of DTT (lane 4, Figure 6A), indicating that the labeling was due to [^{35}S] *S*-thiolation. Two prominent [^{35}S] *S*-thiolated species were observed in this analysis at 76 and 94 kDa (lane 2, Figure 6A).

To address whether diamide treatment induced cPKC-α [^{35}S] *S*-thiolation in the cells, cPKC-α was immunoprecipitated from aliquots of the cell lysates analyzed in Figure 6A and then analyzed analogously for cPKC-α [^{35}S] *S*-thiolation. Western analysis of cPKC-α in the immunoprecipitates and in the cell lysates indicated that cPKC-α migrated at 82 kDa and was recovered to similar extents under all treatment and lysis conditions (Figure 6A and B, lower panels). In the analysis of cPKC-α [^{35}S] *S*-thiolation (Figure 6B, upper panel), no [^{35}S]-labeled bands were evident when cPKC-α was immunoprecipitated from the lysate of untreated cells (lane 1, Figure 6B). In contrast, a pronounced [^{35}S]-labeled band was observed at the migration position of cPKC-α (82 kDa) when cPKC-α was immunoprecipitated from the lysate of diamide-treated cells (lane 2), and the [^{35}S]-labeled 82 kDa band disappeared when the diamide-treated cells were lysed in the presence of DTT (lane 4) (Figure 6B). The [^{35}S] *S*-thiolated bands at 76 and 94 kDa in the immunoprecipitated cPKC-α sample (lane 2, Figure 6B) appear to represent

residual amounts of the predominant [^{35}S] *S*-thiolated bands observed in the cell lysate from diamide-treated cells (lane 2, Figure 6A) that were carried over with immunoprecipitated cPKC-α. The enrichment of the 82-kDa [^{35}S] *S*-thiolated species by cPKC-α immunoprecipitation, which is evident from the lack of detection of this radiolabeled band in the analysis of the cell lysate (Figure 6A), and the comigration of the 82-kDa radiolabeled species with cPKC-α in this system indicate that the 82-kDa [^{35}S] *S*-thiolated species is cPKC-α. Therefore, the results in Figures 5 and 6 demonstrate that cPKC-α is [^{35}S] *S*-thiolated in NIH3T3 cells in response to diamide treatment, under conditions in which the oxidant induces robust, DTT-reversible inactivation of the isozyme.

DISCUSSION

In this paper, we show that purified cPKC-α is *S*-glutathiolated in vitro in response to a mild oxidative stimulus and that the modification inactivates the isozyme. The evidence for this is as follows. The thiol-specific oxidant diamide weakly inactivated purified recombinant human cPKC-α and the cPKC activity of a PKC isozyme mixture purified from rat brain. In each case, the inactivation was markedly potentiated by 100 μM GSH, which by itself did not affect PKC activity. The only oxidative modifications that are induced in proteins by diamide are disulfide bridge formation between protein thiols and, in the presence of GSH, protein *S*-glutathiolation (*23*); both of these modifications are DTT-reversible (*23*). Thus, the predicted oxidative modifications of cPKC-α that would account for diamide inactivation of cPKC-α and GSH potentiation of the inactivation are, respectively, disulfide bridge formation between cPKC-α thiols and cPKC-α *S*-glutathiolation. Consistent with this prediction, the inactivating effects of diamide against cPKC-α and their potentiation by GSH were both fully reversed by DTT. Likewise, DTT reversibility was demonstrated for diamide inactivation of cPKC catalysis by the purified isozyme mixture and its potentiation by GSH. Furthermore, diamide induced DTT-reversible, covalent labeling of cPKC-α by GSH, i.e., cPKC-α *S*-glutathiolation, and this occurred in association with GSH potentiation of cPKC-α inactivation. GSH labeling of cPKC-α and GSH potentiation of cPKC-α inactivation exhibited a similar dependence on the diamide concentration. Taking into consideration the restricted oxidant activity of diamide (*23*), the observations summarized here clearly demonstrate that cPKC-α can be inactivated by oxidant-induced *S*-glutathiolation.

