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Protein kinase C-mediated down-regulation of MDR3 mRNA expression in Chang liver cells

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Abstract

MDR3 is a phospholipid translocator homologous to MDR1 P-glycoprotein. MDR3 localizes to the canalicular membrane and contributes to the secretion of bile. To elucidate the role of protein kinase C in the regulation of *MDR3* gene expression, we investigated the effect of phorbol 12-myristate 13-acetate (PMA) on the level of MDR3 mRNA in human Chang liver cells by a reverse transcription–polymerase chain reaction method. The steady-state expression of MDR3 mRNA was decreased by PMA after treatment for 8–20 hr and at concentrations of 1–100 nM. PMA also decreased the doxorubicin-induced expression of MDR3 mRNA. 4α-Phorbol 12,13-didecanoate, a negative control compound, did not decrease the expression at these concentrations. The down-regulatory effect of PMA was partially suppressed by the protein kinase C inhibitors 2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)maleimide (GF109203X) and calphostin C. Furthermore, cycloheximide, a protein synthesis inhibitor, antagonized the effect of PMA. From these results, it was suggested that the level of MDR3 mRNA was negatively regulated by a protein kinase C- and protein synthesis-dependent system and that the system regulated both the stable and inducible expression of MDR3 mRNA.

Keywords: MDR3; Phorbol 12-myristate 13-acetate; Doxorubicin; Protein kinase C; mRNA; Chang liver cell

1. Introduction

The human multidrug resistance 3 gene (*MDR3*) is a member of the superfamily of ABC (ATP-binding cassette) transporters. *MDR3* is homologous to the *MDR1* gene encoding P-gp, a membrane transporter for hydrophobic cationic drugs. *MDR3* encodes a phospholipid translocator (flippase) specific for phosphatidylcholine. MDR3 P-gp localizes in the canalicular membrane of hepatocytes [1]. The defected MDR3 P-gp is associated with a subtype of progressive familial intrahepatic cholestasis (PFIC-3) [2–4], which is a recessive genetic disorder with a poor prognosis. In the mouse, the *mdr2* gene is analogous to human *MDR3* [5,6]. Most of the phosphatidylcholine lost from the bile of *mdr2*-knockout mice was rescued by the transduction of *MDR3* [7].

Phosphatidylcholine is a major phospholipid found in bile, and is thought to play a critical role in solubilizing bile acid and cholesterol, which are excreted into bile independent of the excretion of phospholipids [8]. Experimental evidence that cholate feeding of *mdr2*-knockout mice induces pronounced liver injury [9] strongly supports the pathological findings in PFIC-3. Therefore, it was suggested that a lack of phosphatidylcholine would reduce the transfer of these bile components from the canalicular membrane to bile causing cholestasis and injury to the plasma membrane of hepatocytes.

MDR3 is located 34 kbp downstream of *MDR1* on chromosome 7q21 [10]. The promoter region contains several consensus sequences where liver-specific C/EBP and HNF5 transcription factors, AP-1, or Sp1 may bind, but the 5′-flanking region of *MDR3* contains neither a TATA box nor a CAAT box [11]. The mouse *mdr2* gene also lacks TATA or CCAAT boxes [12]. From experiments with the rat *mdr2* gene, it has been shown that Sp1 has a role in the regulation of *mdr2* expression [13]. There are several reports of

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Abbreviations: MDR, multidrug resistance; P-gp, P-glycoprotein; PCR, polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate; 4α-PDD, 4α-phorbol 12,13-didecanoate; and PKC, protein kinase C.

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changes in *mdr2* expression levels caused by pharmacological agents and bile salts [14–17]. Up-regulation of *mdr2* gene expression was observed in fibrate- (ciprofibrate, clofibrate, and bezafibrate) [14] or peroxisome proliferator- (2,4,5-trichlorophenoxyacetic acid) treated mice [15]. Furthermore, up-regulation was observed in pravastatin-treated rats [16] and in cholic acid-fed mice [17]. These studies showed that the expression of *MDR3* was not stable and was changed by chemical compounds, as *MDR1* gene expression was up-regulated by doxorubicin [18]. Thus, the function as well as the regulation of *MDR3* gene expression appears to be physiologically very important.

Some bile acids act through the activation of PKC [19–22]. In the present study, we investigated the effects of PMA and doxorubicin, known to up-regulate MDR1 expression, on the expression of MDR3 mRNA.

