Metal-induced metallothionein gene expression can be inactivated by protein kinase C inhibitor

Chih-Wen Yu, Je-Hsin Chen, Lih-Yuan Lin*

Institute of Radiation Biology, National Tsing Hua University, Hsinchu Taiwan, ROC

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Abstract The effects of protein kinase C (PKC) inhibitors on the metallothionein (MT) gene expression induced by metals were investigated. When PKC inhibitor (H7 or chelerythrine) was administered to Cd resistant, MT gene-amplified Chinese hamster ovary (Cd^R) cells, the induction of MT mRNA by Cd or Zn was blocked. Treating the Cd^R cells with a PKA-specific inhibitor, HA1004, did not cause an inhibition of metal-induced MT gene transcription. The inhibitory effect was effectuated by adding inhibitors within 40 min of exposing the cells to Cd. Apparently, AP1 was not involved in this down-regulatory effect of PKC inhibitor on MT gene expression since the inducibility of MT promoter was blocked by H7 even in the absence of the AP1-binding sequence. For Cd-treated cells, Cd accumulation in the cell was similar with or without H7 treatment. However, H7 markedly reduced cellular Zn accumulation when the cells were treated with Zn. Cycloheximide treatment increased the level of MT mRNA. This elevation can also be blocked by treating the cell with PKC inhibitor. Results in this study suggest that PKC participates in the process of metal-induced MT gene expression.

Key words: Metallothionein; Protein kinase C; Gene expression; Cadmium; Zinc

1. Introduction

Metallothioneins (MTs) are sulfur-rich, metal-binding proteins that exist ubiquitously in organisms [1]. Four families of MTs have been identified. MTI and MTII are inducible proteins and implicated in metal detoxification [2,3]. MTIII is a brain-specific, non-inducible protein identified in Alzheimer's disease-related studies [4,5]. The unique biological function of MTIII is its ability to inhibit neuronal growth. MTIV is a family of MTs that is present in squamous epithelium cells. Although it has been proposed that MTIV is involved in maintaining Zn homeostasis [6], its exact function remains unclear. MTIII and MTIV have been isolated from mammalian tissues; whether these proteins can be found in other species remains unknown.

The expression of MTI and MTII genes is regulated primarily at the transcriptional level [7,8]. Several homologous repeats, i.e. metal responsive elements (MREs), have been identified in the 5′-flanking region of MT genes. They can only be activated upon metal stimulation [9,10]. Binding of a transcriptional factor (MTF-1) to this region is needed for the basal and metal-induced activities of MT promoters in mammalian cells [11,12]. MTF-1 contains a zinc finger domain that is responsible for binding with MREs. MTF-1 is only active when Zn ions are present in the zinc finger domain. This protein also has an acidic, a proline-rich and a serine/threonine-rich domain that are involved in transcription activation [11,13]. It has been proposed that MTF-1 serves as a metal sensor and modulates the activity of the MT promoter in response to changes in cellular Zn ion concentration [14]. However, a recent study indicates that an inhibitor might be present and bind with MTF-1 [15]. The inhibitor is dissociated from MTF-1 after Zn binding.

For other inducers, different control mechanisms are involved. Among them, protein kinase C (PKC) plays a prominent role in the transcriptional regulation. For instance, heme is a unique compound that can activate the transcription of the MT genes [16]. The induction mechanism of heme-hemopexin has been demonstrated to be associated with the action of PKC [17]. In addition, a PKC activator, phorbol ester, is also known to be an inducer of MT [18,19]. These findings indicate that the signal transduction pathway of PKC is critical in the transcriptional regulation of MT genes for several chemicals. In this work, we demonstrated that inactivation of PKC markedly affects the metal-induced MT gene expression.

2. Materials and methods

2.1. Cell culture

The CHO K1 and Cd^R cells were cultured as monolayers at 37°C in McCoy's 5A medium supplemented with 10% fetal bovine serum, 0.22% sodium bicarbonate, 100 units/ml penicillin and 100 μg/ml streptomycin, in an atmosphere of 5% CO_2. Before transfection, the cells were cultured at a density of 5×10^5 cells/60 mm petri dish for 18 h.

