Bacterial lipopolysaccharide can induce manganese superoxide dismutase (MnSOD) gene expression in a variety of cells. Paclitaxel (taxol) shares many properties of lipopolysaccharide. Here we report that paclitaxel can induce MnSOD gene expression in human lung adenocarcinoma cell line A549 in a time- and dose-dependent manner. Additional anticancer drugs, vinblastine and vincristine, also induced MnSOD gene expression. We have shown previously (Das, K. C., and White, C. W. (1997) J. Biol. Chem. 272, 14914–14920) that these drugs can activate protein kinase C (PKC). The PKC agonists thymeleatoxin (0.5 μM) and 12-deoxyphorbol 13-phenylacetate 20-acetate (dPPA; 10 nM) potently induced MnSOD gene expression. Calphostin C and GF109203X, both specific inhibitors of PKC, each inhibited MnSOD gene expression by anticancer agents. Down-regulation of PKC by prolonged treatment with phorbol 12-myristate 13-acetate (PMA) also inhibited induction of MnSOD by anticancer drugs, indicating an important role of PKC in MnSOD signaling by these agents. Of 11 PKC isoenzymes, only PKCδ translocated to the cell membrane after stimulation with anticancer drugs. By contrast, dPPA, PMA, and thymeleatoxin caused translocation of PKCα, β1, δ, and μ isotypes. Anticancer drug-stimulated cells also had increased total PKC activity in membrane and cytosolic fractions. Thus, paclitaxel, vinblastine, and vincristine each specifically activate PKCδ, whereas PMA, thymeleatoxin, and dPPA activate multiple isoenzymes. PKCδ was the only isoform activated by each agent in both groups of compounds effective in MnSOD induction.

Paclitaxel, also known as taxol, a diterpene isolated from the bark of Taxus brevifolia, has potent antiproliferative action against tumor cells in vitro and in vivo (1). These effects relate to the ability of taxol to bind tubulin, promote microtubule assembly, and stabilize microtubules by bundle formation (2–4). In addition, taxol exhibits cell cycle-independent, lipopolysaccharide (LPS)1-like effects, including macrophage activation (5), down-regulation of tumor necrosis factor (TNF) receptors (5), and initiation of synthesis and secretion of TNF (6). Taxol also can induce tyrosine phosphorylation of microtubule-associated protein kinases (6) and enhance interferon-γ-mediated induction of nitric oxide synthase and secretion of nitric oxide (7), a macrophage tumoricidal factor. The gene controlling these responses to taxol is closely linked to the gene controlling responses to LPS (5).

Manganese superoxide dismutase (MnSOD) is an inducible enzyme that catalyzes the dismutation of superoxide radical. Regulation of MnSOD gene expression is incompletely understood. In prokaryotic cells (8–10) and some eukaryotic cell types (11–14), MnSOD is induced by oxidative stress. In addition, cytokines, such as TNF or interleukin 1 (IL-1), as well as LPS, can induce MnSOD gene expression (15–17), and an oxidant mechanism for this response has been proposed (18). Because LPS can induce MnSOD gene expression (17), we investigated whether taxol shares this property of LPS in human lung adenocarcinoma (A549) cells. Because TNF and IL-1 are both potent activators of MnSOD gene expression, taxol also could induce secretion of TNF or IL-1, which could, in turn, activate MnSOD gene expression. Such effects also could result from the actions of taxol on the cytoskeleton. To evaluate these possibilities, we assessed the effect of an additional class of antitumor drugs, vinca alkaloids, which do not induce TNF or IL-1 secretion. The mechanism of action of vinca alkaloids relates to their inhibition of microtubule formation in the mitotic spindle, resulting in an arrest of the cell division at metaphase (19). Thus, the mode of action, structure, and function of taxol is different than that of vinca alkaloids, although they do share the property of exerting their action on the microtubule system of the cells.