2. Materials and methods

2.1. Cell culture

Human Chang liver cells were cultured in RPMI 1640 medium supplemented with 5% (v/v) heat-inactivated fetal bovine serum and 100 μg/mL of kanamycin in 35-mm plastic dishes in the presence of 5% CO_2 at 37° until confluent. Then appropriate concentrations of agents were added to the culture medium, and the cells were cultured for 20 hr or as otherwise mentioned. Hydrophobic agents were dissolved in dimethyl sulfoxide and added to the culture medium at 0.1% (v/v).

2.2. Agents

PMA, 4α-PDD, calphostin C, and cycloheximide were purchased from Wako Pure Chemicals. Doxorubicin hydrochloride was obtained from the Sigma Chemical Co. 2-[1-(3-Dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide (GF109203X) was obtained from BIOMOL Research Laboratories.

2.3. RNA preparation and analysis

After cultivation of the cells, total RNA was extracted using Isogen-LS (Wako Pure Chemicals). Then cDNA was prepared by incubation of the RNA with Molony murine leukemia virus (MMLV) reverse transcriptase (200 units, Gibco BRL) and random primers (200 ng, Gibco BRL) at 37° for 60 min in 50 μL of Tris–HCl buffer (20 μM, pH 8.4) containing 50 μM KCl, 2.5 μM $MgCl_2$, 0.1 μg/mL of bovine serum albumin, 10 μM dithiothreitol, 0.5 mM deoxynucleotides, and RNase inhibitor (30 units, Promega). Following inactivation of the enzyme by incubation at 70° for 10 min, PCR was conducted as described below. For MDR3 mRNA amplification, 40 cycles of PCR, consisting of 30 sec at 94° for denaturation, 45 sec at 63° for annealing, and 45 sec at 72° for extension, were carried out with 1.5 U of Taq polymerase (Takara) in 50 μL of PCR solution (2 μL of cDNA solution, 10 μM primers, 2.5 mM dNTPs, 5 μL of 10x PCR reaction buffer) in a programmable thermal controller (PTC-100, MJ-Research, Inc.). Thirty cycles of PCR for β-actin mRNA as an internal standard were similarly performed. PCR primers for the amplification of MDR3 cDNA were synthesized by Pharmacia Japan. Their sequences were as follows: sense, 5′-GGCTTCAGCCGGCATTTTCA-3′; antisense, 5′-GCAGCATCTGTGGCAAGTCT-3′ (+2168 to +2187 and +2446 to +2465 relative to the translation start site). PCR primers for human β-actin were purchased from Stratagene. The PCR products (10 μL) were separated on a 2% agarose gel and visualized with ultraviolet light after immersion in an ethidium bromide solution (1 μg/mL) for 20 min. Densitometric analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

2.4. Inhibition of protein synthesis and mRNA expression

Cells were preincubated in the absence or presence of cycloheximide (20 μg/mL) for 2 hr. Then PMA (100 nM) was added to the medium, and the cells were incubated for 8 hr. Total cellular RNA levels were measured at the beginning and end of the PMA treatment. MDR3 mRNA levels were quantified as mentioned above.

2.5. Statistical analysis

All experiments were performed at least three times. Data are given as averages ± SD. Significance ($P < 0.05$) was determined by Student's *t*-test.

3. Results

3.1. Down-regulation of MDR3 mRNA in PMA-treated Chang liver cells

As shown in Fig. 1, human Chang liver cells expressed MDR3 mRNA at the same level as MDR1 mRNA. The mRNA level of MDR1 has been reported to be increased by PMA through the activation of PKC [23–25]. After treating the cells with 100 nM PMA for 20 hr, the level of MDR1 mRNA was strongly increased as reported, but the MDR3 mRNA level was strongly decreased. Thus, it was found that PMA decreases the expression of MDR3 mRNA. Next, we examined the concentration-dependency of the action of PMA, as shown in Fig. 2. The MDR3 mRNA levels were suppressed by PMA in a concentration-dependent manner in the range of 1–100 nM, and the level was inhibited significantly even at a concentration of 1 nM. We also examined the time-dependency of the effect of PMA on MDR3

