Evaluating the role of dibutyl-cAMP and Ca^{++} as an MT inducer in a clonal rat hepatoma cell

M.S. Yang, K.L. Wan and Dengyun Chen

Department of Biology, Hong Kong Baptist University, Kowloon, Hong Kong

Metallothioneins (MT) is a low molecular weight, cysteine-rich, metal-binding protein that can be induced when cells and tissues are exposed to metal such as Cd, Zn, Cu and Hg. MT could also be induced by non-metallic stimuli including physiological, chemical and pathological stresses. It has been suggested that signalling molecules such as cAMP or Ca^{++} might also induce MT. In the present study, the mode of induction of MT in the RH-35 cells was investigated with reference to the integrity of the cells. The degree of MT induction was measured by the Cd-saturation assay in which total Cd bound to the heat-stable MT was determined by the graphite furnace atomic absorption spectrometry. It was demonstrated that Zn and Cd could markedly increase cellular MT content. Alpha-amanitin, which blocks DNA transcription, can significantly decrease the Cd induced MT induction. MT induction was also lowered by cycloheximide, which blocks protein translation. However, dibutyl-cAMP, Ca^{++} and Ca^{++} in the presence of ionomycin were unable to induce cellular MT. Using this model, the results supported that notion that MT induction might not be mediated by cAMP or Ca^{++} in this cell line.

Introduction

Metallothionein (MT) is a low molecular weight (~ 7000 Da), cysteine rich (1/3 of the 61 amino acid composition), heat-stable, metal binding protein. Under normal condition, cellular MT content is low. The protein level is increased when cells are exposed to metals such as Cd, Hg, Zn or Cu. Thus, the protein has been considered a biochemical marker for metal exposure. However, studies indicated that MT could also be induced by a number of physiological stimuli, chemicals and pathological stresses [1,2]. Thus, in addition to metals, a number of factors, such as signalling molecules, were said to induce MT. To evaluate the relative importance of the different molecules in regulating MT induction, it is necessary to determine their induction potency. In a previous study, we explored the use of a clonal cell line in assessing the potency of nutritional elements in induce MT [3]. In the present study, regulation of MT induction was investigated by using chemicals that either block the transcriptional or translational process, or simulated the action of second messengers.

Materials and methods

Preparation of cells

The RH-35 cell is a clonal hepatoma cell line [4]. It possesses the MT gene that is inducible by Cd, Zn and Cu [5]. In a previous study [3], the cell line was used to study MT induction by various nutrients. In the present study, the cells were maintained in the same ways as that described previously [3]. Cellular MT content was measured after a 2-day treatment with medium containing a) $ZnCl_2$ (0, 4, 8, 12 or 16 mg/L), b) $CdCl_2$ (0, 0.18, 0.36 or 0.9 mg/L), c) α-amanitin (0.02, 0.1 or 0.5 mg/L) with and without 0.9 mg/L $CdCl_2$; d) cycloheximide (0, 0.1, 0.5, 1, 5 and 10 mg/L) for 2 h before addition of 0.9 mg/L $CdCl_2$, e) 100 µM dBcAMP both in the presence or absence of α-amanitin; and f) 2 mM $CaCl_2$ in the presence of ionomycin (0, 1.0 or 1.5 µM). In all cases, cell number, cell viability and total cellular protein content were measured [3]. Heat-stable protein for quantifying cellular MT content was prepared by heating the total cellular protein to 85 °C for 5 min as that described previously [3].

Determination of MT

MT content was determined by a modified Cd-binding assay [6]. Briefly, an aliquot of different volume of the heat-stable protein was added to 10 mM Tris-HCl, pH 7.4 to make up to a volume of 200 µl. $CdCl_2$ was added to obtain a final concentration of 2 µg/ml. After mixing thoroughly, 100 µl of 2 % bovine hemoglobin solution (Type II, sigma Chemical Co., St. Louis, Mo. USA) was added. The solution was heated at 100 °C for 2 min and cooled rapidly. This procedure was repeated again. The precipitated hemoglobin, to which free Cd bound, was removed by centrifugation. The MT bound Cd in the supernatant was measured by graphite furnace atomic absorption spectrometry (GFAAS) at a wavelength of 228.8 nm. To avoid contamination, all solutions were made with double-distilled water (Millipore). Plastic ware was used whenever possible. All glassware and plastic