Protein kinase C (PKC) is a calcium- and phospholipid-dependent protein kinase that is important in signal transduction pathways regulating cell growth (20). In humans, the PKC superfamily consists of at least 11 isoenzymes, which differ in intracellular location, tissue distribution, and activating cofactors (21, 22). Members of the “classical” group of isoenzymes (cPKCs, cPKCa, cPKCbβ1, cPKCbβII, and cPKCγ) contain conserved amino acid sequences within their regulatory domains, which confer responsiveness to calcium, diacylglycerol, and phorbol esters. In contrast, “novel” group members (nPKCs, nPKCa, nPKCb, nPKCd, nPKCe, nPKCh, and nPKCμ) are structurally similar to the cPKCs but lack the functional groups that appear to mediate Ca2+ binding. The least understood are the atypical PKCs ζ and η. These isotypes differ significantly in structure from the other two classes (21, 22). Furthermore, these isoenzymes have been reported not to respond to phorbol esters in vivo or in vitro.
Protein kinase activators such as the phorbol 12-myristate 13-acetate (PMA; 12-O-tetradecanoylphorbol 13-acetate) can induce MnSOD gene expression in a variety of cell lines (23, 24), and this effect occurs at the level of transcription (24). PMA activates PKC nonspecifically, including multiple classical PKCs, as well as nPKCδ and nPKCe. Hence, the precise signaling mechanism by PKC isozymes in MnSOD gene expression by PMA is not known. We recently reported the activation of PKC in response to antitumor drugs in the pulmonary adenocarcinoma cell line A549 (25). In the present study, we have identified specific activation and membrane translocation of PKCδ in these cells in response to antitumor drugs. We also investigated the effect of specific inhibitors of protein kinase C, such as calphostin C and GF109203X, on MnSOD gene expression by these antitumor agents. Calphostin C, a specific inhibitor of PKC, which binds to its regulatory region (26), and GF109203X, which specifically inhibits by binding to the catalytic region of PKC (27), both prevented the induction of MnSOD gene expression by antitumor drugs.

MATERIALS AND METHODS

Isolation of RNA and Northern Blot Analysis—The human MnSOD gene was obtained from ATCC in a PHMNSOD4 plasmid in Escherichia Coli HB101. Plasmids were amplified in E. Coli and were purified with Qiagen plasmid preparation kit (Qiagen Inc., Chatsworth, CA). The cDNAs were isolated from the vectors by treatment with EcoRI and gel-purified. cDNAs were labeled with a random-primed DNA labeling kit (Life Technologies, Inc.). Total RNA was isolated from cells by guanidine isothiocyanate lysis and cesium chloride centrifugation of the lysate (147,000 g, 20–25 °C) in an ultracentrifuge (Beckman Instruments) by a modification of the methods of Maniatis et al. (28). Total RNA was quantified spectrophotometrically. Twenty micrograms of RNA was resolved by electrophoresis in a 1% agarose, 2.5% formaldehyde-gel in buffer containing 20 mM Tris-base, 1 mM EDTA and 0.5 mM sodium chloride, 0.075 M sodium citrate, pH 7.0, 5× Denhardt’s solution, 50 µg/ml salmon sperm DNA, and 0.1% SDS at 42 °C. Blots were hybridized with cDNA for MnSOD labeled to a specific activity of 2–7 × 107 cpm using [γ-32P] dCTP (ICN) in hybridization solution at 42 °C overnight, and then were washed in 0.3 M sodium chloride, 0.03 M sodium citrate, and 0.1% SDS at 42 °C. Autoradiographs were made by exposing blots to x-ray film (Eastman Kodak Co.) at −70 °C with intensifying screens. Some of the blots were exposed to phosphor screens (Molecular Dynamics), and densitometry was performed on phosphor screens by Macintosh computer using Molecular Dynamics Image Quant version 3.3 software.

Preparation of Membrane and Cytosolic Fractions for Western Blotting—Culture dishes (100 mm) of A549 cells were placed on ice, washed twice with phosphate-buffered saline, and scraped into homogenization buffer (1 ml; 20 mM HEPS, 5 mM EGTA, 10 mM 2-mercaptoethanol, 100 µM leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Cells were homogenized in a precooled Dounce homogenizer (Type b pestle). The lysate was centrifuged (3 min, 50,000 g) to remove intact cells. The supernatant was then centrifuged again (1 h, 100,000 g). The resulting supernatant was designated cytosolic fraction and stored at −70 °C for further analysis. The membrane pellet was washed in homogenization buffer and then suspended in the same buffer containing 0.5% Triton X-100 and incubated on ice for 30 min with intermittent vortexing. The suspended pellet fraction also was centrifuged (30 min, 14,000 × g). The supernatant was stored at −70 °C as the membrane fraction. Proein was measured by the Bradford method (29).

