Biochemical Pharmacology, Vol. 52, pp. 393–399, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00240-7

Selective Inhibition of the Effects of Phorbol Ester on Doxorubicin Resistance and P-Glycoprotein by the Protein Kinase C Inhibitor 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine (H7) in Multidrug-Resistant MCF-7/Dox Human Breast Carcinoma Cells

Chang-Ho Ahn,† Jae-Yang Kong,‡ Won Chul Choi‡ and Myung-Sil Hwang‡*

*DIVISION OF ONCOLOGY AND ‡DIVISION OF RESEARCH AND TESTING, CENTER FOR DRUG EVALUATION AND RESEARCH, FOOD AND DRUG ADMINISTRATION, ROCKVILLE, MD 20857, U.S.A.

ABSTRACT. The possible regulation of the multidrug-resistant (MDR) phenotype and P-glycoprotein by protein kinase C (PKC) was investigated in the doxorubicin (Dox)-resistant MCF-7 cell line (MCF-7/Dox). In a clonogenic assay, cells exposed to 100 nM phorbol 12-myristate 13-acetate (PMA) for 1 hr were about 3-fold more resistant to Dox than were cells exposed to Dox alone. The PKC inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7, 30 μM) completely blocked the PMA-induced effect, but did not reverse the MDR phenotype. Complete down-regulation of PKC from MCF-7/Dox cells by 24-hr preincubation with PMA did not alter the degree of Dox resistance. Intracellular accumulation of [^{14}C]Dox decreased from a baseline of 28 pmol/10^6 cells to 15 pmol/10^6 cells in the presence of 100 nM PMA. The reduced Dox accumulation in the presence of PMA was not blocked by pretreatment of cells with H7. Following a 24-hr pretreatment with PMA, the cells accumulated almost equal amounts of [^{14}C]Dox in the absence or presence of PMA. Cells from PMA-treated colonies showed significantly higher levels of expression of P-glycoprotein when compared with those from control colonies. H7 did not affect the basal level of P-glycoprotein in cells from control colonies or PMA-induced overexpression of P-glycoprotein in cells from PMA-treated colonies. Upon stimulation with PMA (100 nM), PKC α and β translocated to the cell membrane and nucleus and PKC δ and ε to the perinuclear membrane and the nucleus, respectively. H7 (30 μM) completely inhibited PMA-induced translocations of PKC δ and ε, whereas it only partially blocked the translocations of PKC α and β. These results suggest that PMA appears to alter Dox resistance and intracellular Dox accumulation in a PKC-dependent manner and to induce increased expression of P-glycoprotein in MCF-7/Dox cells. Differential effects of H7 on the PMA-induced changes suggest that different isoforms of PKC may be involved in cell growth and drug accumulation processes as well as P-glycoprotein expression. BIOCHEM PHARMACOL 52;3:393–399, 1996.

KEY WORDS. multidrug resistance; protein kinase C; P-glycoprotein; phorbol ester; breast cancer

Resistance to anticancer drugs develops in many cancer cells during the course of chemotherapy and is accompanied frequently by multidrug cross-resistance among anticancer drugs, particularly those derived from natural origin (for reviews, see Refs. 1–4). This MDR§ phenomenon has been a major obstacle to effective cancer chemotherapy. In various cancer cells, one of the major mechanisms of MDR is a net decrease in intracellular drug accumulation through increased efflux, increased sequestration into intracellular organelles, or decreased uptake. The increased drug efflux is associated with an overexpression of a membrane glycoprotein, P-gp, which functions as an efflux pump [5]. Divergent classes of chemicals modulate MDR [6]. Although calcium channel blockers and cyclosporine derivatives such as verapamil, cyclosporin A and PSC 833 have been shown to reverse MDR through direct interaction with P-gp [7–9], hormones [10], solubilizing agents [11, 12] and inhibitors of PKC [13, 14] also modulate MDR.

The activities and amount of PKC are higher in MDR MCF-7/Dox cells than in the parent, drug-sensitive MDR-7 cells [15, 16]. Activation of PKC results in an MDR phenotype in human cancer cells [17]. Although the mechanism(s) of PKC-mediated alteration of the MDR phenotype is not fully known, phosphorylation of P-gp is induced by

†* Corresponding author: Dr. Chang H. Ahn, Division of Oncology, HFD-150, CDER, Food and Drug Administration, 5600 Fishers Lane, Rockville, MD 20857. Tel. (301) 594-5754; FAX (301) 594-0499.

§ *Abbreviations:* MDR, multidrug resistance; PKC, protein kinase C; Dox, doxorubicin; P-gp, P-glycoprotein; PMA, phorbol 12-myristate 13-acetate; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; and 4α-PDD, 4α-12,13-didecanoate.

Received 27 June 1995; accepted 7 February 1996.

PKC activated by phorbol ester [18–20], suggesting a direct modulatory role of PKC on the drug efflux function of P-gp itself and P-gp-regulated ion channels [21]. To define better a potential role of PKC in the MDR phenotype as well as the functions and expression of P-gp, we have examined the effects of PMA-induced activation of PKC in the presence or absence of the PKC inhibitor H7 on colony-forming ability, Dox accumulation, and P-gp expression in MCF-7 and MCF-7/Dox cells.