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ware were soaked in 5 % nitric acid overnight and washed with double-distilled water prior to use. The procedure was able to reduce metal contamination adequately to give a good signal-to-noise ratio. Furthermore, the method was examined in our laboratory and showed that after the procedure, Cd was the only metal bound to the protein [7]. Calculation of tissue MT content was based on the equation described in [6] assuming that the molecular weight of MT is 7000 and each mole of MT binds to 7 moles of Cd. All studies were carried out in triplicates. Differences between values were evaluated by ANOVA followed by the Duncan's Multiple Range Test. Significant difference between groups was set at $p < 0.05$.

Results and discussion

Table I shows the cell number, cell viability and cellular protein content when cells were treated with different chemicals. Cell number, which indicates the condition of cell growth, were unchanged in most cases except when cells were treated with 0.5 mg/L α-amanitin and with cycloheximide above 1 mg/L. At these concentrations, cell death was also detected. Change was also observed in cellular protein content. Zn, at concentrations above 8 mg/L caused a significant increase in cellular protein content. Cd at 0.18 mg/L, also caused a significant increase in cellular protein content but protein content decreased when Cd reached 0.9 mg/L. $CaCl_2$ both in the presence or absence of ionomycin, α-amanitin and cycloheximide in the presence of Cd, caused significant decrease in cellular protein content.

Figure 1 shows MT content after treatment with Zn and Cd. Both Zn and Cd caused a concentration-dependent increase in cellular MT content. MT content increased from the control values of 0.03 pg/cell to 0.8 and 0.6 pg/cell after treatment with 16 mg/L Zn and 0.9 mg/L Cd, respectively. Cellular MT content reported in this study was much lowered when compared to that reported previously [3]. It has been well established that although the values obtained by the polarographic method were slightly higher than those by the Cd-saturation assay, the MT content measured by the two methods were proportional to the amount of MT analyzed [8]. Studies in our laboratory also demonstrated that using the same sample, the MT content measured by the polarographic method was higher than that measured by the Cd-binding assay. However, there was a good correlation between the different concentrations of MT measured by the two methods (unpublished results). The results presented in figure 1 demonstrated that using the Cd-binding assay, Cd is shown to be a stronger MT inducer than Zn, and that it took 12 mg/L Zn in order to increase MT to a level as high as that with 0.36 mg/L Cd.

Figure 2 shows the effect of α-amanitin on Cd induced MT induction. Alpha-amanitin alone was unable to cause change in cellular MT content but it could suppress Cd induced MT at concentrations of 0.1 mg/L. The result is consistent with the notion that the action of Cd is at the level of gene transcription. At 0.5 mg/L, the action of α-amanitin was not apparent. However, the result is difficult to interpret since it is confound by a significant decrease in cell number and increase in cell death.

Figure 3 shows that cycloheximide at concentrations between 0.1 to 10 mg/L could also suppress the Cd-induced MT induction. The change was accompanied by a decrease in both cell number and cellular protein content with significant cell death seen only at 5 and 10 mg/L (Tab. I). In reference to the change in total cellular protein content,