Western Blotting of PKC Isoenzymes—Cytosolic and membrane proteins were fractionated in a 7.5% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose in a Mini Trans-blot apparatus (Bio-Rad). The membrane was air-dried and blocked for 1 h with Tris-buffered (50 mM, pH 7.5) saline containing 5% nonfat dry milk or 5% bovine serum album and 0.05% Tween 20. The membrane was incubated with isoenzyme-specific protein G-purified rabbit anti-PKCα (Life Technologies), β, βII, γ, δ, ε, η, ζ, π, and θ (Santa Cruz Biotechnology). PKC isoenzyme-specific bands were visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech) using goat anti-rabbit IgG-horseradish peroxidase (Pierce).

Preparation of Membrane and Cytosolic Fractions for PKC Activity Assay—Confluent A549 cells were incubated with vinblastine (15 µM), vincristine (15 µM), or taxol (56 µM) for 30 min. After incubation, cells were resuspended and 0.5 ml of buffer A (20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 20 mM 2-mercaptoethanol, and 25 µg/ml aprotinin and leupeptin). Cells were disrupted using a precooled Dounce homogenizer with a B-type pestle (15 strokes). An aliquot of homogenate was kept for later protein determination. The homogenate (0.5 ml) was centrifuged at 100,000 × g for 30 min. The supernatant was applied to DE52 ion exchange columns. Columns were prepared by loading DE52 resin (Whatman; 0.125 g/ml in buffer A) into Polyprep columns (Bio-Rad). The column was washed with buffer B (20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, and 20 mM 2-mercaptoethanol). Cytosolic PKC was eluted with 0.1 ml of buffer C (10 mM Tris, pH 7.5, 0.5 mM EGTA, 20 mM 2-mercaptoethanol, and 0.2 mM NaCl). The post-100,000 × g pellet was suspended in 0.5 ml of buffer A containing 0.5% Triton X-100 and incubated on ice for 30 min. After incubation, the membrane fraction was centrifuged at 4000 × g for 30 min, the supernatant was applied to a DE52 column, and the membrane fraction of PKC was eluted as described for cytosolic fractions.

Protein Kinase C Activity Measurement—PKC activity was determined using the PKC assay kit of Life Technologies based on the measurement of the phosphorylation of acetylated myelin basic protein as described by Yasuda et al. (30). The DEAEl eluate (25 µl) was assayed for PKC activity using 50 µM acetylated myelin basic protein as substrate. Activity of PKC was expressed as pmol/min/mg total protein.

Statistical Analysis—Statistical analysis was done in a microcomputer with JMP statistical software (SAS Institute, Cary, NC). Means were compared by one-way analysis of variance followed by Tukey’s test for multiple comparisons, and p < 0.05 was considered significant.

RESULTS

Effects of Vinblastine and Vincristine on MnSOD mRNA Expression: Time Course and Dose Effect—To evaluate the effect of antitumor drugs vinblastine and vincristine on expression of MnSOD mRNA, we incubated confluent A549 cells with vinblastine (50 µM) or vincristine (50 µM) for various intervals (1–8 h). After exposure to either agent, steady state levels of MnSOD mRNA increased in a time-dependent manner. These increases began after 4 h and became significant after 4–8 h (Fig. 1A). The level of B-actin mRNA remained unchanged for each of the compounds examined. Vinblastine was more potent in induction of MnSOD mRNA after 6 and 8 h relative to vincristine. To determine the effect of more prolonged incubation (6–24 h), A549 cells were incubated with less toxic, lesser concentrations of vinblastine (15 µM) or vincristine (15 µM). As shown (Fig. 1, B and C), MnSOD mRNA increased maximally after 6–12 h, declining thereafter. Throughout these time courses, cells remained viable as determined by exclusion of trypan blue dye exclusion and phase contrast microscopy.