2.2. Plasmid construction, DNA transfection and CAT assay

The 5′-flanking region of CHO MTII gene (−925 to +62 relative to the CAP site; see accession number M61714) was blunt-end ligated with a pCAT-Basic (Promega, Madison, WI) plasmid that had been digested at the *Xba*I site and filled in by Klenow enzyme. The recombinant DNA was digested with *Hin*dIII to yield pMTII-253 [20]. The plasmid pMTII-142 was obtained by digesting pMTII-253 with *Pst*I and *Sal*I, followed by a nested deletion with a ExoIII/mung bean nuclease kit (Stratagene). Transfections of the plasmids were conducted by the cationic liposome method. The liposomes were prepared by the procedures of Rose et al. [21]. Five μg of promoter-chloramphenicol acetyltransferase (CAT) gene construct was cotransfected with 5 μg pSV-β-galactosidase plasmid as an internal control into the cells in each experiment so that the transfection efficiency could be adjusted. Optimal transfection efficiency was obtained when 30 μl of liposome was used. Sixteen h after transfection, the medium was replaced by a fresh medium. The cells were harvested 24 h after medium replacement, and chemicals were added to the medium 10 h before cell harvest. The cells were removed by a rubber policeman and lysed by three successive cycles of freezing and thawing in liquid nitrogen and a 37°C water bath. Cell debris were removed by centrifugation, and the β-galactosidase activity in the supernatant was

*Corresponding author. Fax: 886-35-719744.
E-mail: lylin@rb.nthu.edu.tw

Abbreviations: CAT, chloramphenicol acetyltransferase; MT, metallothionein; PKC, protein kinase C

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determined. Cell extracts (30 μl) were heated at 60°C for 10 min, and 0.25 M Tris-HCl (pH 7.8) was added to make the final volume of 100 μl. One μl of [^{3}H]acetyl CoA (0.25 μCi/μl), 2 μl of 125 mM chloramphenicol and 147 μl of H_2O were then mixed with the sample, followed by 3 ml of scintillation fluid (Econofluor-2, Du Pont). Radioactivity in the sample was determined at various times thereafter by a liquid scintillation analyzer (Packard Instrument Comp., Model 1600 CA). Radioactivity at five different time intervals was plotted against the reaction time. A linear line can be obtained after regression. The slope of the line was used to determine CAT concentration using a standard curve generated with known amount of CAT.

2.3. RNA extraction and Northern blot analysis

Cells were washed twice with chilled PBS, and 1 ml of the same buffer was added to the petri dish. Cells were then removed using a rubber policeman and collected into 1.5 ml microcentrifuge tubes. Following centrifugation at 2000 rpm for 5 min at 4°C, cell pellets were resuspended in 0.5 ml of NTE (100 mM NaCl, 10 mM Tris-Cl, pH 7.5, 1 mM EDTA). After adding 25 μl of Nonidet P-40 and standing on ice for 10 min, the samples were spun at 12000 rpm for 5 min in an Eppendorf microcentrifuge. Supernatants were then transferred to new tubes and 50 μl of 10% SDS was added. The supernatants were successively extracted with 0.6 ml of water saturated phenol and a phenol/chloroform/isoamyl alcohol (25:24:1) solution. RNA in the supernatant was precipitated with 1 ml of ethanol at −70°C for 1 h. The RNA was pelleted by centrifugation at 12000 rpm for 10 min, and resuspended in diethylpyrocarbonate-treated water. Equal amount of RNA from each treatment was separated by electrophoresis on a 1.2% formaldehyde agarose gel and transferred onto a nitrocellulose membrane. Hybridization was conducted as described by Ausubel et al. [22] using Chinese hamster metallothionein-II cDNA [23] as probe. The 18S rRNA was used as an internal standard for normalization of the amount of RNA applied.

2.4. Others

Metal contents in the samples were determined as described [24]. *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide (HA1004), 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7), chelerythrine chloride and other chemicals were purchased from Sigma (St. Louis, MO). Modified and restriction enzymes were obtained from Promega (Madison, WI).

3. Results

3.1. Effect of PKC inhibitors on the expression of MT mRNA

To investigate whether PKC is involved in the regulation of metal-induced MT gene transcription, PKC inhibitor (H7) was applied to cadmium resistant CHO cells (Cd^R) 2 h before administering Cd. Comparing with the parental CHO K1, the Cd^R cells have higher tolerance to metal toxicity and their MT genes have been amplified 60- to 100-fold [25]. As indicated in Fig. 1A, Cd markedly induced the synthesis of MT mRNA. The MT mRNA expression was significantly reduced upon H7 administration. The MT mRNA level, as compared with samples without inhibitor treatment, reduced to 30.2 ± 4.6%, 24.2 ± 7.4%, 20.1 ± 6.5% and 15.1 ± 4.9% (mean ± standard deviation of three samples) when 50, 100, 200 and 300 μM of H7 was employed, respectively. This inhibitory effect is not cell-type specific since a similar result was obtained when rat thyroid cells (GH_3) were treated with 50 μM of H7 (unpublished result).