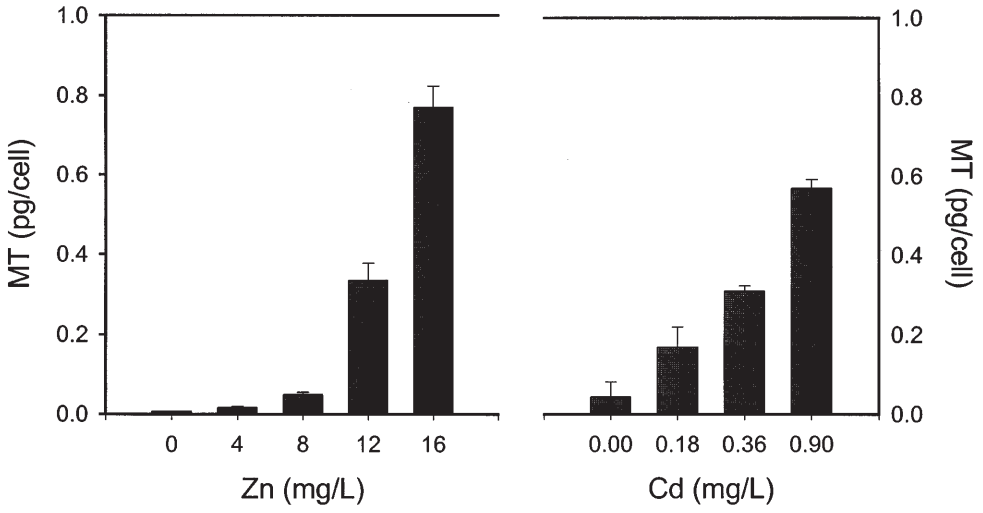

Figure 1. Induction of MT by different concentrations of $ZnCl_2$ and $CdCl_2$.

Table I. Cell number, cell viability and cellular protein content in the RH-35 cells after different treatments.

Incubation medium	*Cell number ($\times 10^6$ cells ± SD)*	*Cell viability % ± SD*	*Cellular protein content (pg/cell ± SD)*
a) Medium alone	8.40 ± 0.82	99.20 ± 0.58	100.09 ± 5.39
b) Effect of metals			
+$ZnCl_2$			
4 mg/L	8.82 ± 0.12	95.67 ± 0.32	111.87 ± 10.74
8mg/L	8.60 ± 0.63	96.25 ± 1.85	122.86 ± 4.06*
12 mg/L	7.95 ± 0.32	96.75 ± 0.79	129.49 ± 3.72*
16 mg/L	7.99 ± 0.20	95.46 ± 1.84	140.47 ± 10.52*
+ $CdCl_2$			
0.18 mg/L	8.19 ± 0.36	95.62 ± 0.72	120.37 ± 5.03*
0.36 mg/L	9.23 ± 0.30	95.62 ± 0.72	103.30 ± 10.04
0.90 mg/L	9.37 ± 0.67	95.10 ± 2.92	86.82 ± 8.37*
c) Effect of α-amanitin			
0.02 mg/L	8.20 ± 0.14	96.54 ± 0.60	97.41 ± 8.04
0.1 mg/L	8.43 ± 0.35	97.62 ± 1.55	93.19 ± 10.04
0.5 mg/L	6.81 ± 0.24*	83.34 ± 1.21*	95.00 ± 11.08
With 0.9 mg/L $CdCl_2$			
0.02 mg/L	9.01 ± 0.56	93.15 ± 1.05	77.17 ± 6.09#
0.1 mg/L	9.48 ± 0.72	93.71 ± 1.51	76.55 ± 1.45#
0.5 mg/L	6.55 ± 0.30*	83.73 ± 1.57*	75.56 ± 6.29#
d) Effect of different concentrations of cycloheximide with 0.9 mg/L $CdCl_2$			
0.1 mg/L	8.43 ± 0.21	98.75 ± 0.21	72.08 ± 4.56#
0.5 mg/L	8.35 ± 0.25	94.41 ± 1.51	72.01 ± 4.56#
1 mg/L	7.33 ± 0.16*	95.72 ± 1.99	69.86 ± 1.85#
5 mg/L	5.40 ± 0.12*	91.71 ± 1.94*	65.02 ± 4.31#
10 mg/L	5.08 ± 0.44*	88.51 ± 1.98*	57.43 ± 6.24#
e) Effect of dBcAMP (100 μM)			
dBcAMP only	9.17 ± 0.53	98.63 ± 0.99	99.44 ± 2.02
with 0.1 mg/L α-amanitin	8.73 ± 0.56	94.68 ± 0.43	100.09 ± 5.54
f) Effect of Ca++			
$CaCl_2$ (2 mM)	8.83 ± 0.45	97.50 ± 1.10	81.84 ± 4.80*
with 1 mM ionomycin	7.94 ± 0.74	97.36 ± 1.60	78.85 ± 1.71*
with 1.5 μM ionomycin	8.41 ± 0.12	97.69 ± 1.11	80.14 ± 1.11*

