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PKC isozyme *S*-cysteinylation by cystine stimulates the pro-apoptotic isozyme PKCδ and inactivates the oncogenic isozyme PKCε

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Protein kinase C (PKC) is a family of ten isozymes that play distinct and in some cases opposing roles in cell growth and survival. We recently reported that diamide, a diazene carbonyl derivative which oxidizes thiols to disulfides through addition/displacement reactions at the diazene bond, induces potent GSH-dependent inactivation of several PKC isozymes, including the oncogenic isozyme PKCε, via *S*-glutathiolation. PKCδ, a pro-apoptotic isozyme, was distinguished by its resistance to inactivation. In this report, we show that PKC-regulatory *S*-thiolation modifications produced by physiological disulfides elicit opposing effects on PKCδ and PKCε activity. We report that PKCδ is stimulated 2.0–2.5 fold by GSSG, $(Cys–Gly)_2$ and cystine, under conditions where PKCγ and PKCε are fully inactivated by cystine, and PKCα activity is affected marginally or not at all by the disulfides. Focusing on cystine, we show that DTT quenches cystine-induced PKCδ stimulation and PKCγ and PKCε inactivation, indicative of oxidative regulation. By analyzing DTT-reversible isozyme radiolabeling by $[^{35}S]$cystine, we demonstrate that PKCγ, PKCδ and PKCε are each $[^{35}S]$ *S*-cysteinylated in association with the concentration-dependent regulation of isozyme activity by cystine. The restricted reactivity of cystine, together with the effects of DTT and thioredoxin on cystine-induced PKC isozyme regulation reported here, indicate that the cystine-induced PKC-regulatory effects entail isozyme *S*-cysteinylation. We recently hypothesized that antagonism of tumor promotion/progression by small cellular thiols may involve PKC regulation via oxidant-induced *S*-thiolation reactions with PKC isozymes. The findings of cystine-induced PKC isozyme regulation by *S*-cysteinylation reported here offer correlative support to the hypothetical model. Thus, PKCδ, a potent antagonist of DMBA–TPA-induced tumor promotion/progression in mouse skin, is stimulated by *S*-cysteinylation, PKCε, an important mediator of the tumor promotion/progression response, is inactivated by *S*-cysteinylation, and PKCα, which is not influential in DMBA–TPA-induced tumor promotion/progression, is not regulated by cystine. Furthermore, PKCγ has oncogenic activity, and *S*-cysteinylation inactivated PKCγ and PKCε similarly. These findings provide evidence that *S*-cysteinyl acceptor-sites in PKC isozymes may offer attractive targets for development of novel cancer preventive agents.

Abbreviations: $(Cys–Gly)_2$, Cys–Gly disulfide; DAG, *sn*-1,2-dioleoylglycerol; DTT, dithiothreitol; GSH, glutathione; GSSG, glutathione disulfide; PKC, protein kinase C; [Ser25]PKC(19–31), RFARKGALRQKNV.

Introduction

Protein kinase C (PKC) is a family of ten isozymes that play distinct and in some cases opposing roles in cell growth, survival and differentiation (1). The isozyme PKCε stimulates the growth of fibroblasts and renders the cells tumorigenic when it is overproduced by enforced expression (2,3). The oncogenic activity of PKCε in fibroblasts has been shown to result from its engagement of the mitogenic Erk1/2 activation pathway through direct interactions with Raf1 that stimulate Raf1 kinase activity (4,5). The importance of PKCε to cell growth regulation and transformation has also been demonstrated in epithelial tissues *in vivo*. Overexpression of PKCε in the epidermis of transgenic mice markedly enhances carcinoma formation in response to topical treatment with a carcinogen and a phorbol ester PKC activator and renders the tumor cells metastatic (6,7). In contrast, overexpression of PKCδ in the epidermis of the mice has the opposite effect of suppressing tumor formation in response to these agents (8). Consistent with this, PKCδ has been shown to play a pro-apoptotic role in mitochondrial-dependent apoptotic pathways in epithelial and other types of cells in culture (9–12). Because most human malignancies are of epithelial origin (7), these observations offer a rationale for cancer prevention strategies that enhance PKCδ or antagonize PKCε function.

The most well understood mechanism of PKC activation entails phosphatidylserine-dependent binding of the second-messenger *sn*-1,2-diacylglycerol to tandem Cys-rich binding regions in the kinase regulatory domain (1). In addition, pro-oxidant conditions support PKC regulation by redox signaling (13). For example, hydrogen peroxide treatment of COS7 cells activates PKC isozymes by a lipid-independent mechanism that entails stabilization of the phosphotyrosine content of the isozymes at conserved sites in the catalytic domain (14). In addition, reversible oxidative modifications of Cys residues in the PKC structure profoundly influence the kinase activity (13,15). Human PKC isozymes contain 16–28 Cys residues including one or two Cys-rich regions in the regulatory domain, with six Cys per region, and five to eight catalytic domain Cys (13). In an analysis of seven PKC isozymes (α, $β_1$, $β_2$, γ, δ, ε, ζ), we have shown that the thiol-specific oxidant diamide, which is a diazenecarbonyl derivative, supports *S*-glutathiolation of each isozyme, i.e. the disulfide linkage of glutathione (GSH) to kinase Cys residues (PKCS-SG), and that *S*-glutathiolation at 1–3 redox-sensitive Cys residues is sufficient to inactivate fully the kinase activity of most of the isozymes, including PKCε (16,17). PKCδ was unique among the isozymes in its resistance to inactivation. A several-fold higher diamide concentration was required to achieve PKCδ inactivation by *S*-glutathiolation, whereas the other isozymes examined were similarly sensitive to the GSH-dependent inactivation mechanism (17). The selectivity of the diamide-induced PKC *S*-thiolation reaction was indicated by the inability of cysteine to substitute for GSH as the *S*-thiolating ligand for any of the isozymes analyzed (17).

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Induction of disulfide bridge formation by diamide entails the addition of a thiol, e.g. GSH, to the diazene bond followed by its displacement by a second thiol species, e.g. a reactive protein thiol, yielding a mixed disulfide (18). Because the diazene-based chemistry of disulfide bridge formation by diamide is not shared by physiological or environmental agents, the demonstration of diamide-induced PKC regulation by *S*-glutathiolation (16,17) left open the question of whether PKC-regulatory *S*-thiolation modifications could be produced by reaction mechanisms that commonly occur in biological systems. In this report, we show that thiol–disulfide exchange with the physiological disulfide cystine produces opposing effects on the activity of the oncogenic isozyme PKCε and the pro-apoptotic isozyme PKCδ through *S*-cysteinylation of the isozymes. Cystine-induced isozyme *S*-cysteinylation stimulated PKCδ 2–3-fold and fully inactivated PKCε. These results suggest that the anticancer effects of small physiological thiols such as cysteine (19) may include PKC regulation by *S*-thiolation under pro-oxidant conditions. Furthermore, the *S*-cysteinylation sites in the isozyme structures may offer novel targets for the development of cancer preventive or therapeutic agents that stimulate the pro-apoptotic isozyme PKCδ or inactivate the oncogenic isozyme PKCε.