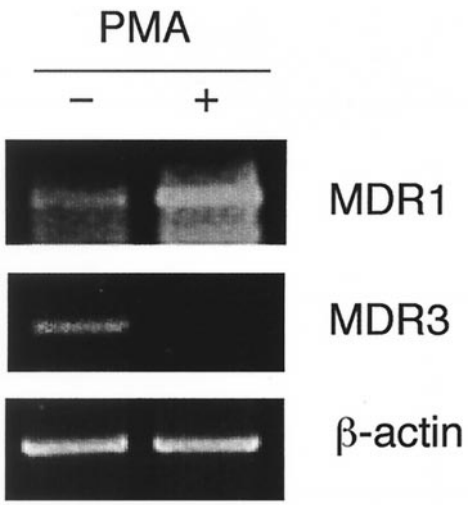

Fig. 1. Expression of MDR1 and MDR3 mRNA in Chang liver cells and the effect of prolonged treatment with PMA on these mRNA levels. Cells were incubated in the absence or presence of 100 nM PMA for 20 hr. Data are typical of one of at least three separate experiments.

mRNA levels in Chang liver cells, as shown in Fig. 3. The MDR3 mRNA level was decreased significantly after 8 and 20 hr of treatment with PMA, whereas it was not changed after 3 hr of treatment. These results suggested that the

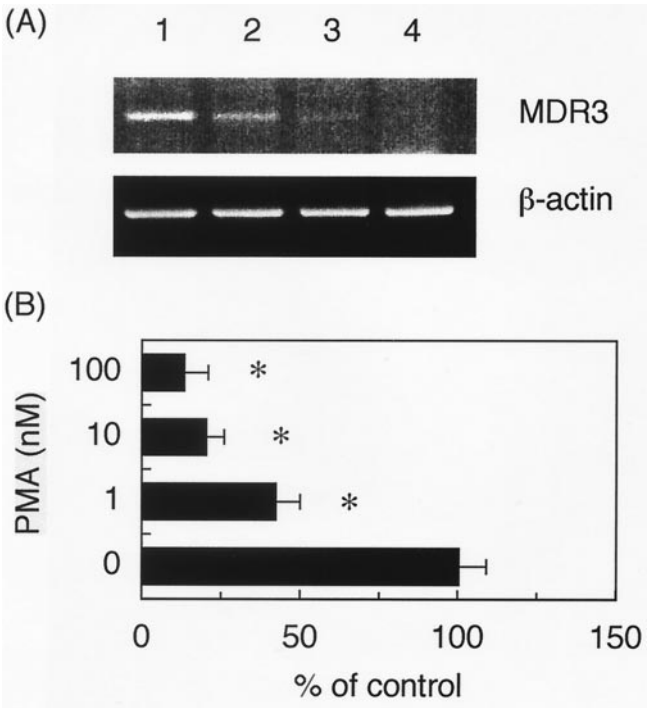

Fig. 2. Concentration-dependency of the effect of PMA on MDR3 mRNA levels in Chang liver cells. (A) Cells were incubated in the absence or presence of 1, 10, or 100 nM PMA for 20 hr. Lane 1, control; lane 2, 1 nM PMA; lane 3, 10 nM PMA; and lane 4, 100 nM PMA. Data are typical of one of three separate experiments. (B) The amount of MDR3 mRNA/β-actin mRNA in PMA-treated cells relative to control is shown in a column graph. Data are averages ± SD (N = 3). Key: (*) $P < 0.05$, compared with the control.