To evaluate the dose response of vinblastine and vincristine on steady state levels of MnSOD mRNA, A549 cells were incubated with various concentrations of each agent for 4 h. As demonstrated (Fig. 2A), cells treated with only 10 µM vinblastine demonstrated maximal elevation of MnSOD mRNA, whereas higher concentrations caused lesser elevation of MnSOD mRNA. By contrast, exposure to vincristine resulted in dose-dependent elevation of MnSOD mRNA at concentrations of 10–100 µM (Fig. 2B).

Effects of Taxol on MnSOD mRNA: Time Course and Dose Response—Exposure to taxol increased steady-state levels of MnSOD mRNA in a time- and dose-dependent manner (Fig. 3). During relatively brief incubation (6 h), taxol caused elevation of steady state levels of MnSOD mRNA in a dose-dependent manner up to 56 µM. Exposure to higher taxol concentrations (147 µM) caused little or no increase beyond that with 56 µM. During more prolonged incubation, taxol (56 µM) increased steady state levels of MnSOD mRNA within 4 h, with maximal elevation occurring after 12 h. After 18 h, mRNA levels began to decline, and significant cytotoxicity, as determined by trypan blue dye exclusion and phase contrast microscopy, was appar-
Expression of β-actin mRNA remained unchanged until after 18 h of incubation, when it began to decline.

Effects of Actinomycin D and Cycloheximide on Anticancer Drug-induced MnSOD mRNA—The transcriptional inhibitor actinomycin D (1 μg/ml, 1 h preincubation) prevented the increase in MnSOD gene expression induced by anticancer drugs (Fig. 4A). This finding suggests that induction of MnSOD gene expression by anticancer drugs is transcriptional rather than attributable to an increased stability of mRNA. By contrast, the translational inhibitor cycloheximide did not inhibit taxol-induced elevation of MnSOD mRNA, suggesting no requirement...
for new protein synthesis for the response (Fig. 4B). Rather, cycloheximide, by itself, caused induction of MnSOD in A549 cells. This induction has been described previously (17). In addition, cycloheximide potentiated the increases in MnSOD mRNA caused by each anticancer drug. Based on these results, it does not appear that taxol-induced cytokine synthesis is involved in MnSOD expression caused by this agent. Effects of Protein Kinase C Inhibitors on MnSOD mRNA Expression—To further evaluate the role of PKC, we examined the effects of various activators of PKC on anticancer drug-induced MnSOD gene expression. Among the more nonspecific PKC activators such as PMA (21), N-(6-phenylhexyl)-5-chloro-1-naphthalenesulfonamide (SC-9) (31), and N-heptyl-5-chloro-1-naphthalenesulfonamide (SC-10) (31), only PMA strongly induced MnSOD gene expression (Fig. 6A). The effects of more specific PKC isoenzyme activators such as thymelea toxin (Thy), which is known to activate classical PKC isoforms α-γ (32), and deoxyphorbol 13-phenylacetate 20 acetate (dPPA), a purported cPKCβI isoenzyme-specific activator (33), also were examined. Both of these agents caused potent elevation of MnSOD gene expression (severalfold greater than the effects of vinblastine, vincristine, or taxol; Fig. 6A). Prolonged incubation of cells with PKC activators such as PMA is known to deplete cells of cytosolic PKC (23). PMA, unlike diacylglycerol, does not readily decompose and thereby stimulates PKC translocation continuously. To further confirm the role of PKC in anticancer drug-induced MnSOD gene expression, A549 cells were depleted of cytosolic PKC by incubating with PMA (20 ng/ml) for 24 h. After exposure to PMA, A549 cells were incubated with