Materials and methods

Purified human recombinant PKC isozymes (α, β_1, γ, δ, ε, ζ) were purchased from Pan Vera Corporation (Madison, WI). [γ-^{32}P]ATP, [^{35}S]cystine, Hyperfilm MP and Amplify fluorographic reagent were 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 *sn*-1,2-dioleoylglycerol (DAG) from Avanti Polar Lipids (Alabaster, AL). Slide-A-Lyzer® Dialysis Cassettes (0.5 ml capacity; 7000 MW cut-off) (Pierce Chem, Rockford, IL) were employed in dialysis experiments. Molecular weight markers and all other SDS–PAGE reagents were purchased from Bio-Rad (Hercules, CA); gels were run on the Bio-Rad Mini-Protean II System. Purified *Escherichia coli* thioredoxin reductase was purchased from American Diagnostica (Greenwich, CT), and recombinant *E.coli* thioredoxin and NADPH from Calbiochem-Novabiochem (La Jolla, CA). GSH, GSSG, Cys–Gly, (Cys–Gly)$_2$, cysteine, cystine, cystamine, γ-Glu-Cys, DTT, 2-mercaptoethanol, diamide, bovine brain PS (>98% pure), ATP, histone III-S, G-25 Sephadex, protease inhibitors, buffers, chelators and all other reagents were purchased from Sigma.

Analysis of the regulation of PKC isozyme activity by disulfides and other thiol-specific oxidants

To analyze effects of thiol-specific oxidants on PKC isozyme activity, PKC isozyme thiols were first refreshed by pretreatment of the kinase (5 µg) with 2 mM DTT for 30 min at 4°C (total volume = 500 µl), and then excess reducing agent was removed from the isozyme by gel filtration chromatography on a 2 ml G-25 Sephadex column at 4°C, as previously described (16,17). Next, the kinase (500 ng) was preincubated with the oxidant under analysis in the buffer employed for G-25 chromatography (total volume = 100 µl). For experiments with diamide, the kinase was incubated with diamide and small thiols (GSH, etc.) specified in the Results section in 20 mM Tris–HCl, pH 7.5, for 5 min at 30°C, placed on ice, and assayed. For analysis of the effects of disulfides such as GSSG and cystine on PKC isozyme activity, the isozyme was preincubated with the disulfide in 50 mM Tris–HCl, 200 mM NaCl, pH 8.0, for 20–30 min at 30°C, placed on ice, and assayed. All preincubation mixtures contained 1 mM EDTA and 1 mM EGTA. In some experiments, DTT reversal of the oxidant-induced changes in isozyme activity was measured. This was done by conducting a second preincubation of the kinase with/without 10 mM DTT (10 min, 30°C) immediately following kinase preincubation with disulfides, etc. In experiments that measured thioredoxin-mediated reversal of disulfide-induced PKC isozyme regulation, the first preincubation period with the disulfide was followed by a 1 h preincubation at 30°C with/without 10 µM thioredoxin–0.2 µM thioredoxin reductase–0.4 mM NADPH or, for comparison, 10 mM DTT.

The PKC activity of the preincubated isozyme was measured by an established assay procedure (16,17) immediately after placing the sample on ice at the end of the preincubation period. Kinase assay mixtures 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, 30 µg/ml DAG, 50 µM [Ser25]PKC(19–31), 6 µM [γ-^{32}P]ATP (5000–8000 c.p.m./pmol) and 40–100 ng of the PKC isozyme under analysis (16,17). $CaCl_2$ was present only in assays of PKCα, PKCβ_1 and PKCγ; DAG was omitted from assays of PKCζ (17). PS and DAG were added to the reaction mixtures as sonicated dispersions. Where indicated, the peptide substrate was replaced by 50 µg/ml histone-III-S. Assays (10 min, 30°C) were initiated by the addition of [γ-^{32}P]ATP and terminated on phosphocellulose paper as previously described (16,17).

Non-reducing SDS–PAGE analysis of PKC isozyme [^{35}S] S-cysteinylation

PKC isozyme thiols were refreshed and the purified isozymes gel filtered. The isozymes were then incubated with 0.1–2.5 mM [^{35}S]cystine (40–250 mCi/mmol) in 80 mM Tris–HCl, pH 8.2, 1 mM EDTA, 1 mM EGTA for 20 min at 30°C (1 µg PKC in a total volume of 200 µl). The incubation period was terminated by the addition of non-reducing SDS–PAGE sample buffer, and samples were boiled and then run on 10% SDS–PAGE (300 ng PKC isozyme/lane). Gels were stained with Coomassie dye to visualize MW markers, rinsed with deionized water, soaked in Amplify fluorographic reagent for 30 min, and exposed to Hyperfilm MP with an intensifying screen. Band intensities in autoradiograms were quantitated by densitometric analysis.

Measurement of the stoichiometry of PKC isozyme [^{35}S] S-cysteinylation

To measure the stoichiometry of PKC isozyme [^{35}S] *S*-cysteinylation by [^{35}S]cystine, PKC isozymes were covalently modified and then dialyzed extensively for removal of unbound radiolabel. In these experiments, PKC isozyme thiols were first refreshed and the purified isozymes gel-filtered. The isozymes were incubated with 2.5 mM [^{35}S]cystine in 80 mM Tris–HCl, pH 8.2, 1 mM EDTA, 1 mM EGTA, 8% glycerol for 20 min at 30°C followed by continued incubation (10 min) with/without 10 mM DTT. Incubation mixtures were then pipetted into Pierce 0.5 ml Slide-A-Lyzer® Dialysis Cassettes (15–18 pmol isozyme in a sample volume of 205 µl per cassette), and dialyzed under bubbling nitrogen against 80 mM Tris–HCl, pH 8.2, 1 mM EDTA, 1 mM EGTA, 8% glycerol (5.8 l) for a total of 26 h at 4°C (three buffer changes); this procedure produced minimal post-dialysis changes in sample volume (<5%). The stoichiometry of PKC [^{35}S] *S*-cysteinylation was calculated as the c.p.m. recovered post-dialysis from the isozyme sample that lacked DTT minus the c.p.m. recovered from the corresponding DTT-containing sample, divided by the pmol PKC in the sample (pmol [^{35}S]cysteine equivalents per pmol PKC).

Results

We recently reported that diamide inactivates several PKC isozymes by inducing *S*-glutathiolation of ~1–3 redox-sensitive Cys residues within the isozyme structures (16,17,20). In this report, as an initial approach to dissect the structural features within GSH (γ-Glu–Cys–Gly) that are involved in the PKC-inactivating *S*-thiolation mechanism, we investigated whether dipeptides corresponding to the NH_2 terminus (γ-Glu–Cys) or COOH terminus (Cys–Gly) of the tripeptide could potentiate diamide-induced PKC isozyme inactivation. We focused on PKCα, because it has the most fully characterized diamide-induced *S*-glutathiolation inactivation mechanism (16), and on PKCδ and PKCε, in light of their prominent, opposing roles in phorbol ester-induced tumor promotion/progression (6–8). Our previous studies established that PKCα and PKCε are equally sensitive to inactivation by diamide-induced *S*-glutathiolation, whereas PKCδ is resistant to the inactivation mechanism and responds biphasically to diamide, whether administered alone or in combination with GSH (17).