MATERIALS AND METHODS

Materials

PMA, 4α-PDD, calf thymus histone IIIS, phosphotidylserine, biotinylated secondary antibody, and rabbit anti-mouse IgG were purchased from Sigma (St. Louis, MO); H7 and monoclonal antibody specific to PKC were obtained from Seikagaku America (Rockville, MD), and polyclonal antibody was from Gibco-BRL (Gaithersburg, MD). [^{14}C]Dox, [γ-^{32}P]ATP, ^{125}I-protein A, and the ECL protein detection system were purchased from Amersham (Arlington Heights, IL); polyclonal antibody to P-gp was obtained from Oncogene Science (Uniondale, NY); and bicinchoninic acid (BCA) protein assay reagents were purchased from Pierce (Rockford, IL). Other chemicals were reagent grade.

Cell Culture and Clonogenic Assay

Cells were cultured in RPMI 1640 containing 10% fetal bovine serum. MCF-7 and MCF-7/Dox cells were provided by Dr. Kenneth Cowan (NCI, Bethesda, MD). For colony-forming assays, cells (1×10^3 cells/well) were plated in RPMI 1640 with 10% fetal bovine serum in 6-well culture plates. After 24 hr, the PKC inhibitor H7, phorbol ester, and Dox were added sequentially. Cells were incubated for 2 hr and then washed three times in PBS. Fresh medium was added and the cells were incubated for 7 days. Colonies larger than 2 × 2 mm were counted, and the clonogenic survival rate was calculated from the control. Average colonies in the control group were 104 ± 9. PMA did not affect the viability of these cells when examined by the trypan blue exclusion assay.

Measurement of PKC Activity

PKC was prepared as described previously [22]. Briefly, aliquots of cytosolic and membrane PKC fractions eluted from a DEAE-Sephacel column were assayed for enzymatic activity with histone type IIIS (1 mg/mL) as substrate, by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP. Enzyme activity was expressed as nanomoles of ^{32}P incorporated into histone per minute per milligram of homogenate protein. Protein content was determined by the method of Bradford [23] and the BCA-based method [24, 25].

Immunoblot Analysis of PKC and P-gp

Immunoreactivity of PKC with monoclonal or polyclonal antibodies was carried out essentially as described previously [26], and the immunoreactive protein band was detected with ^{125}I-protein A or the ECL protein detection system and visualized by autoradiography or chemiluminescence. Detection of immunoreactive P-gp was carried out by running PAGE and semi-dry transblotting onto the nitrocellulose membrane of 10–50 μg cell homogenate. Nonspecific binding sites of the nitrocellulose membrane were blocked by 0.1% Tween 20 in a buffer containing 100 mM Tris base and 0.9% sodium chloride, pH 7.5. The blocked membrane was incubated with an antibody raised against P-gp (10 μg/mL) and washed three times. The nitrocellulose membrane was incubated with 2.5 mg/mL biotinylated secondary antibody for 30 min, and then immunoreactive bands were visualized on X-ray film by chemiluminescence.

Immunocytochemistry of PKC

Monolayered MCF-7/Dox cells on culture slides were treated with 100 nM PMA in the presence or absence of 30 μM H7 and then fixed with 3% formaldehyde and rinsed with PBS three times. After permeabilizing cell membranes with ice-cold 0.5% Triton X-100 for 10 min and incubating them for 1 hr at room temperature in blocking solution containing PBS, 1% (v/v) normal goat serum, and 0.3% Triton X-100, cells were treated with PKC isozymic antibody (1–10 μg/mL) in the blocking solution and incubated overnight at 4° in a humidified chamber. After washing five times with PBS, cells were incubated with biotinylated goat anti-rabbit IgG for 2 hr at room temperature and then reacted with streptavidine Texas red-conjugated anti-rabbit IgG in PBS following washing five times with PBS. After rinsing five times with PBS and once with deionized water, a mounted specimen was observed under a fluorescent microscope (Nikon Optiphot).

Intracellular Drug Accumulation

MCF-7 and MCF-7/Dox cells were incubated with [^{14}C]Dox (0.1 μM for MCF-7 cells and 0.3 μM for MCF-7/Dox cells) (55 mCi/mmol) in the presence or absence of PMA (100 nM) and/or H7 (30 μM). Incubation was terminated at designated times. Cells were washed twice with PBS and then solubilized in a liquid scintillation fluid. The intracellular concentrations of Dox were determined by measuring radioactivity of the fixed cells in a Beckman 9000 scintillation counter. The average radioactivity of at least six experiments was 1050 cpm/pmol Dox.

RESULTS

The effects of activation and down-regulation of PKC on the colony-forming ability of MCF-7/Dox cells were examined. When measured by both enzyme activity and immu-

noblot analysis in the presence of 100 nM PMA, PKC was maximally relocated to the particulate fraction from the cytosol at 45 min and was not detectable at 24 hr (Fig. 1). This translocation process of PKC is required for its enzymatic activity. In clonogenic assays, MCF-7/Dox cells were about 110-fold less sensitive to Dox-induced inhibition of colony formation than parent MCF-7 cells (Table 1). When MCF-7/Dox cells were pretreated with 100 nM PMA for 1 hr, the IC_{50} value of Dox increased from 12.5 ± 1.1 to 34.0 ± 2.1 μM (Table 1). Unlike PMA, non-tumor promoting phorbol ester 4α-PDD was ineffective. Though H7 (30 μM), a PKC inhibitor, alone did not alter the extent of the MDR phenotype of MCF-7/Dox, it completely inhibited the PMA-induced increase in Dox resistance. After a 24-hr pretreatment with 100 nM PMA to induce total down-regulation of PKC, Dox resistance of MCF-7/Dox cells was not altered by the presence or absence of PMA or H7. The colony-forming ability of parent MCF-7 cells was not altered by a 1-hr pretreatment with PMA or H7 (data not shown). These results suggest that PMA-induced potentiation of Dox resistance of MCF-7/Dox cells is mediated specifically through PKC activation and that this PKC-specific effect can be inhibited by H7.