To further demonstrate that this inhibitory effect is due primarily to the inactivation of PKC, a more potent and selective PKC inhibitor, chelerythrine, was used. This inhibitor blocks PKC activity when present at the micromolar level, whereas more than 100-fold higher concentration is needed for exerting a similar effect on other protein kinases [26]. As Fig. 1B reveals, the induction of MT mRNA by metal was inhibited by chelerythrine. The ratio of MT gene expression after treating the cells with chelerythrine was 93.6 ± 14.2%, 82.6 ± 6.5%, 62.3 ± 11.4% and 18.8 ± 14.7% (mean ± standard

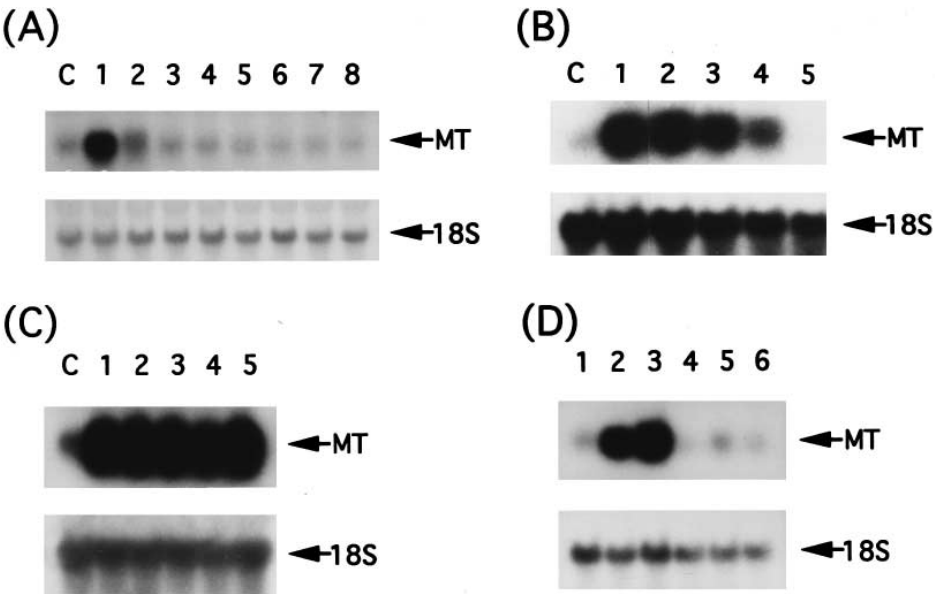

Fig. 1. Effect of protein kinase inhibitors on the MT mRNA expression induced by metals. Various protein kinase inhibitors were administered to the Cd^R cells 2 h before metal treatments. Cd (5 μM) or Zn (100 μM) was then given to the cells for an additional 10 h, and MT mRNAs were analyzed by Northern hybridization. The cells were treated with an increasing amount of H7 (A), chelerythrine (B) or HA1004 (C) before Cd induction. The dose of H7 used was 0, 50, 100, 150, 200, 300, 400 and 500 μM, as indicated by number 1 to 8, respectively. The dose of chelerythrine used was 0, 5, 7.5, 10 and 15 μM, as indicated by number 1 to 5, respectively. The HA1004 used was 0, 25, 50, 100 and 200 μM, as indicated by number 1 to 5, respectively. Control samples without adding Cd or protein kinase inhibitor are designated as C. The effect of H7 on MT mRNA expression induced by Zn is presented in panel D. The cells were treated with metal in the absence (lane 1 to 3) or presence (lane 4 to 6) of 200 μM H7. Lane 1 and 4: no metal added; lane 2 and 5: Cd added; lane 3 and 6: Zn added. The 18S rRNA (18S) was used as an internal standard for quantitation of RNA loading in each lane.