Figure 1 (panel C) shows that Cys–Gly (100 µM) (open circles) is nearly as effective as GSH (open triangles) in potentiating diamide-induced PKCε inactivation (filled circles). The lack of effect of Cys–Gly (open circles) on PKCε activity at the lowest diamide concentration examined (20 µM diamide) indicates that the PKCε-regulatory effects of Cys–Gly are diamide-dependent (Figure 1, panel C). In contrast with Cys–Gly, 100 µM γ-Glu–Cys (filled triangles) failed to potentiate diamide-induced PKCε inactivation (Figure 1, panel C). Similarly, diamide-induced PKCα inactivation (filled circles) was potentiated by 100 µM GSH (open triangles) and Cys–Gly

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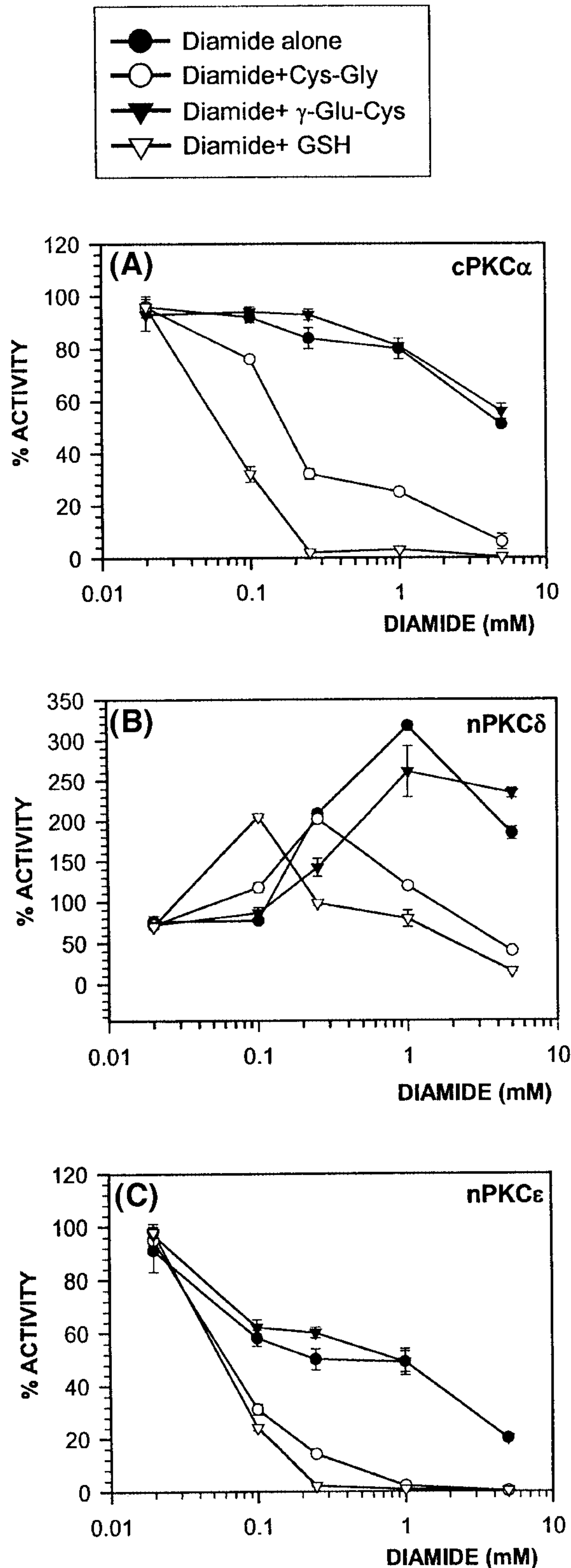
(open circles) but not by 100 μM γ-Glu–Cys (filled triangles) (Figure 1, panel A). The results of the PKCδ analysis appear complicated due to the biphasic responses of the isozyme to diamide with/without thiol peptides (Figure 1, panel B). However, by comparing the curves it can be discerned that the responses of PKCδ to diamide alone (filled circles) and diamide plus γ-Glu–Cys (filled triangles) are similar, indicating that the effects of γ-Glu–Cys were negligible in this system. Furthermore, the curves corresponding to diamide plus Cys–Gly (open circles) and diamide plus GSH (open triangles) are distinct from the diamide alone curve (filled circles) but resemble each other, consistent with analogous diamide-potentiating effects of Cys–Gly and GSH on PKCδ activity. Thus, Cys–Gly contains structural features of GSH that are necessary and sufficient for potentiation of diamide-induced regulatory effects on PKCα, PKCδ and PKCε.

Diamide-induced protein *S*-glutathiolation entails the formation of diamide–GSH adducts that react with protein thiols to form *S*-glutathiolated protein species (18). The potentiation of diamide-induced PKC isozyme regulation achieved by 100 μM GSH and Cys–Gly in Figure 1 is most likely reflective of the abilities of diamide–GSH and diamide–Cys–Gly adducts to react with regulatory PKC-thiols, although it may also involve formation of adducts between PKC-thiols and diamide that react with GSH and Cys–Gly. Given that diamide is neither a physiological nor an environmental agent in biological systems (18), this raised the critical question of whether PKC could be *S*-thiolated by the thiol peptides through delivery mechanisms other than adduct formation with diamide. Therefore, as a second approach to evaluate structural features of GSH that may be involved in PKC isozyme regulation by *S*-thiolation, we compared the PKC regulatory effects of the physiological disulfides GSSG, $(Cys–Gly)_2$, and cystine (21,22).



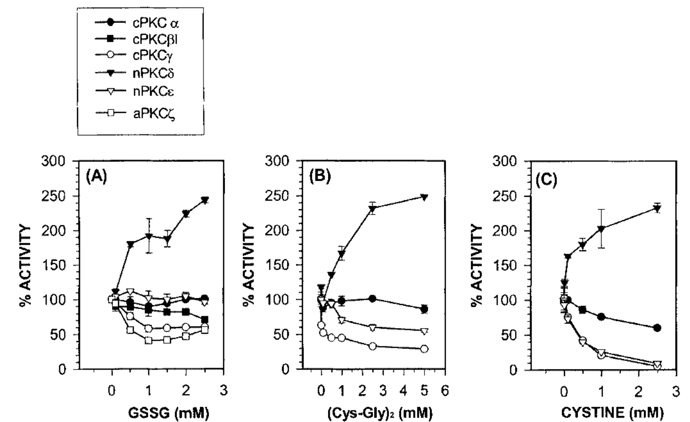


Fig. 2. Regulatory effects of physiological disulfides on PKC isozyme activity. PKC isozymes were preincubated with GSSG (30 min) (**A**), $(Cys–Gly)_2$ (20 min) (**B**), or cystine (20 min) (**C**) in 50 mM Tris–HCl, 200 mM NaCl, pH 8.0, 1 mM EDTA, 1 mM EGTA at 30°C, and then assayed. Each assay mixture contained 60 ng of the PKC isozyme under analysis. For other experimental details, see Materials and methods. In (**A**), 100% activity values are 28 pmol ^{32}P/min (cPKCα), 1.4 pmol ^{32}P/min (cPKCβ_1), 3.6 pmol ^{32}P/min (cPKCγ), 1.4 pmol ^{32}P/min (nPKCδ), 8.6 pmol ^{32}P/min (nPKCε) and 3.3 pmol ^{32}P/min (aPKCζ). Similar 100% activity levels were achieved when the corresponding isozymes were analyzed in (**B**) and (**C**). Each data point is the average ± SD of assays done in triplicate. The results shown were reproduced in an independent analysis.