To examine further the possible mechanism of the increased resistance of MCF-7/Dox by PMA treatment, intracellular [^{14}C]Dox accumulation in parent MCF-7 and MCF-7/Dox cell lines was measured. Dox accumulation in MCF-7 cells reached a peak at 12 hr, whereas it maintained a plateau after 1 hr in MCF-7/Dox cells. The slope of the initial phase of Dox accumulation was about 2-fold steeper in MCF-7 cells than in MCF-7/Dox cells. The Dox accumulations in MCF-7 cells were 2.3-fold higher at 1 hr and about 6.4-fold higher at 12 hr than those in MCF-7/Dox cells (Fig. 2). These findings suggest that there are qualitative and quantitative discrepancies between these two cell lines in regard to intracellular drug accumulation as a net balance of uptake and efflux.

When MCF-7/Dox cells were pretreated with 100 nM PMA for 1 hr, Dox accumulation decreased from 28 to 15 pmol/10^6 cells (Fig. 3A), that is, about a 50% reduction ($P < 0.05$). Neither Dox accumulation nor PMA-induced re-

TABLE 1. Effects of Dox on the colony-forming ability of MCF-7 and MCF-7/Dox cells in the presence and absence of PKC activator and inhibitor

Treatment	IC_{50}* (μM)
MCF-7 cells	0.11 ± 0.05
MCF-7/Dox cells	12.5 ± 1.1
+ PMA (100 nM)	34.0 ± 2.1
+ H7 (30 μM)	10.7 ± 1.2
+ PMA (100 nM) + H7 (30 μM)	10.0 ± 0.8
+ 4α-PDD (1 μM)	11.9 ± 1.0
MCF-7/Dox cells pretreated with PMA for 24 hr	10.8 ± 1.4
+ PMA (100 nM)	12.1 ± 2.2
+ H7 (30 μM)	10.3 ± 1.7
+ PMA (100 nM) + H7 (30 μM)	10.5 ± 1.3

* IC_{50} is the doxorubicin concentration required to inhibit 50% of colony formation of these cells. Values are means ± SD of four separate experiments.

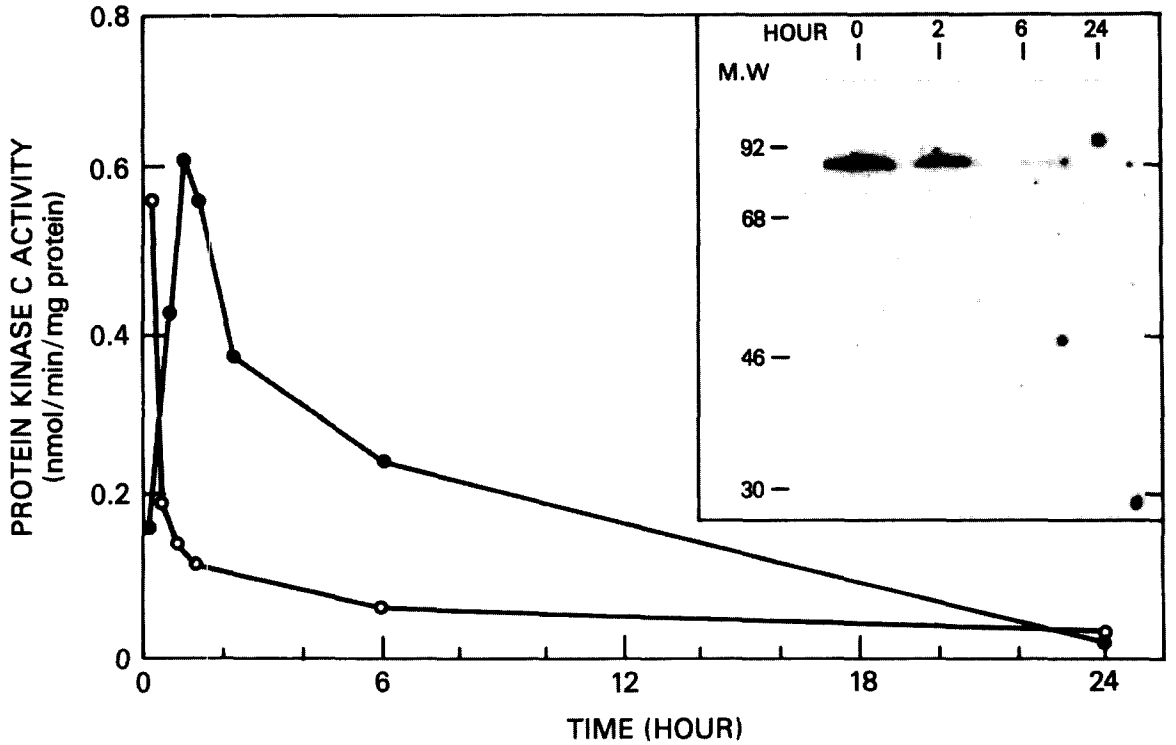

FIG. 1. Translocation and down-regulation of PKC in MCF-7/Dox cells. Cells were treated with 100 nM PMA, and activities of PKC at the designated time points were measured in the cytosol (○) and the particulate (●) as described in Materials and Methods. Inset: Immunoblot analysis of time-dependent down-regulation of PKC in the presence of 100 nM PMA.