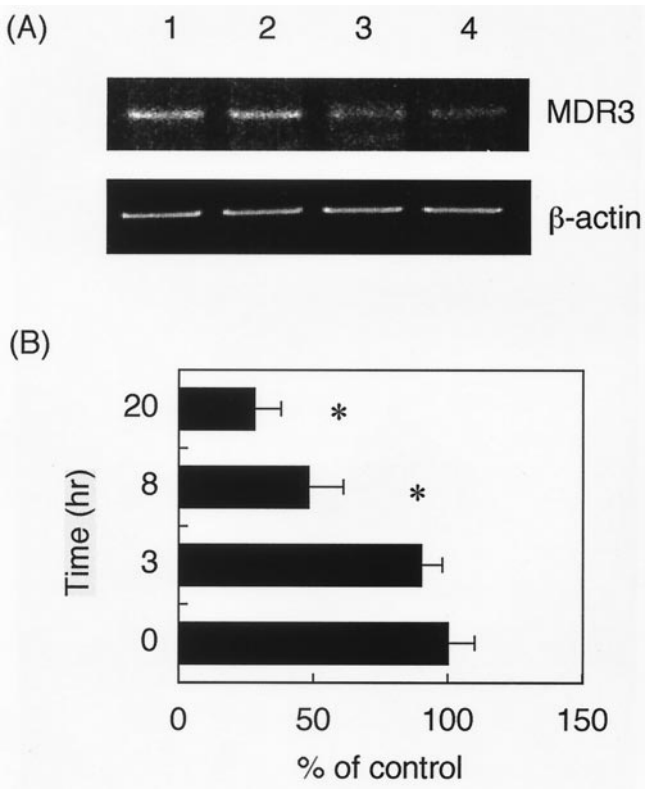

Fig. 3. Time-dependency of the effect of PMA on MDR3 mRNA levels in Chang liver cells. (A) Cells were treated with 100 nM PMA for 0, 3, 8, or 20 hr. Lane 1, 0 hr; lane 2, 3 hr; lane 3, 8 hr; and lane 4, 20 hr. Data are typical of one of three separate experiments. (B) The amount of MDR3 mRNA/β-actin mRNA in the PMA-treated cells relative to control is shown in a column graph. Data are averages ± SD (N = 3). Key: (*) $P < 0.05$, compared with the 0-hr control.

down-regulation of MDR3 mRNA levels was caused by PMA after a lag time of several hours and that the action was concentration-dependent.

From the above results, we questioned whether the PMA-induced down-regulation of MDR3 mRNA might be phosphorylation- and PKC-mediated. Hence, we investigated the effects of 4α-PDD, a negative control compound for PMA, on MDR3 mRNA expression. As shown in Fig. 4, 4α-PDD did not decrease the mRNA level of MDR3 at 100 nM, the usual concentration for a control compound. Thus, it was suggested that the PMA-induced decrease in the MDR3 mRNA level was caused by activation of PKC by PMA. Although 4α-PDD at higher concentrations slightly decreased the MDR3 mRNA level, this may reflect a nonspecific membrane action. Next, we examined the effects of two selective inhibitors for PKC, GF109203X and calphostin C. As shown in Fig. 5, GF109203X at 100 nM significantly reversed the PMA-induced reduction in the level of MDR3 mRNA. In addition, as shown in Fig. 6, calphostin C at 10 nM also partially reversed the PMA-induced decrease in the expression of MDR3 mRNA. These results indicate that the down-regulation of MDR3 mRNA expression by PMA is mediated through a PKC-dependent phosphorylation reaction.

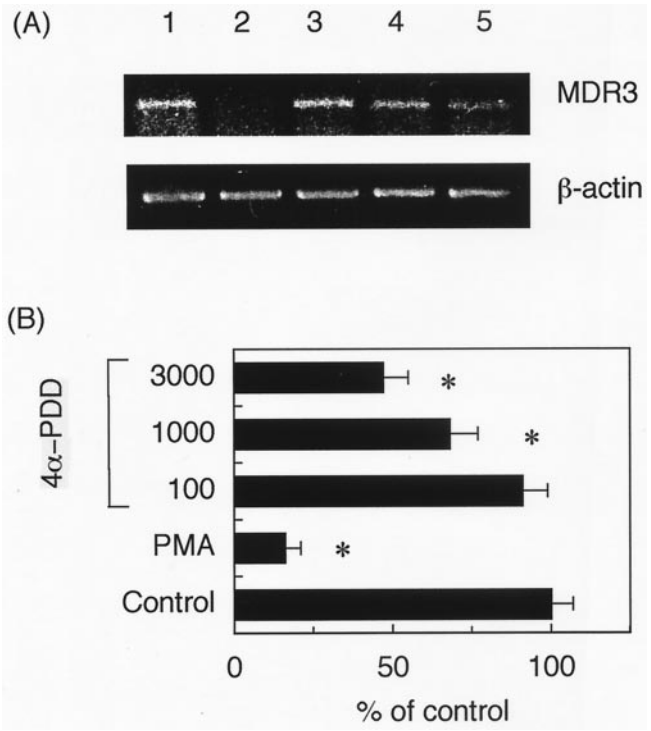

Fig. 4. Effect of 4α-PDD on MDR3 mRNA levels in Chang liver cells. (A) Cells were treated with 100 nM PMA or 0.1, 1, or 3 μM 4α-PDD for 20 hr. Lane 1, control; lane 2, 100 nM PMA; lane 3, 100 nM 4α-PDD; lane 4, 1 μM 4α-PDD; and lane 5, 3 μM 4α-PDD. Data are typical of one of three separate experiments. (B) The amount of MDR3 mRNA/β-actin mRNA in drug-treated cells relative to control is shown in a column graph. Data are averages ± SD (N = 3). Key: (*) $P < 0.05$, compared with the control.