Fig. 1. Potentiation of diamide-induced PKC isozyme regulation by GSH and GSH-related dipeptides. PKC isozymes were preincubated with diamide (0.02–5 mM) alone or in the presence of thiol peptide (100 μM Cys–Gly, γ-Glu–Cys, or GSH) in 20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA for 5 min at 30°C, and then assayed. Each assay mixture contained 45 ng of the PKC isozyme under analysis. Other experimental details are provided in Materials and methods. 100% activity values shown correspond to 28 pmol ^{32}P/min (cPKCα), 1.5 pmol ^{32}P/min (nPKCδ), and 10 pmol ^{32}P/min (nPKCε). Each data point is the average ± SD of assays done in triplicate. The results shown were reproduced in an independent analysis.

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Figure 2 shows that the lipid cofactor-stimulated activity of PKCδ (filled triangles) was enhanced up to ~2.5-fold by incubating the isozyme with GSSG (panel A), $(Cys–Gly)_2$ (panel B), and cystine (panel C) for 20–30 min at 30°C. Concentrations required to enhance PKCδ activity 2.0-fold were 1.8 ± 0.1 mM GSSG, 1.9 ± 0.1 mM $(Cys–Gly)_2$, and 0.93 ± 0.01 mM cystine (averaged values ± SE from graphical analysis of Figure 2 and an independent analysis). In addition to PKCδ, several other PKC isozymes were surveyed for effects of GSSG (0.1–2.5 mM) on lipid cofactor-stimulated isozyme activity. GSSG effects were either modestly inactivating (PKCγ, PKCζ) or negligible (PKCα, $PKC\beta_1$, PKCε) (Figure 2, panel A).

To compare PKC isozyme responses to $(Cys–Gly)_2$, we returned to the original focus on PKCα, PKCδ and PKCε but widened it to include PKCγ, because it was the most sensitive of the phorbol ester-responsive isozymes surveyed to inactivation by GSSG (Figure 2, panel A). As was the case for GSSG, the effects of $(Cys–Gly)_2$ on the isozymes examined other than PKCδ were only partially inactivating (PKCγ, PKCε) or negligible (PKCα) (Figure 2, panel B). In contrast, cystine fully inactivated PKCγ (open circles) and PKCε (open triangles), while it had modest inactivating effects against PKCα (filled circles) (Figure 2, panel C). Cystine inactivated PKCγ and PKCε with IC_{50}s of 350 ± 50 μM and 370 ± 20 μM respectively (averaged values ± SE of the IC_{50} obtained from the graph in Figure 2 and an independent analysis). Thus, cystine was the most potent PKC-regulatory disulfide in the analysis, and it produced opposing effects on the activity of the pro-apoptotic isozyme PKCδ (9–12) versus the growth-stimulatory isozymes PKCγ (23) and PKCε (2,3) (Figure 2). Intriguingly, from the perspective of structure–activity relationships, the results in Figure 2C are in sharp contrast with the complete lack of effect of oxidized cysteine on PKC isozyme activity, when delivered as a diamide adduct (17) rather than a disulfide.

To determine whether PKCδ-stimulatory effects of GSSG, $(Cys–Gly)_2$ and cystine were selective for the lipid cofactor-stimulated form of the isozyme, we utilized a histone kinase assay of PKCδ activity, because the lipid dependence of purified PKC isozyme activity is especially pronounced with histone as substrate (Chu and O'Brian, unpublished observations). Figure 3 (panels A–E, first versus third bar) shows that the histone kinase activities of PKCγ, PKCδ and PKCε were stimulated 9–50-fold by lipid cofactors. In panels A–C, comparison of the basal PKCδ activity recovered after preincubation alone (first bar) versus with GSSG, $(Cys–Gly)_2$, or cystine (second bar) indicates that the disulfides did not stimulate basal PKCδ activity. In contrast, each disulfide enhanced the lipid cofactor-stimulated histone–kinase activity of PKCδ ~2.0-fold (Figure 3, panels A–C, third versus fourth bar), i.e. to about the extent observed for the peptide kinase activity of PKCδ in Figure 2. Thus, the PKCδ-stimulatory effects of GSSG, $(Cys–Gly)_2$ and cystine are selective for the lipid cofactor-activated form of the isozyme. Figure 3 also shows that 2 mM cystine inactivated the lipid cofactor-stimulated histone kinase activities of PKCγ (panel D) and PKCε (panel E) with about the same efficacy as it achieved against the peptide kinase activities of the isozymes in Figure 2.

To establish whether the regulation of PKC isozyme activity by the physiological disulfides involves an oxidative mechanism, we first evaluated whether DTT could quench the observed PKC-regulatory effects. We previously reported that >2 mM GSSG and >4 mM GSH each antagonized the activity of purified PKC isozymes in a DTT-insensitive manner, indicative of a non-oxidative/reductive (non-redox) inhibitory mechanism (24). Those studies were done by preincubating purified PKC with GSH/GSSG in 20 mM Tris–HCl, pH 7.5 (5 min, 30°C) (24), whereas the analyses in Figures 2 and 3 utilize a higher ionic strength (50 mM Tris–HCl, 200 mM NaCl, pH 8.0), to dampen potentially inactivating ionic interactions between PKC and GSH/GSSG, and a longer preincubation period (20–30 min, 30°C), to provide more time for thiol–disulfide exchange reactions to occur.