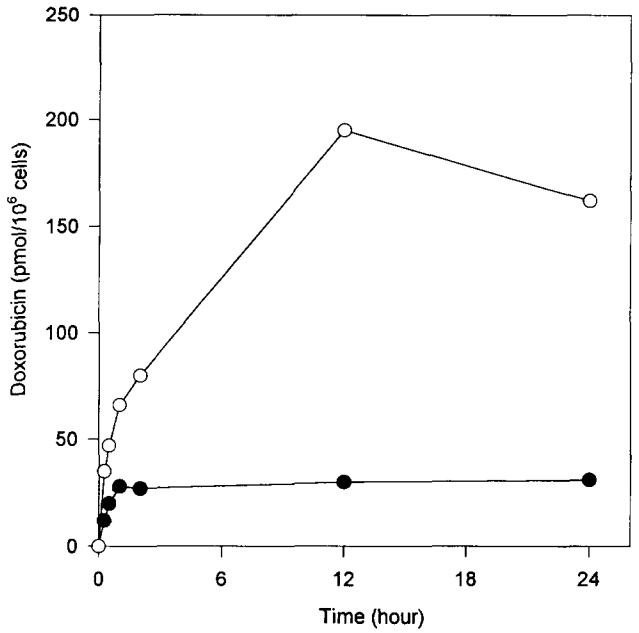

FIG. 2. Dox accumulation in MCF-7 and MCF-7/Dox cells. MCF-7 (○) and MCF-7/Dox (●) cells were incubated for up to 24 hr with 0.1 and 0.3 μM [^{14}C]Dox, respectively, and the radioactivity of the cells was measured at the designated time points. Each time point represents a mean of at least three different experiments.

duction of Dox accumulation in MCF-7/Dox cells was affected by 30 μM H7. To examine if the PMA-induced effect on Dox accumulation in MCF-7/Dox was PKC dependent, the cells were preincubated with PMA for 24 hr to down-regulate completely the enzyme and then treated the same way as in Fig. 3A. This treatment did not result in reduction of Dox accumulation, suggesting that the reduction of Dox accumulation was mediated by a PKC-dependent mechanism that could not be blocked by H7 (Fig. 3B).

Expression of P-gp in MCF-7/Dox cells obtained at the end of the colony formation assay was measured. Cells from colonies pretreated with 100 nM PMA expressed several-fold higher levels of P-gp when compared with cells from colonies with no treatment, while P-gp expression was not altered in cells from colonies pretreated with 30 μM H7 (Fig. 4). In cells pretreated with both PMA and H7, overexpression of P-gp was still observed, and the extent of the overexpression was not altered when compared with that of PMA-pretreated cells. The intracellular drug accumulation of cells obtained from PMA-treated colonies was qualitatively similar to cells from control colonies (data not shown). Though it was not inhibited by H7, the overexpression of P-gp by PMA may be one of the contributing factors for the increased MDR phenotype following pretreatment with PMA.

As an indication of its activation and for its action upon stimulation, PKC translocates to the cell membrane, nucleus, or possibly other cellular organelles. An effective inhibitor of PKC should be able to inhibit this translocation process. Translocation of PKC isozymes in MCF-7/Dox cells was examined in the presence or absence of PMA with or without H7 by immunofluorescent microscopy. Upon stimulation with PMA (100 nM), immunoreactive PKC α, β, δ, ε, and ζ, but not PKC γ, were detected (Fig. 5). PKC α and β appeared to translocate to the cell membrane and nucleus, and PKC δ and ε to the perinuclear membrane and the nucleus, respectively. As expected, PKC ζ was not affected by treatment with PMA. H7 (30 μM) completely

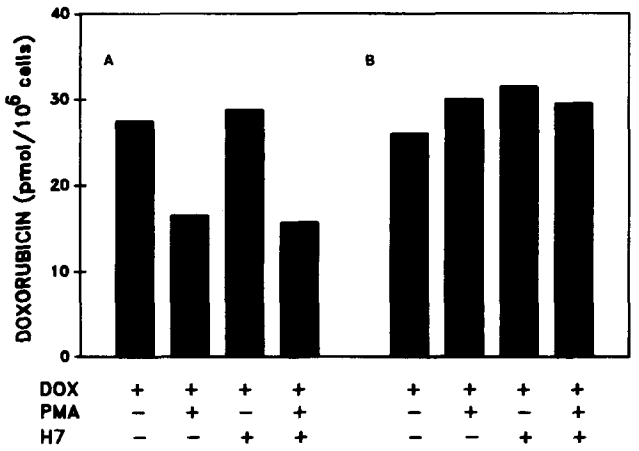

FIG. 3. Effects of PMA and H7 on Dox accumulation in MCF-7/Dox cells. (A) Cells were incubated with 0.3 μM [^{14}C]Dox in the presence or absence of 100 nM PMA for 1 hr with or without 30 μM H7 (B) Cells were pretreated for 24 hr with 100 nM PMA for complete down-regulation of PKC and then further incubated with 0.3 μM [^{14}C]Dox in the presence or absence of 100 nM PMA for 1 hr with or without 30 μM H7. A set of representative data of at least three different experiments is shown.