anticancer drugs, cPKC activators, or generalized activators of PKC. As shown in Fig. 6A, previous treatment of cells with PMA inhibited the increase in MnSOD mRNA by anticancer drugs or PKC activators. In a similar experiment, when PMA was depleted by PMA (20 ng/ml) for 24 h and then taxol was added and cells were incubated for 6 h, the induction of MnSOD mRNA by taxol was inhibited in comparison with the effect of taxol exposure alone (Fig. 6B). These findings with calphostin C (1 h) before exposure to anticancer drugs inhibited elevation of MnSOD mRNA expression (Fig. 5). To further verify the role of PKC, we incubated A549 cells with another inhibitor of PKC, the bisindoylmaleimide GF109203X, which binds to the catalytic region of the enzyme (27). As seen in Fig. 5, GF109203X (25 μM) completely inhibited elevation of MnSOD mRNA. Thus, both of the PKC inhibitors prevented anticancer drug-induced elevation of MnSOD gene expression, indicating a role for PKC in this process. FIG. 4. Effects of actinomycin D and cycloheximide on anticancer drug-induced MnSOD mRNA expression. A, after preincubation with actinomycin D (Act. D, 1 μg/ml) for 1 h, cells were exposed to vinblastine (Vbl, 15 μM), vincristine (Vcr, 15 μM), or taxol (56 μM) for 4 h. Results from cells exposed to these agents were compared with untreated cells (Control) or cells treated with actinomycin D alone (Act. D). Total RNA was isolated and Northern analysis was performed as described under “Materials and Methods.” Transcripts for MnSOD (4 and 1 kb; upper panel) and β-actin (lower panel) are shown. B, A549 cells were preincubated with cycloheximide (10 μg/ml) for 1 h. After incubation, cells were exposed to vinblastine (Vbl, 15 μM), vincristine (Vcr, 15 μM), or taxol (56 μM) for 4 h. Results from cells exposed to these agents were compared with untreated cells (Con) and to those treated with cycloheximide alone. Total RNA was isolated and Northern analysis was performed as described under “Materials and Methods.” Transcripts for MnSOD (4 and 1 kb; upper panel) and β-actin (lower panel) are shown. FIG. 5. Effect of protein kinase C inhibitors on elevation of MnSOD mRNA by anticancer drugs. After preincubation with protein kinase C inhibitors GF109203X (25 μM) or calphostin C (Calphos C, 1 μM) for 1 h, cells were exposed to vinblastine (Vbl, 15 μM), vincristine (Vcr, 15 μM), or taxol (56 μM) for 6 h. Total RNA was isolated and Northern analysis was performed as described under “Materials and Methods.” Transcripts for MnSOD (4 and 1 kb; upper panel) and β-actin (lower panel) are shown.
Effects of Anticancer Drugs on Translocation of PKC Isoenzymes—In general, PKC is located in the cytosol of unstimulated cells. After stimulation, PKC isoenzymes are translocated to the membrane region (20). Hence, translocation of a particular PKC isoenzyme to the membrane fraction provides one indicator of its activation. To determine the specific PKC isotype(s) involved in signaling for anticancer drug-induced MnSOD gene expression, we incubated A549 cells with anticancer drugs and detected PKC isoenzymes by Western blot. As shown in Fig. 6, there is already some membrane-associated PKCδ in the control cells. Thus, to conclusively establish the translocation of PKCδ to the membrane by anticancer drugs, we treated A549 cells with various doses of anticancer drugs and detected PKCδ by Western blot. As shown in Fig. 7, PKCδ is translocated to the membrane in a concentration-dependent manner. As in Fig. 6, translocation of PKCδ was more pronounced with taxol than with vinblastine or vincristine.