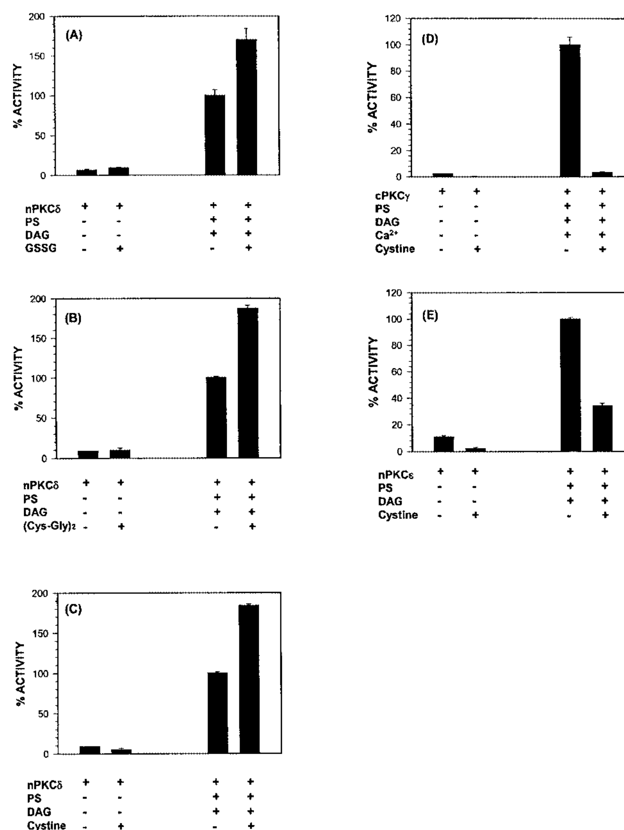

Fig. 3. Regulatory effects of cystine and other physiological disulfides on lipid cofactor-stimulated versus basal PKC isozyme activity. nPKCδ was preincubated with 2 mM GSSG (30 min) (**A**), 5 mM $(Cys–Gly)_2$ (20 min) (**B**), or 2 mM cystine (20 min) (**C**). cPKCγ (**D**) and nPKCε (**E**) were preincubated for 20 min with 2 mM cystine. All preincubations were done in 50 mM Tris–HCl, 200 mM NaCl, pH 8.0, 1 mM EDTA, 1 mM EGTA at 30°C. The histone kinase activities of the preincubated isozymes were assayed, as shown, in the absence or presence of the PKC-stimulatory cofactors 30 μg/ml PS, 30 μg/ml DAG and 0.2 mM Ca^{2+}. Each assay mixture contained 95 ng of the PKC isozyme under analysis. Other experimental details are provided in Materials and methods. 100% activity is the isozyme activity observed under control conditions in the presence of stimulatory cofactors. 100% activity values shown correspond to 6.3 pmol ^{32}P/min (nPKCδ) (**A**), 1.5 pmol ^{32}P/min (nPKCδ) (**B**), 1.5 pmol ^{32}P/min (nPKCδ) (**C**), 0.67 pmol ^{32}P/min (cPKCγ) (**D**) and 3.9 pmol ^{32}P/min (nPKCε) (**E**). Each data point is the mean ± SD of triplicate determinations; the results shown were reproduced in an independent analysis.

Figure 4 (panels A–C) shows that, under conditions where preincubation with GSSG, $(Cys–Gly)_2$, and cystine each stimulated PKCδ activity 2.0–2.5-fold in Figure 2, co-incubation with 10 mM DTT fully quenched the stimulatory response, while 10 mM DTT alone had no effect on PKCδ activity. In addition, replacement of the disulfide agent with the corresponding reduced thiol (GSH, Cys–Gly, cysteine) at twice the

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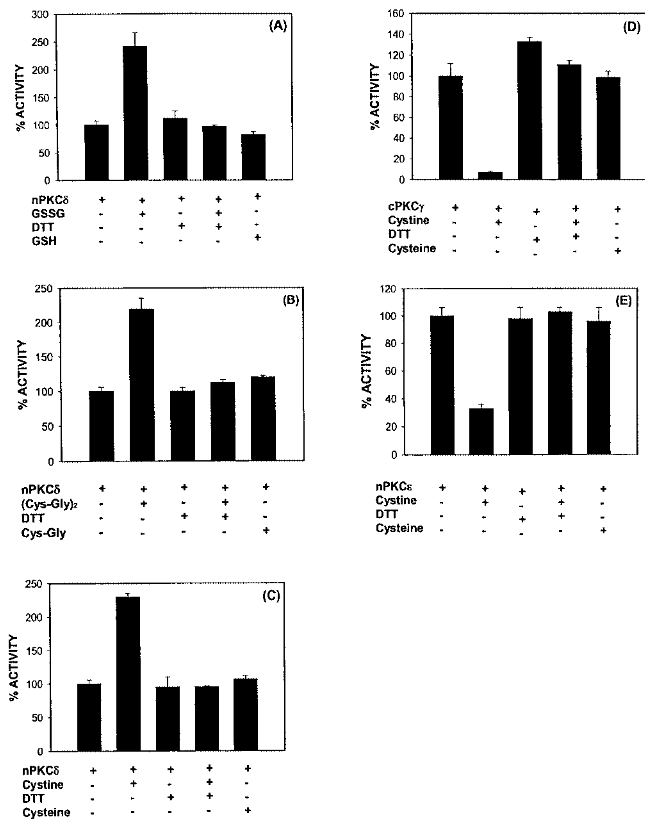

Fig. 4. Cystine and other physiological disulfides regulate PKC isozyme activity by an oxidative mechanism. nPKCδ was pre-incubated with/without 2.0 mM GSSG (**A**), 5.0 mM $(Cys–Gly)_2$ (**B**), or 2.5 mM cystine (**C**). cPKCγ (**D**) and nPKCε (**E**) were pre-incubated with 2 mM cystine. All preincubations were done in 50 mM Tris–HCl, 200 mM NaCl, pH 8.0, 1 mM EDTA, 1 mM EGTA at 30°C; preincubation periods were 30 min (**A**) and 20 min (**B–E**). Where indicated, the preincubation mixtures contained 10 mM DTT or the reduced amino-acid (cysteine) or peptide (Cys–Gly or GSH) at twice the concentration of the disulfide reagent in the analysis. The samples were assayed for PKC isozyme activity immediately following the preincubation period. Each assay mixture contained 45 ng of the PKC isozyme under analysis. 100% activity is defined as the PKC activity observed in the absence of disulfide and thiol reagents; 100% activity values shown correspond to 0.48 pmol ^{32}P/min (nPKCδ) (**A**), 0.33 pmol ^{32}P/min (nPKCδ) (**B**), 0.68 pmol ^{32}P/min (nPKCδ) (**C**), 5.3 pmol ^{32}P/min (cPKCγ) (**D**) and 12 pmol ^{32}P/min (nPKCε) (**E**). Each data point represents the mean ± SD of triplicate determinations; the results shown were reproduced in an independent analysis.

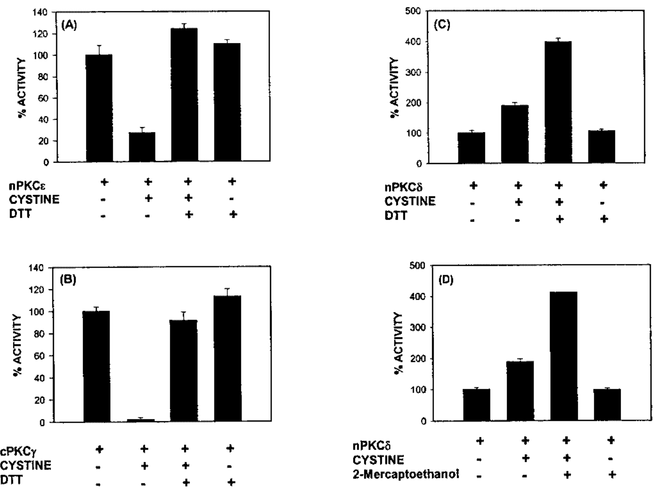

Fig. 5. DTT reverses PKCε and PKCγ inactivation while potentiating PKCδ stimulation by cystine. PKC isozymes were subjected to two successive preincubations in 50 mM Tris–HCl, 200 mM NaCl, pH 8.0, 1 mM EDTA, 1 mM EGTA at 30°C. In the first preincubation (20 min), cystine was present, where indicated, at 2.0 mM (**A, B, D**) or 2.5 mM (**C**). At the initiation of the second preincubation period (10 min), 10 mM DTT (**A, B, C**) or 2.5 mM 2-mercaptoethanol (**D**) was added, where indicated. Isozymes were assayed immediately after the second preincubation period. Each assay mixture contained 50 ng of the isozyme under analysis. 100% activity values shown correspond to 5.7 pmol ^{32}P/min (nPKCε) (**A**), 2.4 pmol ^{32}P/min (cPKCγ) (**B**), 0.69 pmol ^{32}P/min (nPKCδ) (**C**) and 0.85 pmol ^{32}P/min (nPKCδ) (**D**). Each data point is the mean ± SD of triplicate determinations; the results shown were reproduced in an independent analysis.

disulfide concentration, e.g. 2.0 mM GSSG versus 4.0 mM GSH, had no effect on PKCδ activity (Figure 4, panels A–C). These results provide evidence that the disulfide bridge in GSSG, $(Cys–Gly)_2$, and cystine plays a critical role in the stimulation of PKCδ activity by these agents. Because cystine was the only disulfide agent in Figure 2 to markedly inactivate PKC isozymes, we confined the analysis of disulfide agent-mediated PKC isozyme inactivation to cystine. Figure 4 (panels D and E) shows that 10 mM DTT quenched cystine-induced PKCγ and PKCε inactivation and produced only modest or negligible effects on the isozyme activity in the absence of cystine. In addition, cysteine affected neither PKCγ nor PKCε activity, when administered at twice the concentration of cystine. Taken together, the results in Figure 4 provide evidence that cystine stimulates the activity of PKCδ and inactivates PKCγ and PKCε by oxidative mechanisms.