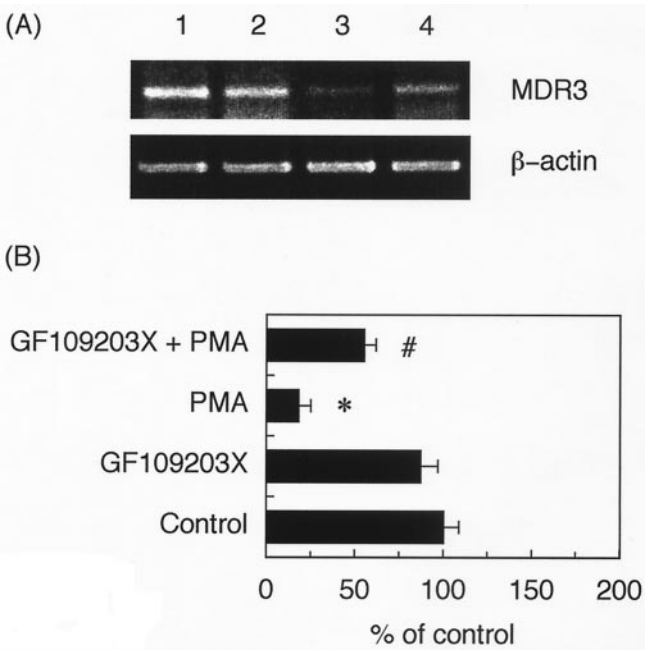

Fig. 5. Effects of GF109203X on the down-regulation of MDR3 mRNA expression by PMA in Chang liver cells. (A) Cells were treated with 100 nM PMA in the absence or presence of 100 nM GF109203X for 20 hr. Lane 1, control; lane 2, 100 nM GF109203X; lane 3, 100 nM PMA; and lane 4, 100 nM GF109203X and 100 nM PMA. Data are typical of one of three separate experiments. (B) The amount of MDR3 mRNA/β-actin mRNA in drug-treated cells relative to control is shown in a column graph. Data are averages ± SD (N = 3). Key: (*) $P < 0.05$, compared with the control; and (#) $P < 0.05$, compared with PMA alone.

3.2. Effects of a protein synthesis inhibitor on MDR3 mRNA levels

Although the down-regulation of MDR3 mRNA by PMA was indicated to be mediated by PKC, it was unclear whether the down-regulation was caused only by the phosphorylation of a regulatory protein. To investigate whether the down-regulation of MDR3 mRNA by PMA might also be modulated by the synthesis of some regulator protein, cells were preincubated in the absence or presence of 20 μg/mL of cycloheximide, a protein synthesis inhibitor, for 2 hr. Then the cells were incubated with 100 nM PMA in the presence of 20 μg/mL of cycloheximide for 8 hr. As shown in Fig. 7, the down-regulation of MDR3 mRNA was inhibited by cycloheximide. This suggested that the down-regulation of MDR3 mRNA by PMA is mediated through the synthesis of some regulator protein.

3.3. Down-regulation by PMA of MDR3 mRNA levels in doxorubicin-treated cells

MDR1 mRNA expression has been shown to be increased by doxorubicin treatment in a concentration-dependent manner. There have been no previous reports on the effects of doxorubicin and other anthracyclines on MDR3 mRNA levels. Hence, the effects of 0.1 and 1.0 μM doxorubicin on MDR3 mRNA expression were studied, as shown in Fig. 8. MDR3 mRNA was increased by treatment with these concentrations of doxorubicin. Thus, it was shown that MDR3 mRNA, like MDR1, was inducible by doxorubicin. Based on this observation, we investigated whether PMA suppressed the expression of MDR3 mRNA induced by doxorubicin. As shown in Fig. 9, in the presence of PMA, the expression of MDR3 mRNA was down-regulated in the doxorubicin-treated cells as well as in the control cells.