Effect of Anticancer Drugs on PKC Activity of Membrane and Cytosolic Fractions—Because we had found that PKCδ was translocated to the membrane fraction, we also sought to determine whether the total PKC activity was increased in the membrane fraction in response to anticancer drugs. PKC translocation after stimulation with natural agonists is both transient and tenuous. By contrast, translocation attributable to phorbol ester treatment is much more persistent. This is because diacylglycerol is rapidly metabolized, whereas phorbol esters are very slowly degraded (21). A moderate increase in PKC activity did occur in the membrane fraction after treatment with vinblastine, vincristine, or taxol (Table I). dPPA also caused a modest increase in PKC activity in the membrane fraction. Treatment with PMA or Thy caused a 5- to 6-fold increase in membrane PKC activity with concomitant loss of cytosolic activity (Table I). Thus, because PMA and Thy activate PKC most isoforms of PKC, their potent membrane translocation of PKCs was not surprising. In comparison with anticancer agents, phorbol ester PKC activators caused more pronounced elevation of PKC activity in the membrane fraction.

DISCUSSION

In the present investigation, we demonstrate that microtubule-active anticancer drugs, including taxol, vinblastine, and vincristine, induce MnSOD gene expression in the lung adenocarcinoma cell line A549. Previously, taxol was shown to induce TNF-α and IL-1 gene expression (5). Thus, it was possible that taxol induced one or both of these cytokines, which subsequently caused MnSOD gene expression. To evaluate this possibility, cells were pretreated with the inhibitor of translation cycloheximide. Cycloheximide did not prevent elevation of MnSOD mRNA by taxol. Therefore, elevation of MnSOD mRNA by taxol was not dependent on new synthesis of protein(s) such as TNF or IL-1. We also evaluated the effect of additional antitumor drugs that do not induce cytokine gene expression and that, although acting on microtubules, differ in structure and mode of action from taxol. These included vinblastine and vincristine. Both of these agents also increased MnSOD mRNA. Therefore, anticancer drugs caused MnSOD gene expression by a mechanism other than cytokine up-regulation. In contrast to the effects of cycloheximide, actinomycin D inhibited MnSOD gene expression caused by each of the drugs, suggesting that the effect occurs at the level of transcription.

Protein kinase C activation can induce MnSOD mRNA in endothelial and other cell types (23, 24). Because PKC activity could be increased by anticancer drugs (25), we examined the effects of various PKC inhibitors on MnSOD gene expression caused by these agents. As shown, elevated MnSOD gene expression could be inhibited by specific PKC inhibitors calphostin C and GF109203X. Calphostin C binds to the regulatory...
role for specific isoenzymes of PKC, we treated cells with the cPKC specific activator Thy (32) and the purported cPKCβI-specific activator dPPA (33). Thymeleatoxin, an analog of mezerein isolated from Thymelea hirsuta L., is known to activate PKCα-γ with an ED₅₀ of 100 nm, but it does not activate PKCδ and ε in vivo at concentrations as high as 1 μM (32). dPPA is a phorbol ester that is very unstable and undergoes hydrolysis of its position 20 acetate group to produce 12-deoxyphorbol 13-phenyl acetate (33). dPPA in its native form is a specific agonist of the PKCβI isotype, but its hydrolysis product is a potent agonist of PKCα, β, γ, δ, and ε isotypes (34). In our experiments, only freshly prepared dPPA and thymeleatoxin were used. As demonstrated, dPPA and thymeleatoxin potently induced MnSOD mRNA increases severalfold greater than those resulting from exposure to anticancer drugs. Previously, PMA, an activator of PKC, also was shown to induce MnSOD potently (23, 24), and our experiments confirmed this effect. However, the specific PKC isoform involved in PMA-induced MnSOD gene expression is not known. In contrast to PMA, SC-9 and SC-10, which are less extensively characterized activators of PKC (32), both failed to cause MnSOD expression in A549 cells. Prolonged exposure to relatively high concentrations of PMA can deplete cytosolic PKC. When A549 cells were continuously stimulated with PMA (20 ng/ml) for 24 h, followed by exposure to the anticancer drugs thymeleatoxin, dPPA, and PMA, there was inhibition of elevation of MnSOD gene expression. Taken together, these results demonstrate that PKC activation is crucial in signaling of MnSOD gene expression both by phorbol esters and anticancer drugs.

To conclusively establish the specific isoenzyme responsible for signaling the elevation in MnSOD gene expression caused by anticancer drugs and phorbol esters, we prepared membrane and cytosolic extracts from treated A549 cells and identified membrane translocation of isoenzymes using specific antibodies. As demonstrated (Fig. 7), A549 cells constitutively identified membrane translocation of isoenzymes using specific antibodies and Western blotting as described under “Materials and Methods.” PKC isoenzymes were detected using PKC isotype-specific antibodies by Western blotting as described under “Materials and Methods.” PKC isoenzymes were prepared as described under “Materials and Methods.” Membrane and cytosolic fractions of cell lysates were prepared as described under “Materials and Methods.” Both fractions were subjected to DE52 ion exchange. PKC activities are expressed as pmol/min/mg of total cellular protein.