The ability of cystine, but not cysteine, to stimulate PKCδ and inactivate PKCγ and PKCε (Figure 4) suggested PKC isozyme S-cysteinylation as the mechanism responsible for the observed PKC-regulatory effects. Protein S-thiolation is readily reversed by DTT via reduction of the disulfide linkage (25,26). In Figure 5, PKCγ, PKCδ and PKCε were each preincubated with cystine for 20 min at 30°C followed by a second preincubation with/without the addition of 10 mM DTT or 2.5 mM 2-mercaptoethanol (10 min, 30°C), and then assayed. Cystine-induced inactivation of PKCε (panel A) and PKCγ (panel B) was fully reversed by DTT, while DTT had little or no effect on the isozyme activity in the absence of cystine (Figure 5, panels A and B). The DTT reversibility of cystine-induced PKCγ and PKCε inactivation supports isozyme S-cysteinylation as the inactivation mechanism.

Cystine-induced PKCδ stimulation was further enhanced by subsequent incubation of the isozyme with 10 mM DTT (Figure 5, panel C). This phenomenon was also produced when 2.5 mM 2-mercaptoethanol was substituted for 10 mM DTT (Figure 5, panel D). Each reducing agent enhanced cystine-induced PKCδ stimulation to an extent that was ~4-fold over the original activity level. Neither DTT nor 2-mercaptoethanol modified PKCδ activity in the absence of cystine treatment (Figure 5, panels C and D), eliminating a trivial explanation for the observed enhancement of isozyme activity. These results provide evidence that, upon exposure to DTT, cystine-induced structural changes in PKCδ yield to additional changes in the isozyme structure that further enhance activity. However, the results left open the question of whether cystine-induced PKCδ stimulation is associated with isozyme S-cysteinylation and, if so, whether the effects of DTT include reversal of that modification.

To establish whether PKCγ, PKCδ and PKCε were S-cysteinylated (PKC–S–S–Cys) in association with isozyme regulation by cystine, the isozymes were incubated with

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[^{35}S]cystine (0.1–2.5 mM) for 20 min at 30°C and analyzed by non-reducing 10% SDS–PAGE/autoradiography. Figure 6 shows concentration-dependent labeling of PKCγ (panel A), PKCδ (panel B) and PKCε (panel C) by [^{35}S]cystine at 0.1 mM (lane 1), 0.5 mM (lane 2), 1.0 mM (lane 3), and 2.5 mM (lane 4). When DTT was included in the SDS–PAGE sample buffer that was added to isozyme samples incubated with 2.5 mM [^{35}S]cystine, isozyme labeling was abrogated (panels A–C, lane 5). The DTT reversibility of [^{35}S]cystine labeling of the isozymes (panels A–C, lanes 4 versus 5) is indicative of [^{35}S] S-cysteinylation (25,26). The concentration dependence of PKCγ, PKCδ and PKCε [^{35}S] *S*-cysteinylation by [^{35}S]cystine (Figure 6) was in general agreement with the concentration-dependence of the cystine-induced regulatory effects observed for each isozyme (Figure 2, panel C). Thus, cystine-induced PKCγ and PKCε inactivation (Figure 2, panel C) is associated with isozyme *S*-cysteinylation, as predicted from the DTT reversibility of the inactivation (Figure 5, panels A and B). Furthermore, the DTT-reversible labeling of PKCδ by [^{35}S]cystine in Figure 6 (panel B) indicates that cystine-induced PKCδ stimulation (Figure 2, panel C) is associated with isozyme *S*-cysteinylation, and that the structural changes in PKCδ associated with DTT-induced enhancement of S-cysteinylated PKCδ activity (Figure 5, panel C) include reversal of S-cysteinylation.

Having established an association between cystine-induced regulation of PKCγ, PKCδ and PKCε activity with isozyme *S*-cysteinylation, we next employed a selective mode of disulfide reduction to address whether isozyme *S*-cysteinylation was causally linked to the observed effects on isozyme activity. For this purpose, we employed thioredoxin, which is a dithiol–disulfide oxidoreductase that catalyzes the reduction of disulfide linkages when coupled with thioredoxin reductase (27). PKCγ, PKCδ and PKCε were incubated with 2 mM cystine for 20 min at 30°C and then gel-filtered in order to remove excess cystine, which would otherwise interfere with thioredoxin catalysis. The gel-filtered, *S*-cysteinylated isozymes were incubated for 1 h at 30°C with/without thioredoxin–thioredoxin reductase or DTT, and then assayed. In these experiments, the use of isozymes that were gel-filtered after cystine treatment precluded utilization of isozymes that had not been exposed to cystine as internal positive controls. Therefore, as an alternative, activity values observed for the DTT-treated isozymes were normalized to 100%. Table I shows that DTT treatment of gel-filtered, *S*-cysteinylated PKCγ and PKCε induced marked increases in isozyme activity (⩾5-fold) that were in close agreement with the effects of DTT on S-cysteinylated PKCγ and PKCε in Figure 5. The 30% enhancement of the activity of gel-filtered, *S*-cysteinylated PKCδ by DTT (Table I) is consistent with the stimulatory effect of the reducing agent on *S*-cysteinylated PKCδ in Figure 5. Thus, the gel-filtered, *S*-cysteinylated forms of the isozymes in Table I (designated as untreated control), correspond to cystine-inactivated PKCγ and PKCε and cystine-stimulated PKCδ.