4. Discussion

The *MDR3* gene encodes a phospholipid translocator localized on the canalicular membrane of hepatocytes and some other tissues. Although several other transporter proteins are located on the canalicular membrane, MDR3 appears to have a role that cannot be compensated for by others because mutation of the *MDR3* gene is related to genetic cholestasis, with a poor prognosis [2–4].

We showed that human Chang liver cells expressed MDR3 mRNA following treatment with doxorubicin (Fig. 9), and that treatment of the cells with PMA resulted in a

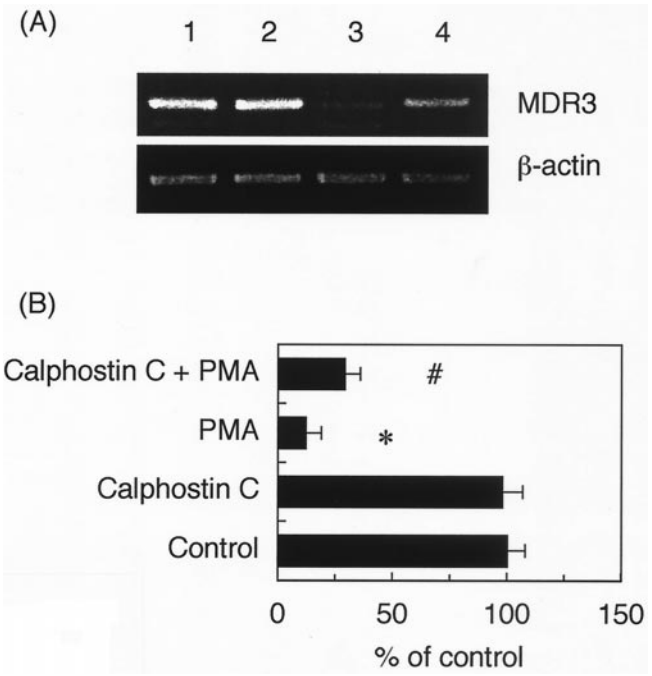

Fig. 6. Effects of calphostin C on the down-regulation of MDR3 mRNA expression by PMA in Chang liver cells. (A) Cells were incubated for 30 min under fluorescent light in the absence or presence of 10 nM calphostin C and then treated with or without 100 nM PMA for 20 hr. Lane 1, control; lane 2, 10 nM calphostin C; lane 3, 100 nM PMA; and lane 4, 10 nM calphostin C and 100 nM PMA. Data are typical of one of three separate experiments. (B) The amount of MDR3 mRNA/β-actin mRNA in the drug-treated cells relative to control is shown in a column graph. Data are averages ± SD (N = 3). Key: (*) $P < 0.05$, compared with the control; and (#) $P < 0.05$, compared with PMA alone.

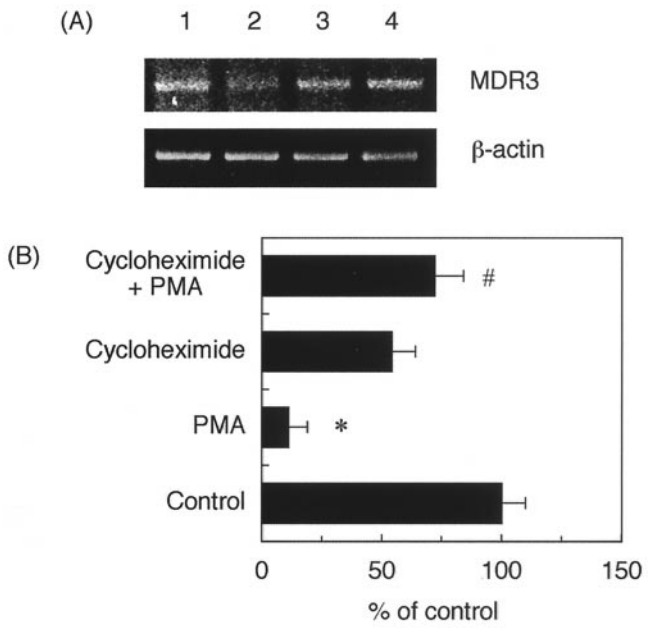

Fig. 7. Effect of PMA on MDR3 mRNA levels in Chang liver cells treated with cycloheximide. (A) Lane 1, control; lane 2, cells treated with 100 nM PMA for 8 hr; lane 3, cells incubated with 20 μg/mL of cycloheximide for 10 hr; and lane 4, cells incubated with 20 μg/mL of cycloheximide for 2 hr and then with 100 nM PMA in the presence of 20 μg/mL of cycloheximide for 8 hr. Data are typical of one of three separate experiments. (B) The amount of MDR3 mRNA/β-actin mRNA in drug-treated cells relative to control is shown in a column graph. Data are averages ± SD (N = 3). Key: (*) $P < 0.05$, compared with the control; and (#) $P < 0.05$, compared with cycloheximide alone.