* indicates that value is significantly different from that treated with MEM only.
# indicates that the value is significantly different from that treated with 0.9 mg/L $CdCl_2$.

cellular MT level change from a control value of 6.5 μg MT/mg protein to 5.4, 2.2 and 0.9 μg MT/mg protein upon treatment with 0.1, 1 and 10 mg/L cycloheximide, respectively. Figure 4 shows that neither 100 μM dBcAMP nor 2 mM $CaCl_2$ in the medium was able to change cellular MT content significantly. In the presence of ionomycin (1 and 1.5 μM), Ca^{++} was able to reduce cellular MT content significantly.

MT induction in response to physiological stress might be mediated through the action of Ca^{++} and cAMP. Data concerning *in vitro* studies on identifying the role of cAMP and

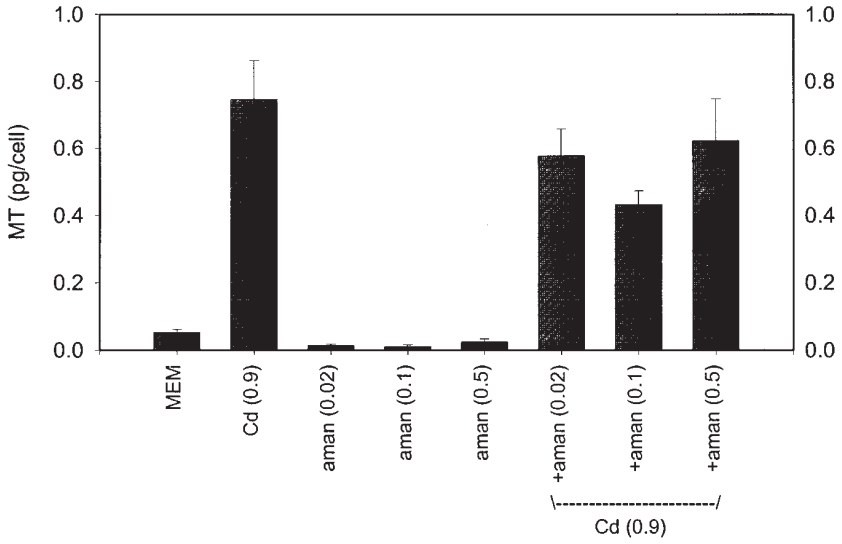

Figure 2. Suppression of Cd induced MT increase by α-amanitin (aman). Brackets indicated the concentrations in mg/L used.

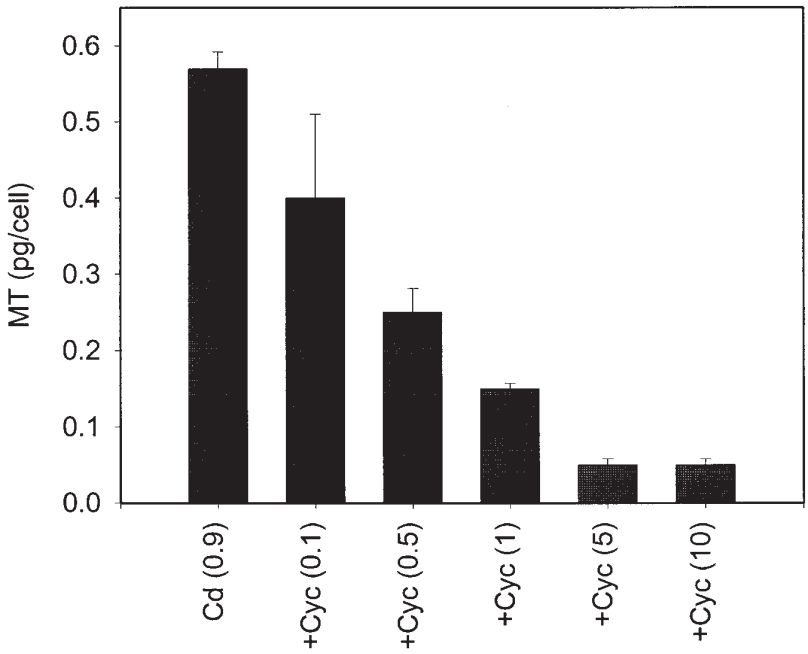

Figure 3. The suppression of MT induction by different concentrations of cycloheximide (cyc). Brackets indicated the concentrations in mg/L used.