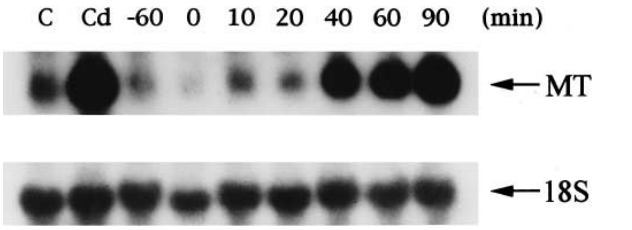

Fig. 2. A time course study of the inhibitory effect of H7 on the Cd-induced MT gene expression. The cells were treated with 5 μM Cd for 2 h and MT mRNA was analyzed by Northern hybridization. H7 (200 μM) was added either at 60 min before (−60), at the same time (0) or 10, 20, 40, 60 or 90 min after Cd administration. The 18S rRNA (18S) was used as an internal standard for RNA quantitation. C: control without Cd and H7; Cd: cells treated with Cd only.

deviation of three samples) of the control when 5, 7.5, 10 and 15 μM of chelerythrine was given, respectively. These findings clearly suggest that PKC is involved in the process of Cd-induced MT gene expression.

Since H7 is also a potent inhibitor of PKA [27], we further treated the cells with a PKA-specific inhibitor, HA1004, to investigate the role of PKA in the MT induction. As Fig. 1C reveals, this chemical has no effect on MT mRNA induction. This finding indicates that PKA is not involved in the MT gene expression induced by metal.

In addition to Cd, Zn is also a very effective inducer of MT genes. The cellular response to Zn was examined to determine whether the effect of PKC on MT induction is metal specific. Cells were treated with Zn and H7, and MT mRNA levels were analyzed. As shown in Fig. 1D, MT mRNA could also be induced by Zn, and H7 could similarly prevent this induction. The result demonstrates that PKC is also involved in the induction of MT gene expression by another potent metal inducer. We used H7 in the following studies due to the stability of the chemical. Although 50 μM of H7 can produce significant inhibitory effect, we used a higher concentration of the inhibitor to ensure that the PKC response could be easily identified.

A time course study was conducted on the effect of the PKC inhibitor on the MT gene expression. The H7 was given to the cells before or after Cd stimulation. The cells were harvested 2 h after Cd exposure, and MT mRNA was analyzed by Northern hybridization. As shown in Fig. 2, metal-induced MT mRNA synthesis can be blocked by H7 at several time intervals. Blockage of MT gene expression occurred not only when the inhibitor was given before the induction by metal, but also at 20 min after stimulation. However, the inhibition became less effective when H7 was given 40 min after Cd induction. The relative MT gene expression (amount of MT mRNA normalized to that of 18S rRNA) increased gradually and became similar to that of cells treated with Cd when H7 was provided 90 min after metal exposure.

Table 1
Effect of PKC inhibitor on cellular metal accumulation

Treatment	Zn (fmole/cell)	Cd (fmole/cell)
Control	1.19 ± 0.03	ND
Cd	1.38 ± 0.18	0.37 ± 0.07
Zn	4.81 ± 0.55	ND
H7	1.27 ± 0.19	ND
Cd+H7	1.14 ± 0.13	0.41 ± 0.13
Zn+H7	1.86 ± 0.05	ND

Cd^R cells were treated with metal in the presence or absence of H7 for 10 h, and cytosolic metal content was measured by atomic absorption spectrophotometry. The concentrations of Cd, Zn and H7 administered to the cells were 5, 100 and 200 μM, respectively. Each value represents a mean ± standard deviation of three samples. ND, not detectable.

3.2. *AP1 is not involved in the action of PKC on metal induction of MT*

Signal transduction via PKC pathway can modulate the expression or phosphorylation status of c-jun/c-fos, resulting in an elevation of AP1 activity [28,29]. The AP1 then further regulates the expression of several genes. Since AP1-binding sites have been identified in the CHO MT promoter regions, it has been speculated that AP1 might be involved in the regulation of MT gene expression via PKC [20,30]. To clarify such a conjecture, CHO MTII promoter with (pMTII-253) or without (pMTII-142) the AP1-binding site was fused with a chloramphenicol acetyltransferase (CAT) gene, and transfected into CHO K1 cells. The CAT activities were then compared for the metal-induced cells with or without H7 treatment. As shown in Fig. 3, the CAT activities increased as the cells carrying pMTII-253 and pMTII-142 promoters were challenged with metal. However, the activities dropped to the basal level when