### Table 1

| Treatment | Cytosol | Membrane |
|-----------|---------|----------|
| None      | 29.7 ± 8.5 | 5.5 ± 0.5 |
| Vinblastine | 57.7 ± 13.9<sup>a</sup> | 35 ± 13.0<sup>a</sup> |
| Vincristine | 100 ± 8.4<sup>b</sup> | 11.5 ± 0.5<sup>b</sup> |
| Taxol      | 62.3 ± 7.6<sup>b</sup> | 6.5 ± 2.5<sup>b</sup> |
| Thymeleatoxin | 10.0 ± 2.5<sup>c</sup> | 27.7 ± 2.7<sup>c</sup> |
| dPPA      | 51.0 ± 9.3<sup>c</sup> | 11.7 ± 5.7<sup>c</sup> |
| PMA       | 8.3 ± 1.2<sup>c</sup> | 33.0 ± 3.0<sup>c</sup> |

<sup>a</sup> Significantly different at *p* < 0.05 (*n* = 3 per condition).

domain of all PKC isoforms with equal potency (26) and, thereby, causes inhibition. GF109203X is a highly selective inhibitor of PKC that binds to the catalytic domain of PKCo, βII, γ, δ, and ε isoenzymes (27). To further evaluate a potential
indicating its activation. On the other hand, PMA, dPPA, and Thy each caused translocation of cPKCα, cPKCβII, and nPKCμ isoforms in addition to that of PKCδ. Thus, dPPA was not a specific activator of PKCβII in A549 cells. These findings are in agreement with those of others, which indicate that dPPA is not a selective activator of PKCβII in whole cell assays (34). These results likely relate to the fact that dPPA metabolism to its product, the nonselective PKC agonist dPP, occurs efficiently in intact cells. Hence, the effects of dPPA are nonspecific, resulting in stimulation of many isoforms, including PKCδ (34). Similarly, Thy, a purported specific activator of cPKCs (32), caused translocation of PKCδ and PKCμ in addition to that of PKCβII and PKCα. Although thymeleatoxin was originally reported to be 200–1000-fold more potent on α-γ isoforms than on the δ, ε, or η isoforms (33), recent reports have indicated that there is only a 5-fold difference in the K for PKCα-ε and η (range, 2.5–12.5 nm). Therefore, this agent cannot be regarded as a specific activator of cPKCs. As we have shown, translocation of PKCδ alone to the membrane was associated with MnSOD expression by anticancer drugs, and this MnSOD expression could be prevented by PKC inhibitors. Although PMA, dPP, and Thy each caused membrane translocation of multiple PKC isoforms, including PKCδ, it seems likely that PKCδ alone was responsible for induction of MnSOD signaling by these compounds. This was concluded because PKCδ was the only isoform activated both by anticancer drugs and by each of the PKC activators (PMA, dPP, and Thy), which effected MnSOD expression. The finding that PKC depletion by PMA pretreatment diminished MnSOD expression by all these agents added further support. This part of the investigation provided another example among the variety of signaling responses that can be evoked because of the multiple PKC isoforms activated by phorbol esters. Low concentrations of those agents caused almost complete translocation of PKCδ, as well as potent induction of MnSOD, in comparison with the anticancer compounds. To conclude, this investigation demonstrates that 1) microtubule-active anticancer drugs cause MnSOD gene expression in pulmonary adenocarcinoma cells, and 2) this effect is mediated by the PKCδ isofrom. The study also suggests that other, less specific PKC-activating agents such as PMA, dPP, and Thy also cause MnSOD expression by translocation of PKCδ. Recent reports continue to indicate that superoxide and MnSOD are potentially important in modulating cell signaling, survival, and proliferation in cancer (35–38).