The inclusion of 10−25 mM DTT in samples of *S*-thiolated proteins is known to indiscriminately and fully reverse *S*-thiolating modifications detected by nonreducing SDS−PAGE analysis, as a result of the conditions of sample preparation, i.e., boiling in the presence of a denaturing detergent (*24*−*26*). Indeed, we observed full reversal of cPKC-α *S*-glutathiolation by 10 mM DTT, using detection methods that entailed SDS−PAGE analysis (Figure 4). In addition, we found that exposing *S*-glutathiolated cPKC-α to 30 mM DTT for 20 min at 30 °C fully restored the catalytic activity of the inactivated isozyme (Table 2). While these results do not allow a direct comparison of the efficacy of DTT in restoring the catalytic activity of *S*-glutathiolated cPKC-α versus reversing the oxidative modification, they do indicate that conditions less rigorous than those employed to completely deglutathiolate cPKC-α via DTT were sufficient to fully restore the catalytic activity of the isozyme. Thus, the results of the DTT reversal experiments are consistent with the conclusion that *S*-glutathiolation is the mechanism underlying GSH-potentiated, diamide-induced cPKC-α inactivation.

To our knowledge, this is the first report to demonstrate *S*-glutathiolation of a protein kinase. cPKC-α *S*-glutathiolation was detected based on the DTT-reversible incorporation of the radiolabel [^{35}S]GSH into the isozyme structure and by an associated change in the migration position of cPKC-α in nonreducing SDS−PAGE. The efficacy of GSH in potentiating diamide inactivation of the catalytic activity of a mixed PKC isozyme preparation (α, β, γ, ϵ, ζ) provides evidence that the observation of cPKC-α inactivation by *S*-glutathiolation reported here may extend to additional PKC isozymes. The ability of diamide to induce potent cPKC-α inactivation via *S*-glutathiolation at diamide concentrations as low as 100 μM indicates that cPKC-α is highly susceptible to this type of inactivating oxidative modification. On the other hand, the weak inactivation of cPKC-α by diamide alone at millimolar concentrations provides evidence that cPKC-α is relatively resistant to inactivation by disulfide bridge formation among cPKC-α thiols.

We tested the significance of the observed inactivation of purified cPKC-α by *S*-glutathiolation by investigating whether diamide treatment of mammalian cells could likewise produce cPKC-α inactivation in association with isozyme *S*-thiolation. Diamide treatment produced concentration-dependent and robust DTT-reversible cPKC-α inactivation in NIH3T3 cells in association with *S*-thiolation of the isozyme. Because the diamide-induced inactivation of cellular cPKC-α was DTT-reversible, the potential oxidative inactivation mechanisms are limited to three categories. The inactivation could entail (i) intermolecular disulfide bridge formation between cPKC-α and other proteins (including cPKC-α itself), (ii) intramolecular disulfide bridge formation within cPKC-α, or (iii) cPKC-α *S*-thiolation.

The first inactivation mechanism, disulfide-linked complex formation of cPKC-α with other cellular proteins, is ruled out because complexes were not detected under treatment conditions that achieved up to 50% DTT-reversible cPKC-α inactivation in NIH3T3 cells (Figure 5). The absence of substantial complex formation is indicated by the similar amounts of cPKC-α immunoreactivity recovered at 82 kDa in the nonreducing Western analysis of lysates from diamide-treated and untreated NIH3T3 cells (Figure 6A, lower panel) and by the lack of diamide-induced, DTT-sensitive cPKC-α immunoreactivity at apparent MWs >82 kDa in the analysis (data not shown). The second inactivation mechanism, intramolecular disulfide bridge formation within cPKC-α, can be ruled out by the limited efficacy of diamide against purified cPKC-α in the absence of GSH, i.e., under conditions that promote intramolecular disulfide bridge formation in proteins. At concentrations ≤1.0 mM, diamide failed to inactivate purified cPKC-α, and 5 mM diamide inactivated cPKC-α <20% (Figure 3B). In contrast, treatment of NIH3T3 cells with 5 mM diamide achieved approximately 50% DTT-reversible inactivation of cellular cPKC-α (Figure 5). Thus, it is clear that the weak inactivation of cPKC-α achieved by diamide induction of intramolecular disulfide bridge formation cannot account for the potent inactivation