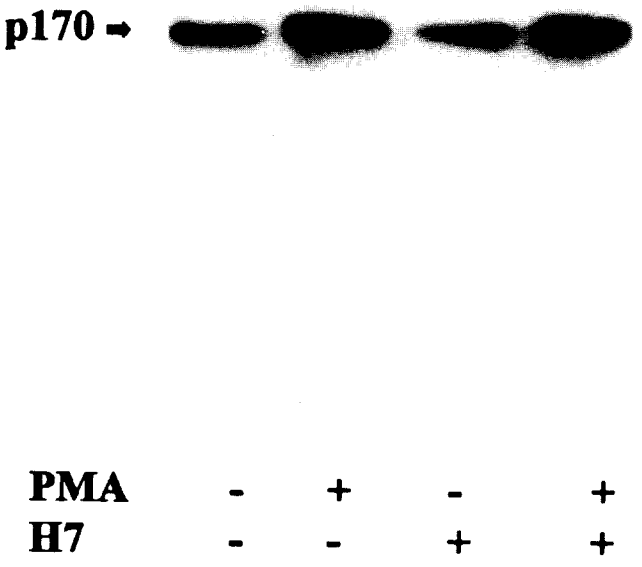

FIG. 4. Expression of P-gp in MCF-7/Dox cells. Levels of P-gp in cells from untreated colonies or colonies pretreated with 100 nM PMA or 30 μM H7, or both were determined by affinity-purified polyclonal antibody to P-gp.

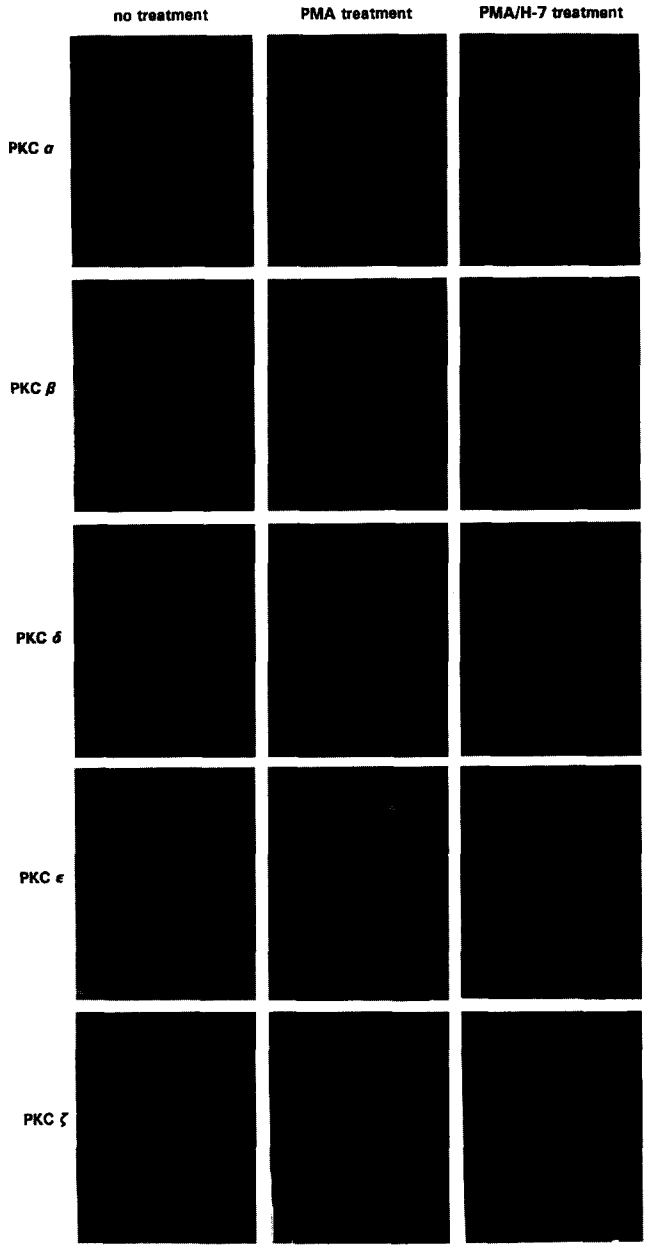

FIG. 5. Immunocytochemical detection of translocation of PKC isozymes induced by PMA and its inhibition by H7. Monolayered MCF-7/Dox cells on culture slides were treated with 100 nM PMA in the presence or absence of 30 nM H7, and immunofluorescent analysis was carried out as described in Materials and Methods. Immunoreactive PKC isozymes were visualized under an immunofluorescent microscope with proper excitation. The exposure time of each PKC isozyme was adjusted depending upon the fluorescent intensity for proper visualization.

inhibited PMA-induced translocations of PKC δ and ϵ, whereas it only partially blocked the translocations of PKC α and β.

DISCUSSION

Activation of PKC by phorbol ester results in increased phosphorylation of proteins including P-gp in MCF-7/Dox cells [19]. A recent study demonstrated that the sites phosphorylated on P-gp by PKC occur at multiple serine positions [27]. Elevated levels of PKC activity and PKC protein were observed previously in MCF-7/Dox cells compared with parent MCF-7 cells [15, 17]. In the present study, phorbol ester treatment increased Dox resistance by 3-fold, increased P-gp expression by several-fold, and decreased intracellular Dox accumulation in MCF-7/Dox cells. These effects induced by phorbol ester were not observed when PKC was down-regulated completely or when non-tumor-promoting phorbol ester was used, indicating that a PKC-dependent modulation of Dox resistance was involved. However, it is also possible that a PMA-mediated mechanism(s) other than activation of PKC may have participated. In contrast to our results of phorbol ester-induced reduction in Dox accumulation in MCF-7/Dox cells, continuous exposure of KB-V1 cells with 200 nM PMA and vinblastine did not induce any observable effect on baseline drug accumulation. Incubation of KB-V1 cells with PMA and calcium channel blockers (e.g. verapamil and tetrandrine), however, does reduce the blocker-induced increases in vinblastine accumulation by about 30% [20]. These observations may be due to differences in PKC or in drug transport mechanism(s) in MDR cell lines of divergent origin.