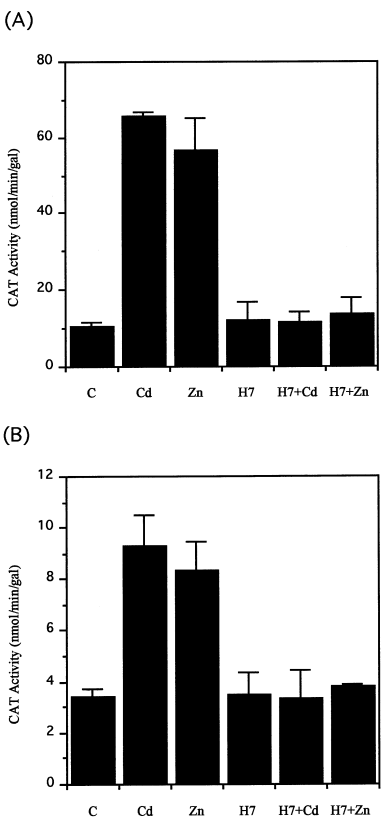

Fig. 3. Effect of H7 on the MT promoter activity. CHO MTII promoter with (pMTII-253, A) or without (pMTII-142, B) the AP1 recognition sequence was inserted in front of a CAT gene and transfected into CHO K1 cells. Ten h before harvest, cells were subjected to the indicated treatments. CAT activity was determined after cell lysis. The concentrations of Cd, Zn and H7 used in this experiment were 5, 100 and 200 μM, respectively. Each value represents a mean ± standard deviation of three samples.

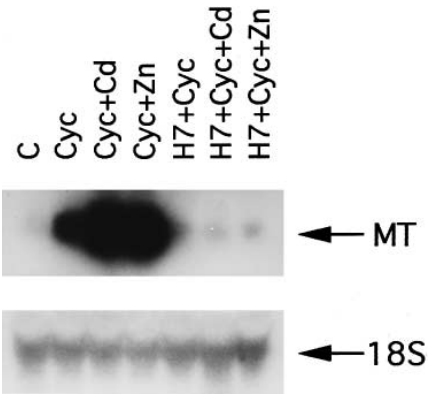

Fig. 4. Effect of protein synthesis inhibitor and protein kinase inhibitor on the expression of MT mRNA. CHO Cd^R cells were treated with cycloheximide (64 μM), Cd (5 μM) or Zn (100 μM) in the presence or absence of H7 (200 μM) for 10 h. H7 was added 2 h before the metal or cycloheximide treatment. The level of MT mRNAs was analyzed by Northern hybridization. C: control; Cyc: cycloheximide.

H7 was administered regardless of the presence or absence of the AP1-binding site in the promoter region. This result demonstrates not only that PKC regulates MT gene expression at the transcriptional level, but also that AP1 is not involved in the PKC action on the induction of MT by metal.

3.3. Effect of PKC inhibitor on the accumulation of metals in the cells

The inhibitory effect of MT gene expression after H7 administration could be due to the blocking of metal transport systems when cells are treated with metal. To address this issue, the accumulations of metals in the CHO Cd^R cells were examined after metal and H7 treatments. Table 1 reveals that H7 did not inhibit Cd accumulation. The zinc content was also not significantly altered when cells were treated with either Cd, H7 or both. However, in contrast to the Cd treatment, Zn accumulation was dramatically reduced in cells treated with H7 and Zn, as compared to cells treated with Zn only. Approximately 3 femtomoles of Zn per cell were lost upon H7 treatment.

3.4. Possible target for the PKC action in MT induction

A model has been proposed by Palmiter [15] in which MTF-1 is usually bound with an inhibitor. Upon metal induction, the inhibitor is released and the MTF-1 becomes effective [15]. Since the involvement of PKC in the MT gene transcription has been demonstrated in this study, the ultimate regulatory site for the PKC action might be at either the putative inhibitor or the MTF-1. To address this issue, cells were treated with cycloheximide. The synthesis of the MTF-1 inhibitor is considered to be cycloheximide sensitive [15,31]. As Fig. 4 indicates, MT mRNA in Cd^R cells was elevated in the presence of cycloheximide. This finding agrees with the proposed mechanism that a labile factor is present in the cells to modulate MT gene expression. The addition of cycloheximide with Cd or Zn to the cells further stimulates the synthesis of MT mRNA. However, H7 blocked the elevation of MT mRNA in the cycloheximide-treated cells regardless of the presence or absence of metal. Results in this study imply that the ultimate target of PKC action is more likely MTF-1 rather than labile factor.