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REFERENCES

1. Wani, M. C., Taylor, H. L., Wall, M. E., Coggon, P., and McPhail, A. T. (1971) J. Am. Chem. Soc. 93, 2325–2327
2. Kumar, N. (1981) J. Biol. Chem. 256, 10435–10441
3. Schiff, P. B., Fast, J., and Horwitz, S. B. (1979) Nature 282, 665–667
4. Schiff, P. B., and Horwitz, S. B. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 1561–1565
5. Ding, A. H., Forteau, F., Sanchez, E., and Nathan, C. F. (1990) Science 248, 370–372
6. Manthey, C. L., Branese, M. B., Perra, Y. P., and Vogel, S. N. (1992) J. Immunol. 149, 2459–2465
7. Kirikae, F., Kirikae, T., Qureshi, N., Takayama, K., Morrison, D., and Nakano, M. (1995) Infect. Immun. 63, 486–497
8. Hassan, H. (1988) Free Radical Biol. & Med. 5, 377–385
9. Privalle, C. T., and Fridovich, I. (1988) J. Biol. Chem. 263, 4274–4279
10. Schiauene, J. R., and Hassan, H. M. (1988) J. Biol. Chem. 263, 4269–4273
11. Forman, H. J., and Fisher, A. B. (1981) Lab. Invest. 45, 1–6
12. Freeman, B. A., Mason, R. J., Williams, M. C., and Crapo, J. D. (1986) Exp. Lung Res. 16, 203–222
13. Krall, J., Bagley, A. C., Mullenbach, G. T., Hallewell, R. A., and Lynch, R. E. (1988) J. Biol. Chem. 263, 1910–1914
14. Oberley, L. W., St. Clair, D. K., Auter, A. P., and Oberley, T. D. (1987) Arch. Biochem. Biophys. 254, 69–80
15. Wong, G. H. W., and Goeddel, D. V. (1988) Science 242, 941–944
16. Masuda, A., Longo, D. L., Kobayashi, Y., Appeliea, E., and Oppenheim J. J. (1988) FASEB J. 2, 3087–3091
17. Visser, G. A., Dougall, W. C., Wilson, J. M., Burr, I. A., and Nick, H. S. (1990) J. Biol. Chem. 265, 2856–2864
18. Wong, G. H. W., Elwell, J. H., Oberley, L. W., and Goeddel, D. V. (1989) Cell 59, 923–931
19. Watanabe, K., and West, W. L. (1982) Fed. Proc. 41, 2292–2299
20. Martelly, I., and Castagna, M. (1989) Curr. Opin. Cell Biol. 1, 206–210
21. Newton, A. C. (1995) J. Biol. Chem. 270, 28485–28498
22. Nishizuka, Y. (1995) FASEB J. 9, 484–496
23. Fujii, J., and Taniguchi, N. (1991) J. Biol. Chem. 266, 23142–23146
24. Whitsett, J. A., Clark, J. C., Wispe, J. R., and Pryhuber, G. S. (1992) Am. J. Respir. Cell Mol. Biol. 6, 481–490
25. Das, K. C., and White, C. W. (1997) J. Biol. Chem. 272, 14914–14920
26. Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M., and Tomita, F. (1986) Biochem. Biophys. Res. Commun. 135, 397–402
27. Toulecle, D., Pianetti, P., Coste, H., Belleveruge, P., Grand-Perret, T., Ajakanes, M., Baudet, V., Boissin, P., Boursier, E., Lorialle, F., Dubemel, L., Charon, D., and Kirilovsky J. (1991) J. Biol. Chem. 266, 15771–15781
28. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
29. Bradford, M. M. (1976) Anal. Biochem. 72, 248–251
30. Yasuda I, Kishimoto, A., Tanaka, S., Tomina, M., Sakurai, A., and Nishizuka, Y. (1990) Biochem. Biophys. Res. Commun. 166, 1220–1229
31. Ito, M., Tanaka, T., Inagaki, M., Naharishie, K., and Hidaka, H. (1986) Biochim. Biophys. Acta 861, 1220–1229
32. Kazanietz, M. G., Arecea, L. B., Bahador, A., Mischak, H., Goodright, J., Mushinski, J. F., and Blumberg, P. M. (1993) Mol. Pharmacol. 44, 298–307
33. Ryves, W. J. (1991) FEBS Lett. 288, 5–9
34. Kifey, S. C., Olivier, A. R., Gordge, P. C., Ryves, W. J., Evans, F. J., Ways, D. K., and Parker, P. J. (1994) Carcinogenesis 15, 319–324
35. Manna, S. M., Zhang, H. J., Yan, T., Oberley, L. W., and Aggarwal, B. B. (1998) J. Biol. Chem. 273, 13245–13254
36. Joneson, T., and Bar-Sagi, D. (1998) J. Biol. Chem. 273, 17991–17994
37. Irani, K., Xia, Y., Zweier, J., Sollott, S., Der, C., Fearn, E., Sundaresan, M., Finkel, T., and Goldschmidt-Clermont, P. J. (1997) Science 275, 1649–1652
38. Zhong, W., Oberley, L. W., Oberley, T. D., and St. Clair, D. K. (1997) Oncogene 14, 481–490