H7 did not block phorbol ester-induced reduction in Dox accumulation and overexpression of P-gp, whereas it completely inhibited the increase in Dox resistance by phorbol ester in clonogenic assay. These findings raise three important points. First, these results suggest that different isoforms of PKC may mediate different cellular processes such as proliferation (colony formation) as well as modulation and expression of P-gp. It is possible that a PKC isoform(s) that is not inhibited completely by H7 may modulate the function of P-gp in MCF-7/Dox cells. In KB-V1 cells, P-gp phosphorylation in cells induced by phorbol ester is not inhibited by H7 [20]. Not all PKC isozymes regulate MDR processes including expression and modulation of P-gp. PKC α increases phosphorylation of P-gp and thus increases P-gp pump function [28]. PKC βI decreases drug accumulation but is not associated with an altered level of P-gp [29], whereas PKC γ does not modulate P-gp function [30]. Likewise, different functions of PKC isozymes in cellular processes such as proliferation and differentiation of various cell types have been studied. PKC α and δ participate in myeloid differentiation induced by PMA, whereas PKC βII has been implicated in the proliferation of these cells [31, 32]. We observed that H7 partially inhibited translocation of PKC α and β and completely blocked translocation of PKC δ and ϵ when MCF-7/Dox cells were preincubated with PMA, which suggests a possible modulatory role of PKC α, β, and/or ζ on pump function and expression of P-gp. Second, the results with H7 suggest that inhibition of PKC activity and modulation of MDR functions may have a weak correlation. Similarly, the differential effects between modulation of MDR and inhibition of PKC activity

by inhibitors of PKC have been studied in various MDR cell lines. Calphostin C, a PKC inhibitor, reverses MDR through P-gp independently of its effect on PKC activity [33]. Effects of staurosporine derivatives and phenothiazines on drug accumulation and PKC activity are not correlated [34, 35]. Staurosporine, a PKC inhibitor that possesses an inhibitory mechanism similar to that of H7 and reverses MDR phenotype, has shown that it acts probably through its inhibition of PKC activity but not through P-gp binding [36]. Likewise, H7 may not bind or be a substrate of P-gp. Unlike these PKC inhibitors, MDR-reversing drugs such as verapamil and cyclosporine A directly interact with P-gp [37, 38]. Third, the role of P-gp in mediating the increased drug resistance induced by phorbol ester may not be critical, since H7 was able to reverse the phorbol ester-induced potentiation of MDR in spite of its ineffectiveness in blocking PKC-specific effects on drug accumulation and P-gp expression. Furthermore, the selective inhibition of phorbol ester-induced effects by H7 reflects complex mechanisms involved with the colony-forming ability of MCF-7/Dox cells, including PKC-dependent modulation. In addition, the discrepancy of ratio between Dox accumulation and Dox resistance of MCF-7 and MCF-7/Dox cells further suggests that factors other than P-gp function may also be important in drug resistance, although there may be additional functions of P-gp such as the sequestration of drug into the cytoplasmic endosomes and lysosomes. In MCF-7/Dox cells, glutathione S-transferase and other free radical scavenging systems, as well as DNA topoisomerase II, are also considered as important mechanisms responsible for MDR [39, 40]. Recent studies have also found that P-gp overexpression alone cannot explain the MDR phenotype in glioblastoma cell lines, and thus P-gp-independent mechanisms of Dox resistance (e.g. glutathione-related detoxifying enzymes) have been suggested [40, 41].

In studies of colony-forming ability and intracellular Dox accumulation, complete down-regulation of PKC did not reverse or alter the MDR phenotype of MCF-7/Dox cells, suggesting that PKC is not a causal mechanism of the MDR phenotype in MCF-7/Dox cells. Considering this result as well as the efficacy of H7 and staurosporine in reversal of MDR [42], PKC inhibitors may not be an effective treatment means of MDR reversal compared with P-gp blockers which completely reverse MDR. However, it is also reasonable to expect that in tumors that have low-level MDR, the modulation of P-gp activity by phosphorylation could have a much larger impact. That is, down-regulation of PKC might reverse MDR in such a clinical setting. Alternatively, modulation of PKC activity by these chemicals may have affected the MDR phenotype of MCF-7/Dox cells through an altered apoptotic process of MCF-7/Dox cells, since both activators and inhibitors of PKC including PMA and H7 are involved with proliferation and apoptosis of various cell types [43–45]. Since PKC-mediated apoptosis appears to be cell-type specific and isozyme dependent, it may be worthwhile to examine the possible effects of activation and inhibition of PKC isozymes on apoptosis in MCF-7/Dox cells in relation to the development and maintenance of the MDR phenotype.

Our study has demonstrated that activation of PKC by phorbol ester further increases Dox resistance and expression of P-gp and decreases intracellular Dox accumulation as well. The PKC inhibitor H7 inhibited the phorbol ester-induced increase in Dox resistance, but not those effects of phorbol ester on functions and expression of P-gp, suggesting involvement of isozyme-specific roles of PKC on cell proliferation and P-gp. Our study also suggests that, unlike P-gp binding MDR inhibitors, PKC inhibitors may not be an effective class of chemicals for circumvention of MDR.