Table I shows that thioredoxin was as effective as DTT in restoring the activity of cystine-inactivated PKCγ, indicating that PKCγ inactivation by cystine entailed isozyme *S*-cysteinylation. Thioredoxin was also effective in restoring the activity of cystine-inactivated PKCε, indicating that PKCε inactivation likewise entailed isozyme S-cysteinylation. The activity of cystine-inactivated PKCε was increased ~3-fold by thioredoxin and 5-fold by DTT (Table I). Interestingly, thioredoxin shared with DTT the property of further enhancing, rather than

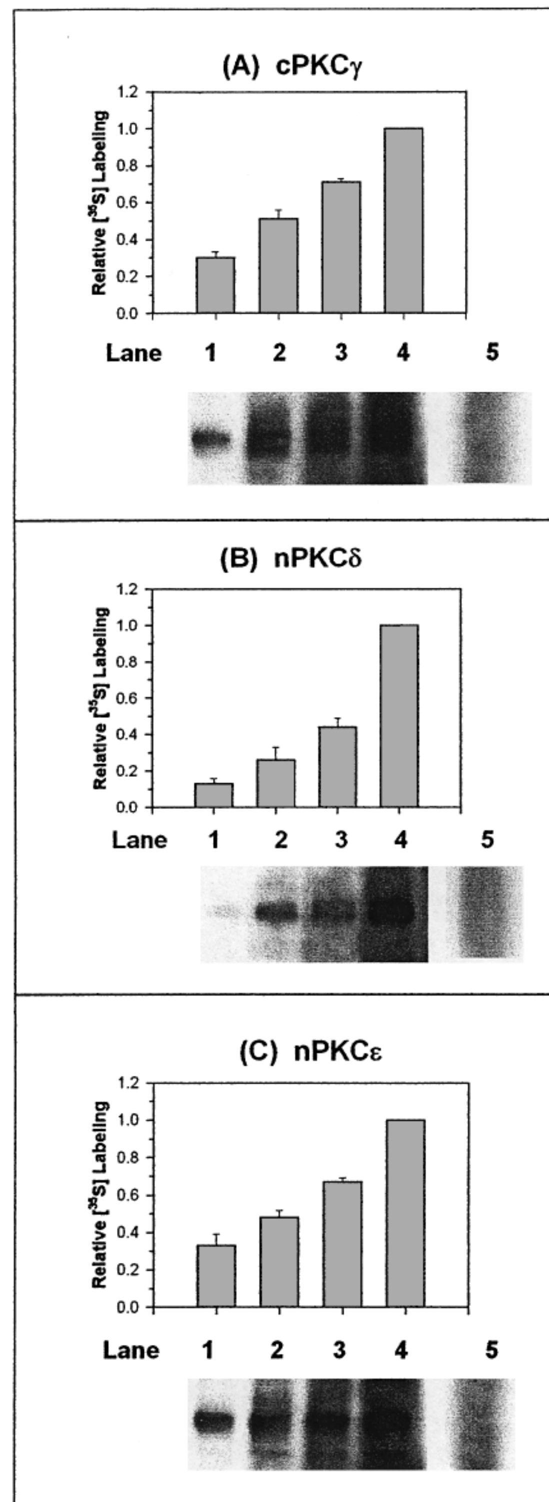

Fig. 6. [^{35}S] *S*-cysteinylation of PKC isozymes by [^{35}S]cystine. PKCγ (**A**), PKCδ (**B**), and PKCε (**C**) were incubated for 20 min at 30°C in 80 mM Tris–HCl, pH 8.2, 1 mM EDTA, 1mM EGTA with [^{35}S]cystine at 0.1 mM (lane 1), 0.5 mM (lane 2), 1.0 mM (lane 3), and 2.5 mM (lanes 4 and 5). After the incubation period was terminated by adding non-reducing SDS–PAGE sample buffer to each sample, DTT was added to samples corresponding to lane 5 (A and C, 5 mM DTT; B, 10 mM DTT). Samples were boiled and analyzed by 10% SDS–PAGE/autoradiography (300 ng PKC isozyme/lane). (The lane designated as 5 is from the same gel as lanes 1–4 but was separated from the heavily labeled lane 4 by skipping one lane). In each panel, the bar graph depicts averaged densitometric values ± SD obtained from the autoradiogram shown and a second, independent analysis.

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Table I. Thioredoxin-mediated effects on PKC isozyme regulation by *S*-cysteinylation

Isozyme	% activity of *S*-cysteinylated PKC treated with/without thioredoxin or DTT		
	Untreated control	Thioredoxin	DTT
PKCγ	0 ± 1	123 ± 14	100 ± 15
PKCδ	78 ± 1	211 ± 11	100 ± 7
PKCε	20 ± 1	58 ± 3	100 ± 6

To measure thioredoxin-mediated effects on PKC isozyme regulation by *S*-cysteinylation, each PKC isozyme (5 μg) was pre-incubated with 2 mM cystine for 20 min at 30°C and then gel filtered for removal of excess cystine. Gel filtered PKC (400 ng) was incubated for 1 h at 30°C, in a total volume of 100 μl, either alone (untreated control) or with 10 μM thioredoxin–0.2 μM thioredoxin reductase–0.4 mM NADPH (thioredoxin) or 10 mM DTT (DTT). At the end of the incubation period, samples were placed on ice and assayed immediately for PKC activity, as described in Materials and methods. Results are calculated by normalizing the activity values observed for the DTT-treated samples to 100%. 100% activity = 1.1 pmol/min/ng PKCγ, 1.0 pmol/min/ng PKCδ, and 3.0 pmol/min/ng PKCε. Each experimental value shown is the average ± SE of two independent analyses done in triplicate.

reversing, cystine-induced PKCδ stimulation (Table I). In fact, thioredoxin stimulated PKCδ activity to twice the extent achieved by DTT (Table I). Thus, two distinct modalities of disulfide reduction, thioredoxin catalysis and exposure to reducing agents, further enhanced the activity of *S*-cysteinylated PKCδ (Table I; Figure 5, panels C and D).

Superoxide treatment has been shown to stimulate the activity of several PKC isozymes by inducing release of Zn^{2+}, which is bound to Cys-rich Zn^{2+} finger structures in the PKC regulatory domain (15). A facile and effective mode of reversing the Zn^{2+} release mechanism of PKC stimulation is incubation of the enzyme with millimolar $ZnCl_2$ (15). To test for involvement of Zn^{2+} release in the mechanism of PKCδ stimulation by S-cysteinylation, we treated PKCδ with/without 2 mM cystine for 20 min at 30°C, under the conditions employed in Figure 2, followed by further incubation with/without 2 mM $ZnCl_2$ for 10 min at 30°C. Incubation with $ZnCl_2$ abrogated PKCδ stimulation by S-cysteinylation and had only minor effects on the activity of unmodified PKCδ. In two independent experiments, a >2.5-fold stimulation of PKCδ by cystine was abrogated to 70 ± 3% of the original activity level by further incubation with $ZnCl_2$. In the absence of cystine treatment, 84 ± 6% of the original activity was recovered after incubation with $ZnCl_2$. These results provide strong evidence that *S*-cysteinylation stimulates PKCδ activity by provoking Zn^{2+} release from the regulatory domain.

To measure the stoichiometries of isozyme [^{35}S] *S*-cysteinylation associated with PKCγ and PKCε inactivation and PKCδ stimulation, the isozymes were incubated with 2 mM [^{35}S]cystine for 20 min at 30°C followed by a further 10 min incubation with/without 10 mM DTT. The isozymes (15–18 pmol/sample) were dialyzed extensively at 4°C (26 h with three changes), and then analyzed for DTT-sensitive incorporation of the radiolabel (c.p.m. in DTT-free isozyme samples minus c.p.m. in DTT-containing samples). For each isozyme, the stoichiometry of [^{35}S] *S*-cysteinylation was close to one; the values were 0.41 pmol [^{35}S]cysteine/pmol PKCγ, 0.60 pmol [^{35}S]cysteine/pmol PKCδ and 0.82 pmol [^{35}S]cysteine/pmol PKCε (Table II). These results provide evidence that selective *S*-cysteinylation of one or at most a few redox-sensitive Cys in PKC isozymes

Table II. Stoichiometry of PKC isozyme [^{35}S] *S*-cysteinylation

Isozyme	pmol [^{35}S]cysteine/pmol PKC
PKCγ	0.41 ± 0.12
PKCδ	0.60 ± 0.17
PKCε	0.82 ± 0.05

Gel filtered PKC isozymes (15–18 pmol) were incubated with 2.5 mM [^{35}S]cystine in 80 mM Tris–HCl, pH 8.2, 1 mM EDTA–1 mM EGTA for 20 min at 30°C and then with/without 10 mM DTT for an additional 10 min at 30°C (final volume = 205 μl). Isozymes were dialyzed against 80 mM Tris–HCl, pH 8.2, 1 mM EDTA, 1 mM EGTA for a total of 26 h (three changes) at 4°C under bubbling nitrogen. Stoichiometries of [^{35}S] *S*-cysteinylation of dialyzed PKC isozymes were calculated by subtracting the c.p.m. in the DTT-treated PKC isozyme sample from the c.p.m. in the isozyme sample lacking DTT. Each experimental value is the average ± SE of three independent experiments done in duplicate.

containing 21–23 Cys residues (13) is sufficient to inactivate PKCγ and PKCε and to stimulate PKCδ.