down-regulation of the mRNA level (Figs. 1–7 and 9). We also showed that 4α-PDD, a negative control compound, was much less active than PMA (Fig. 4), and that the down-regulation by PMA was partially reversed by treatment with selective PKC inhibitors [GF109203X (Fig. 5) and calphostin C (Fig. 6)] and a protein synthesis inhibitor [cycloheximide (Fig. 7)]. Thus, the down-regulation might be mediated via protein phosphorylation by PKC and also by the synthesis of some regulator protein. Although PKC can be down-regulated by long-term treatment with phorbol ester [26–28], the down-regulation of *MDR3* gene expression appeared to depend on the activation of PKC because reduction in the level of MDR3 mRNA caused by PMA was prevented by two PKC inhibitors (Figs. 5 and 6).

While expression of MDR1 mRNA was increased by treatment with either PMA or doxorubicin, expression of MDR3 was induced by doxorubicin and suppressed by PMA. The varied responses of *MDR1* and *MDR3* genes to PMA and doxorubicin suggest that their expression is regulated by diverse pathways and that these agents affect gene expression through different mechanisms. They also indicate that although PMA acts through PKC, as shown in the present study, doxorubicin does not. Recently, Ziemann *et al.* [29] reported that MDR1 overexpression induced by doxorubicin is associated with the generation of reactive oxygen species (ROS) and is markedly suppressed by antioxidants, such as ascorbate, in primary rat hepatocyte cultures. Thus, the induction of *MDR3* gene expression by doxorubicin may also be due to the production of ROS.

The regulatory sequences of the *MDR3* gene, the promoter and enhancer regions, have been analyzed by Borst and co-workers [11] and in other studies [12,13]. Consensus sequences of the AP-1 site, the TPA responsive element (TRE) and the AP-2 site, and several other putative transcription factor binding sites including the Sp1 site were detected in this region. Among these elements, the Sp1 site has been considered a promoter of stable expression of the *MDR3* gene [11–13]. In contrast to the expression of the *MDR1* gene [30], the expression of the *MDR3* gene was down-regulated by PMA. The PKC-mediated pathway may contribute to the transcriptional regulation of *MDR3* via a mode of regulation different from that of *MDR1*.

A case of PKC-mediated down-regulation of gene expression by the m2 muscarinic receptor was reported in human embryonic lung cells. In that case, co-incubation with 4β-phorbol dibutyrate (PDBu) and the protein synthesis inhibitor cycloheximide inhibited the PDBu-mediated reduction of m2 receptor mRNA, whereas the stability of the mRNA was not altered by PDBu treatment [31]. In the case of β_1-adrenergic receptor gene expression in rat C6 glioma cells, a similar phenomenon was observed [32].