References

1. Gottesman M and Pastan I, Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* **62:** 385–427, 1993.
2. Moscow JA and Cowan KH, Multidrug resistance. *J Natl Cancer Institute* **80:** 14–20, 1988.
3. Bradley G, Juranka PF and Ling V, Mechanism of multidrug resistance. *Biochim Biophys Acta* **948:** 87–128, 1988.
4. Hayes JD and Wolf CR, Molecular mechanisms of drug resistance. *Biochem J* **272:** 281–295, 1990.
5. Beck WT, Drug accumulation and binding in P-glycoprotein-associated multidrug resistance. In: *Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells* (Ed. Roninson IB), pp. 215–227. Plenum Press, New York, 1991.
6. Ford JM and Hait WN, Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol Rev* **42:** 155–199, 1990.
7. Silbermann MH, Boersma AWM, Janssen ALW, Scheper RJ, Herweijer H and Nooter K, Effects of cyclosporin A and verapamil on the intracellular daunorubicin accumulation in Chinese hamster ovary cells with increasing levels of drug-resistance. *Int J Cancer* **44:** 722–726, 1989.
8. Coley HM, Twentyman PR and Workman P, Improved cellular accumulation is characteristic of anthracyclines which retain high activity in multidrug resistant cell lines, alone or in combination with verapamil or cyclosporin A. *Biochem Pharmacol* **38:** 4467–4475, 1989.
9. Boesch D, Gaveriaux C, Jachez B, Pourtier-Manzanedo A, Bollinger P and Loo F, *In vivo* circumvention of P-glycoprotein-mediated multidrug resistance of tumor cells with SDZ PSC 833. *Cancer Res* **51:** 4226–4233, 1991.
10. Yang, C-P, DePinho SG, Greenberger LM, Arceci RJ and Horwitz SB, Progesterone interacts with P-glycoprotein in multidrug-resistant cells and in the endometrium of gravid uterus. *J Biol Chem* **264:** 782–788, 1989.
11. Ross DD, Wooten PJ, Tong Y, Cornblatt B, Levy C, Sridhara R, Lee EJ and Schiffer CA, Synergistic reversal of multidrug-resistance phenotype in acute myeloid leukemia cells by cyclosporin A and cremophor EL. *Blood* **83:** 1337–1347, 1994.
12. Woodcock DM, Linsenmeye ME, Chojnowski G, Kriegle AB, Nink V, Webster LK and Sawyer WH, Reversal of multidrug resistance by surfactants. *Br J Cancer* **66:** 62–68, 1992.
13. Posada JA, McKeegan EM, Worthington KF, Morin MJ, Jaken S and Tritton TR, Human multidrug resistant KB cells overexpress protein kinase C: Involvement in drug resistance. *Cancer Commun* **1:** 285–292, 1989.
14. Ford JM, Prozialeck WC and Hait WN, Structural features determining activity of phenothiazines and related drugs for inhibition of cell growth and reversal of multidrug resistance. *Mol Pharmacol* **35:** 105–115, 1989.