of cPKC-α that was observed in the diamide-treated cells. The third inactivation mechanism, cPKC-α *S*-thiolation, is supported by the marked potentiation of diamide-induced inactivation of purified cPKC-α by 100 μM GSH, even at submillimolar diamide concentrations (Figure 3B) and by the observation that cPKC-α is [^{35}S] *S*-thiolated in NIH3T3 cells in response to diamide treatment, under conditions in which the oxidant induces robust, DTT-reversible cPKC-α inactivation (Figures 5 and 6). Collectively, the results provide strong evidence that diamide-induced cPKC-α inactivation in NIH3T3 cells is primarily due to cPKC-α *S*-thiolation.

Variable apparent molecular weights have been observed for some PKC isozymes by SDS−PAGE, and the isozyme phosphorylation state has been shown to contribute to this variability (*8*). Our observation that *S*-glutathiolation of purified cPKC-α shifts the migration position of the isozyme in SDS−PAGE (82 → 88 kDa) provides evidence that oxidative modifications may also contribute to the variability in the apparent molecular weight observed for a given PKC isozyme. The absence of a migration shift by [^{35}S] *S*-thiolated cPKC-α extracted from NIH3T3 cells is not yet accounted for, but a reasonable explanation is that cPKC-α binding proteins or other molecules associated with the isozyme in the cellular milieu may limit access to the thiol oxidation sites in cellular cPKC-α. Partial protection of cellular cPKC-α from oxidative modification could also account for the lesser degree of inactivation of cellular cPKC-α by *S*-thiolation as compared with the purified form of the isozyme. On the other hand, it is also reasonable that the absence of a migration shift by the 3T3-derived, *S*-thiolated cPKC-α species may stem from structural differences between human and murine forms of the isozyme.

Despite numerous reports of both oxidative activation and inactivation of PKC over the past decade, it is only recently that a molecular mechanism of oxidative regulation of PKC has been elucidated (*27*, *28*). Recent studies have established that PKC isozymes can be oxidatively activated in cells by an indirect mechanism that involves oxidant-induced protein-tyrosine kinase (PTK) activation and protein-tyrosine phosphatase (PTP) inactivation (*27*, *28*). These studies have shown that H_2O_2 treatment activates PKC isozymes in cells by inducing the phosphorylation of the catalytic-domain Tyr residues 512 and 523 in subdomain VIII (nPKC-δ) (*27*). The observations in this paper describe for the first time a molecular mechanism that accounts for oxidative inactivation of cellular cPKC-α. Analysis of site-specific Cys → Ala cPKC-α mutants will be required to identify the Cys residue(s) in cPKC-α responsible for *S*-thiolation-mediated inactivation of the isozyme and to identify the endogenous *S*-thiolating species oxidatively attached to those residues in oxidant-treated cells. These studies are underway. The mutants may also provide an approach to defining the role of cPKC-α *S*-thiolation in the oxidative regulation of the isozyme in cells under physiological or pathophysiological conditions, e.g., in models of oxidant-induced tumor promotion (*30*, *31*).

In mammalian systems, cellular GSH levels typically range from 0.5 to 10 mM (*39*). We previously reported that high concentrations of GSH (>3 mM) inhibit PKC isozymes in vitro by a nonredox (oxidation/reduction-independent) mechanism that may be operative in cells under reducing conditions that support high levels of cellular GSH (*22*). The observations in this report may relate to PKC regulation under oxidizing conditions that dampen cellular GSH levels and promote protein *S*-glutathiolation (*17*, *18*). It is well established that PKC is a mediator of tumor promotion and that antioxidants such as GSH antagonize tumor promotion (*3*, *40*). The potent inactivation of cPKC-α achieved by *S*-thiolation suggests that tumor promotion antagonism by GSH and related antioxidants may involve PKC isozyme inactivation via *S*-thiolation in cells exposed to oxidant tumor promoters.

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