Ca^{++} as MT inducer is not abundant. Ca^{++} has not been shown to induce MT by itself, but the action might be mediated through protein kinase C [9]. In the case of cAMP, Nebes *et al*. [10] showed that dBcAMP was able to induce MT-mRNA expression in rat hepatocytes by an increase in the rate of transcription. The inducibility was said to be suppressed in transformed cells [11]. However, Pallauf *et al.* [12] showed that in isolated primary rat hepatocytes, dBcAMP at a level as high as 500 µM, was unable to induce MT. The present study support the notion that dBcAMP and Ca^{++} are poor inducers of MT. Not only was the MT protein not present in this study, we were also unable to detect MT-mRNA under the same experimental conditions (unpublished result). Thus, the failure of induction was not regulated at the stage of translation. Upon analyzing the correlation with ^{65}Zn uptake in rat tissue upon exposed to dBcAMP on MT induction, Dunn and Cousins (1989) showed that the action of cAMP might be related to a redistribution of Zn in tissues. The present experimental design enables one to study MT induction with reference to the cell integrity. Besides metals, cAMP and Ca^{++}, we had also studied the efficacy of cystein and ascorbic acid on MT induction (Yang *et al.*, 1998). MT induction was also said to depend on other elements such as the dexamethasone (Kelly *et al.*, 1997) and the interleukin-6 (Lee *et al.*, 1999).

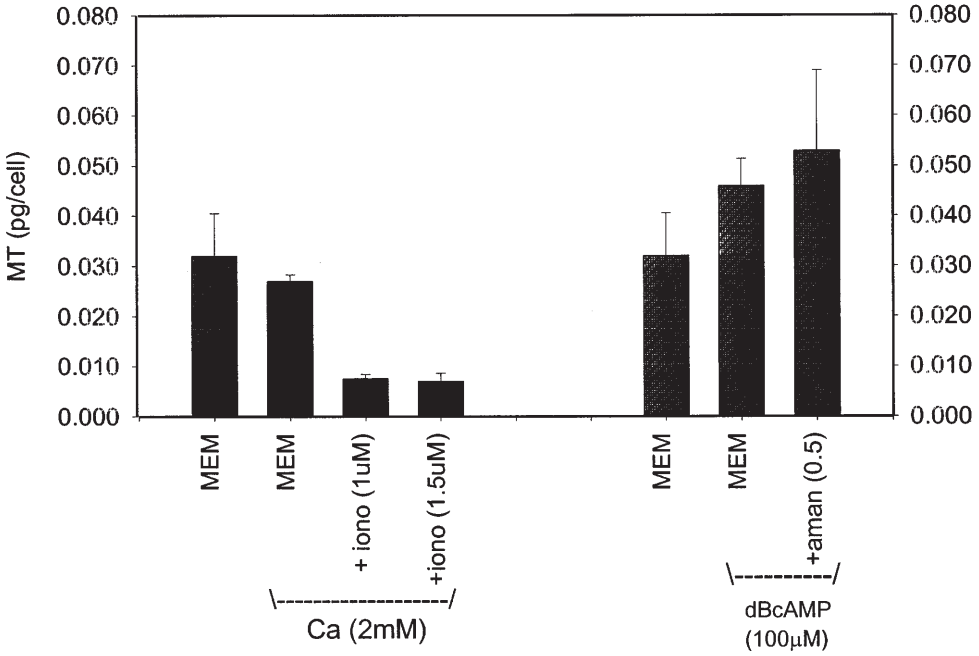

Figure 4. The effect of $CaCl_2$, both in the presence and the absence of ionomycin (iono) and dBcAMP in the presence and the absence of α-amanitin (aman) on MT induction. Brackets indicated the concentrations used.

The present system could be used to investigate the relative contribution of these molecules to MT induction.

Acknowledgement

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