4. Discussion

As generally accepted, induction of MT gene expression in mammalian cells is attributed to the interactions among Zn ion, MTF-1 and MREs. When cells are exposed to metal (e.g. Zn or Cd), the metal (i.e. Zn) either directly activates MTF-1, or the metal (i.e. Cd) replaces Zn from cellular components and allows the free Zn to activate the transcriptional factor, MTF-1. The MTF-1 can then bind MREs and activate the MT gene transcription [15]. In the work reported here, we showed that PKC is also involved in the process of MT gene induction by metals. The participation of PKC in MT gene regulation has been demonstrated in the stimulation of MT gene expression by several chemicals. For instance, TPA, which is a PKC activator, is also an inducer of MT [18,19]. Calcium ionophore, which perturbs cytosolic Ca concentration and results in an activation of PKC, increases the level of MT mRNA [32,33]. A recent investigation confirmed that induction of MT genes by heme-hemopexin is modulated by PKC [17]. However, metal induction of MT gene expression via the regulation of PKC has not been reported. By using PKC inhibitors, MT induction can be eliminated. This effect is further demonstrated to be regulated at the transcriptional level (Fig. 3). This regulation is not due to the action of AP1, since MT expression can be blocked by the PKC inhibitor in the absence of the AP1-binding site at the promoter region (Fig. 3).

Smith et al. [34] reported that cadmium can evoke inositol polyphosphate formation and calcium mobilization via a putative 'Cd receptor'. Signal transduction by those secondary messengers can result in the activation of PKC. The MT induction by Cd is therefore speculated to be controlled by this mechanism. However, an antagonist of the pathway and also a potent MT inducer, Zn, can not exert its inducibility of MT mRNA in the presence of H7. If a common mechanism is shared in the induction of MT by Zn or Cd, the secondary messengers evoked by Cd certainly do not play a role in the activation of MT gene transcription for both metals. Either a different common mechanism that is not mediated by 'Cd receptor', or possibly two separate mechanisms may exist for MT induction by Cd and Zn.

When metal accumulation was examined, the H7 treatment did not affect Cd concentration (Table 1). This finding suggests that a lack of MT mRNA induction is not due to the blocking of Cd transport. However, Zn concentration was significantly reduced with the co-treatment of H7 and Zn. The results demonstrate that Cd and Zn do not share the same route for transport since Cd content is similar for either the presence or absence of H7. If the reduction of MT mRNA by H7 is due to the blocking of Zn transport during Zn stimulation, the roles of the PKC inhibitor on MT induction pathway should be expected to be different for Cd and Zn. Nevertheless, it can not be ruled out that, in addition to Zn transport, the induction of MT by Zn is also related to the PKC activity.

Although MTF-1 is recognized to be a Zn sensor to modulate the activity of MT promoter, another MT regulatory mechanism has been proposed. Palmiter has indicated that MTF-1 is usually bound with an inhibitor when cells are not stimulated by metals [15]. Upon induction by metal, the inhibitor can be released by Zn and consequently causes the activation of the MTF-1. Therefore, whether the ultimate tar-

get of PKC is on the MTF-1 or the putative inhibitor merits investigation. An increase in MT mRNA by cycloheximide treatment (Fig. 4) indicates that reduction in the synthesis of a labile factor (inhibitor) in Cd^R cells allows active MTF-1 to bind MREs and stimulates the expression of MT genes. If the target of PKC is on the labile factor, H7 should not change the MT mRNA level in the cycloheximide-treated cells because the labile factor is absent. However, a decrease in MT mRNA synthesis after administration of both H7 and cycloheximide was observed (Fig. 4). This result implies that the ultimate action of PKC is more likely on the MTF-1 but not on the inhibitor.

Although our results do not directly demonstrate that phosphorylation occurs at the MTF-1, there is evidence that may support this possibility. For example, a recent study using mobility shift assays indicates that nuclear extracts prepared from Zn-treated cells have higher MTF-1-binding activities than those from uninduced cells [14]. This situation remains the same when saturating amounts of Zn ions were added to the in vitro binding reaction. Modification (e.g. phosphorylation) of the MTF-1 to modulate the transcriptional competence is one of the conceivable explanations [35]. When examining the MTF-1 amino acid sequence, several consensus PKC phosphorylation sites [36] at the Zn finger domain can be identified [11,12]. In addition, a serine/threonine-rich domain at the C-terminal of MTF-1 is also a region where phosphorylation can occur. Therefore, activation of MTF-1 by phosphorylation via a PKC-dependent pathway is possible in the process of metal-induced MT gene expression.

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