Finally, we extended the analysis of PKC isozyme regulation by physiological disulfides to the cystine analog cystamine, reasoning that elimination of anionic character in cystine might improve reactivity with PKC-regulatory thiols. Cystamine proved to be several-fold more effective than cystine in the oxidative regulation of PKCγ, PKCδ and PKCε, inducing DTT-reversible inactivation of PKCγ and PKCε with IC_{50}s of 55 ± 5 μM and 95 ± 5 μM and stimulating PKCδ 2-fold in a DTT-sensitive manner at 120 ± 20 μM.

Discussion

In this report, we demonstrate differential responses of several PKC isozymes to cystine and other physiological disulfides. We report that PKCδ is stimulated 2.0–2.5-fold by GSSG, $(Cys–Gly)_2$, and cystine, under conditions where PKCγ and PKCε are fully inactivated by cystine, and PKCα activity is affected marginally or not at all by the disulfides. Focusing on cystine, we show that DTT quenches cystine-induced PKCδ stimulation and PKCγ, ε inactivation, indicative of oxidative regulation. By analyzing DTT-reversible [^{35}S]cystine-induced labeling of the isozymes by non-reducing SDS–PAGE, we demonstrate that PKCγ, PKCδ and PKCε are each [^{35}S] *S*-cysteinylated with a concentration dependence which indicates an association between the cystine-induced regulatory effects on the isozymes and isozyme *S*-cysteinylation.

Cystine-induced inactivation of PKCγ and PKCε was reversed by DTT as well as by thioredoxin, indicating that inactivation entailed disulfide bridge formation. The stable *S*-cysteinylation of PKCγ and PKCε in association with inactivation strongly supports an inactivation mechanism where PKC *S*-cysteinylation serves as a redox switch that turns off PKCγ,ε activity in a thioredoxin-reversible manner. Furthermore, the stoichiometries of PKCγ and PKCε [^{35}S] *S*-cysteinylation are consistent with a single redox-sensitive Cys in each isozyme as the switch, although direct identification of the involved Cys residue(s) by mass spectroscopic or site-specific mutagenesis approaches will be required to ascertain whether this is the case. On the other hand, the data do not exclude a more complicated mechanism in which *S*-cysteinylation of a critical Cys residue(s) in PKCγ,ε is an intermediate step in the inactivation mechanism that, while not in itself inactivating, leads to concerted displacement of the *S*-cysteinyl modification

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by another PKC-thiol and formation of an inactivating intramolecular disulfide bridge in the kinase.

The analysis of PKCδ produced the surprising result that DTT treatment not only failed to reverse cystine-induced PKCδ stimulation but actually enhanced the activity of *S*-cysteinylated PKCδ while having no effect on the activity of native (unmodified) PKCδ. Thioredoxin likewise further enhanced the activity of *S*-cysteinylated PKCδ. These results are intriguing, as they suggest that although PKCδ *S*-cysteinylation is a reversible modification, it may serve as an irreversible mode of kinase stimulation, given that a physiological mechanism of dethiolation, thioredoxin catalysis (26,27), produces structural changes in *S*-cysteinylated PKCδ that further stimulate the kinase activity. A recently described oxidative mode of PKC stimulation entails release of Zn^{2+} from the PKC regulatory domain, where it is bound to Cys-rich Zn^{2+} finger structures (15). In this report, we present evidence that *S*-cysteinylation-mediated stimulation of PKCδ entails induction of Zn^{2+} release from the regulatory domain. These findings offer a logical explanation for the persistence of *S*-cysteinylation-mediated PKCδ stimulation after reversal of the post-translational modification. Furthermore, the stoichiometry of PKCδ *S*-cysteinylation suggests that a single redox-sensitive Cys may mediate kinase stimulation.

The concentrations of cystine required to stimulate PKCδ and inactivate PKCγ,ε *in vitro* are substantially higher than the physiological spectrum of intracellular cystine concentrations (22,28). That said, the robust PKC-regulatory effects achieved by cystine at low stoichiometries of isozyme *S*-cysteinylation suggest the existence of oxidative post-translational modifications that are physiological counterparts to the PKC-regulatory *S*-cysteinyl modifications produced by *in vitro* exposure to cystine. This is also supported by our findings that the physiological disulfide cystamine exerts potent oxidative regulatory effects on PKCγ, PKCδ and PKCε *in vitro* at concentrations <200 μM. The potencies reported here for cystine-induced regulation of PKC isozymes by *S*-cysteinylation are in line with the potencies reported for *S*-thiolation-mediated regulation of other enzymes by physiological disulfides *in vitro*. For example, GSSG inactivates creatine kinase by *S*-glutathiolation with an IC_{50} of 0.5 mM (29), and aldose reductase is inactivated ~80% by *S*-thiolation reactions with 1.5 mM GSSG and 0.4 mM cystine (21). Other studies have utilized 50 mM GSSG to drive *S*-glutathiolation-mediated inactivation of protein tyrosine phosphatase 1B (30) and HIV protease (31).

We have hypothesized that the antagonism of tumor promotion/progression by GSH and other small cellular thiols may involve regulatory effects on PKC through *S*-thiolation reactions with PKC isozymes under pro-oxidant conditions (17). Our observations of cystine-induced PKC isozyme regulation by *S*-cysteinylation offer strong support to the hypothetical model. Thus, an isozyme that potently antagonizes DMBA–TPA-induced tumor promotion/progression when expressed as a transgene in mouse skin (PKCδ) (8) is stimulated by *S*-cysteinylation, an isozyme that has the opposite effect of markedly increasing carcinoma formation when expressed as a transgene in the skin (PKCε) (6) is inactivated by *S*-cysteinylation, and an isozyme that does not influence DMBA–TPA-induced tumor promotion/progression when expressed as a transgene in the skin (PKCα) (32) is not regulated by cystine. Furthermore, PKCγ, while not widely expressed in mammalian tissues (33), is implicated as an oncogene through studies of transfected fibroblasts (23), and *S*-cysteinylation inactivated PKCγ and PKCε similarly. Therefore, *S*-cysteinyl acceptor-sites in PKC isozymes may offer attractive targets for the development of drugs with isozyme-selective PKC-regulatory action for preventive intervention and treatment of proliferative disorders such as cancer.

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