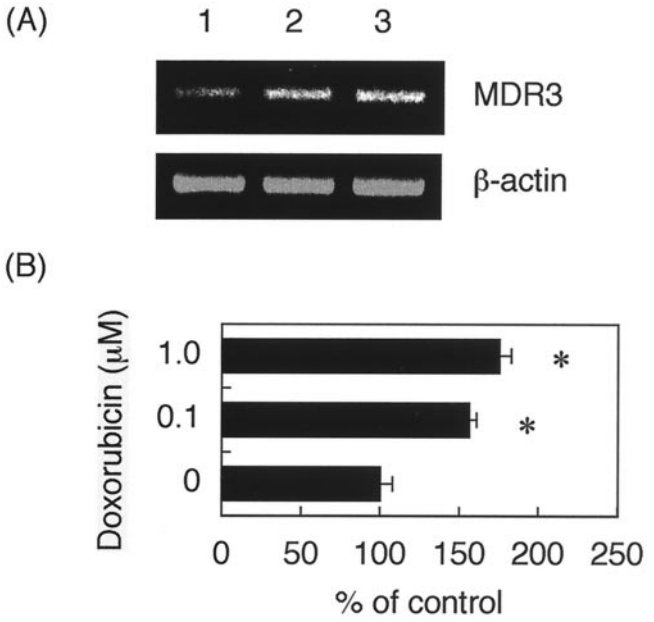

Fig. 8. Induction of MDR3 mRNA expression by doxorubicin in Chang liver cells. (A) Cells were treated with 0.1 or 1 μM doxorubicin for 20 hr. Lane 1, control; lane 2, 0.1 μM doxorubicin; and lane 3, 1 μM doxorubicin. Data are typical of one of three separate experiments. (B) The amount of MDR3 mRNA/β-actin mRNA in doxorubicin-treated cells relative to control is shown in a column graph. Data are averages ± SD (N = 3). Key: (*) $P < 0.05$, compared with the control.

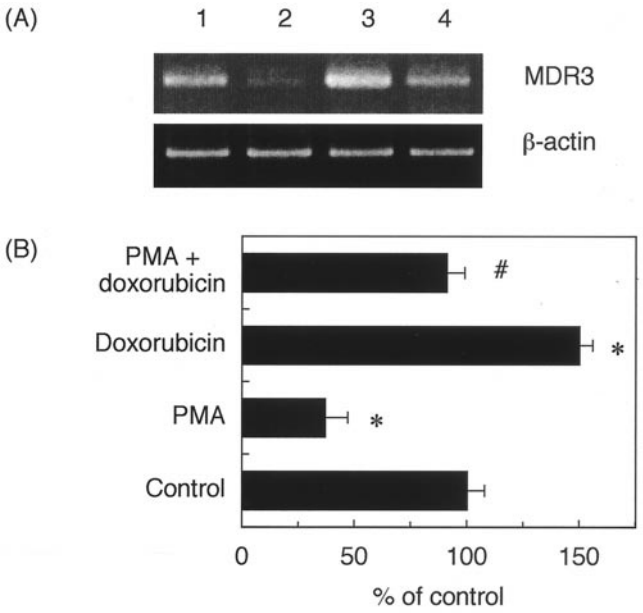

Fig. 9. Inhibitory effect of PMA on the action of doxorubicin in Chang liver cells. (A) Cells were treated with 100 nM PMA in the absence or presence of 1 μM doxorubicin for 20 hr. Lane 1, control; lane 2, 100 nM PMA; lane 3, 1 μM doxorubicin; and lane 4, 100 nM PMA and 1 μM doxorubicin. Data are typical of one of three separate experiments. (B) The amount of MDR3 mRNA/β-actin mRNA in drug-treated cells relative to control is shown in a column graph. Data are averages ± SD (N = 3). Key: (*) $P < 0.05$, compared with the control; and (#) $P < 0.05$, compared with doxorubicin alone.

Thus, in the regulatory systems for other genes, an induction of repressors is proposed to be the mechanism of down-regulation by PKC. With these mechanisms, down-regulation of the gene expression is thought to require both the transcription of some genes and the synthesis of a regulator protein. Thus, the repressor-mediated down-regulation is suggested to start after a latent period during which time the repressor or other regulator protein is synthesized and/or processed. However, it was shown that PKC-dependent phosphorylation of the p97 repressor regulated the transcription of the aldolase A L-type promoter [33]. In this case, the binding of a repressor protein to the repressor element was inhibited by PKC-dependent phosphorylation and resulted in the up-regulation of transcription. In the present study, as already mentioned above, MDR3 mRNA expression was not affected by treatment with cycloheximide alone but the PMA-induced down-regulation of this mRNA was inhibited by selective PKC inhibitors [GF109203X (Fig. 5) and calphostin C (Fig. 6)] and by cycloheximide (Fig. 7). Thus, the transcription of the *MDR3* gene may be negatively regulated by PKC through the synthesis of some regulator protein as is the case for the m2 muscarinic receptor and β_1-adrenergic receptors. The PKC subtype involved in the down-regulation of the *MDR3* gene, however, remains unknown.

It has been reported that PKC activity can be stimulated with insulin and neurotransmitters such as histamine and serotonin [34–36]. Based upon our present findings, that doxorubicin up-regulates MDR3 mRNA levels, it can be hypothesized that some therapeutic agents and hormones may affect MDR3 mRNA expression, bile formation, and the excretion or entero-hepatic circulation of drugs.

Acknowledgments

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