15. Ahn C-H and Kim JK, Characterization of protein kinase C in doxorubicin-sensitive and -resistant human breast MCF-7 cancer cell lines. *J Biomed Res* **1:** 52–58, 1991.
16. Lee SA, Karaszhiewicz JW and Anderson WB, Elevated level of nuclear protein kinase C in multidrug-resistant MCF-7 human breast carcinoma cells. *Cancer Res* **52:** 3750–3759, 1992.
17. Fine RL, Patel J and Chabner BA, Phorbol esters induce multidrug resistance in human breast cancer cells. *Proc Natl Acad Sci USA* **85:** 582–586, 1988.
18. Hamada H, Hagiwara KI, Nakajima T and Tsuruo T, Phosphorylation of the M_r 170,000 to 180,000 glycoprotein specific to multidrug-resistant tumor cells: Effects of verapamil, trifluoperazine and phorbol esters. *Cancer Res* **47:** 2860–2865, 1987.
19. Fine RL, Ozol R, Patel J and Ahn C-H, Phosphoprotein, protein kinase C and second-messenger system changes in human multi-drug resistant cancer cells. In: *Drug Resistance in Cancer Therapy* (Ed. Ozol F), pp. 107–123. Kluwer Academic, New York, 1989.
20. Chambers TC, McAvoy EM, Jacobs JW and Eilon G, Protein kinase C phosphorylates P-glycoprotein in multidrug resistant human KB carcinoma cells. *J Biol Chem* **265:** 7679–7686, 1990.
21. Hardy SP, Goodfellow HR, Valverde MA, Gill DR, Sepúlveda V and Higgins CF, Protein kinase C-mediated phosphorylation of the human multidrug resistance P-glycoprotein regulates cell volume-activated chloride channels. *EMBO J* **14:** 68–75, 1995.
22. Lee YJ, Berns CM, Erdos G, Borrelli MJ, Ahn CH and Corry PM, Effect of 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) on HSP70 and HSP28 gene expression and thermotolerance development in human colon carcinoma cells. *Biochem Pharmacol* **48:** 2057–2063, 1994.
23. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
24. Wiechelman K, Braun R and Fitzpatrick J, Investigation of the bicinchoninic acid protein assay: Identification of the groups responsible for color formation. *Anal Biochem* **175:** 231–237, 1988.
25. Gates RE, Elimination of interfering substances in the presence of detergent in the bicinchoninic acid protein assay. *Anal Biochem* **196:** 290–295, 1991.
26. Fridman F, Lacal JC, Reich R, Bonfil DR and Ahn C-H, Differential effects of phorbol ester on the *in vitro* invasiveness of malignant and non-malignant human fibroblast cells. *J Cell Physiol* **142:** 55–60, 1990.
27. Chambers TC, Pohl J, Raynor RL and Kuo JF, Identification of specific sites in human P-glycoprotein phosphorylated by protein kinase C. *J Biol Chem* **268:** 4592–4595, 1993.
28. Blobe GC, Sachs CW, Khan WA, Fabbro D, Stabel S, Wetsel WC, Obeid LM, Fine RL and Hannun YA, Selective regulation of expression of protein kinase C (PKC) isoenzymes in multidrug-resistant MCF-7 cells. Functional significance of enhanced expression of PKC α. *J Biol Chem* **268:** 658–664, 1993.
29. Fan D, Fidler IJ, Ward NE, Seid C, Earnest LE, Housey GM and O'Brian CA, Stable expression of a cDNA encoding rat brain protein kinase C-βI confers a multidrug–resistant phenotype on rat fibroblasts. *Anticancer Res* **12:** 661–667, 1992.
30. Ahmad S, Trepel JB, Ohno S, Suzuki K, Tsuruo T and Glazer RI, Role of protein kinase C in the modulation of multidrug resistance: Expression of the atypical γ isoform of protein kinase C does not confer increased resistance to doxorubicin. *Mol Pharmacol* **42:** 1004–1009, 1992.
31. Mischak H, Pierce JH, Goodnight J, Kazanietz MG, Blumberg PM and Mushinski JF, Phorbol ester-induced myeloid differentiation is mediated by protein kinase C-α and -δ and not by protein kinase C-βII, -ε, -ζ, and -η. *J Biol Chem* **268:** 20110–20115, 1993.
32. Murray, NR, Baumgardner GP, Burns DJ and Fields AP, Protein kinase C isotypes in human erythroleukemia (K562) cell proliferation and differentiation. Evidence that βII protein kinase C is required for proliferation. *J Biol Chem* **268:** 15847–15853, 1993.
33. Gupta S, Patel K, Singh H and Gollapudi S, Effect of calphostin C (PKC inhibitor) on daunorubicin resistance in P388/ADR and HL60/AR cells: Reversal of drug resistance possibly via P-glycoprotein. *Cancer Lett* **76:** 139–145, 1994.
34. Miyamoti KI, Inoko K, Ikeda K, Wakusawa S, Kajita S, Hasegawa T, Takagi K and Koyama M, Effect of staurosporine derivatives on protein kinase activity and vinblastine accumulation in mouse leukemia P388/ADR cells. *J Pharm Pharmacol* **45:** 43–47, 1993.
35. Pereira E and Garnier-Suillerot A, Correlation between the short-term measurements of drug accumulation in living cells and the long-term growth inhibition. *Biochem Pharmacol* **47:** 1851–1857, 1994.
36. Laredo J, Huynh A, Muller C, Jaffrezou JP, Bailly JD, Cassar G, Laurent G and Demur C, Effect of the protein kinase C inhibitor staurosporine on chemosensitivity to daunorubicin of normal and leukemic fresh myeloid cells. *Blood* **84:** 229–237, 1994.
37. Yusa K and Tsuruo T, Reversal mechanism of multidrug-resistance by verapamil: Direct binding of verapamil to P-glycoprotein on specific sites and transport of verapamil outward across the plasma membrane of K562/ADM cells. *Cancer Res* **49:** 5002–5006, 1989.
38. Foxwell BMJ, Mackie A, Ling V and Ryffel B, Identification of the multidrug resistance-related P-glycoprotein as a cyclosporin binding protein. *Mol Pharmacol* **36:** 543–546, 1989.
39. Mimnaugh EG, Dusre L, Atwell J and Myers CE, Differential oxygen radical susceptibility of Adriamycin-sensitive and -resistant MCF-7 human breast tumor cells. *Cancer Res* **49:** 8–15, 1989.
40. Batist G, Tulpule A, Sinha BK, Katki AG, Myers CE and Cowan KH, Overexpression of a novel anionic glutathione transferase in multidrug-resistant human breast cancer cells. *J Biol Chem* **261:** 15544–15549, 1986.
41. Huet S, Schott B and Robert J, P-glycoprotein overexpression cannot explain the complete doxorubicin-resistance phenotype in rat glioblastoma cell lines. *Br J Cancer* **65:** 538–544, 1992.
42. Efferth T and Volm M, Reversal of doxorubicin-resistance in Sarcoma 180 tumor cells by inhibition of different resistance mechanisms. *Cancer Lett* **70:** 197–202, 1993.
43. Couldwell WT, Hinton DR, He S, Chen TC, Sebat I, Weiss MH and Law RE, Protein kinase C inhibitors induce apoptosis in human malignant glioma cell lines. *FEBS Lett* **345:** 43–46, 1994.
44. Shaposhnikova VV, Dobrovinskaya OR, Eidus LKH and Korystov YN, Dependence of thymocyte apoptosis on protein kinase C and phospholipase A_2. *FEBS Lett* **348:** 317–319, 1994.
45. Wright SC, Zhong J and Larrick JW, Inhibition of apoptosis as a mechanism of tumor promotion. *FASEB J* **8:** 654–660, 1994.