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Induction of zinc metallothionein by calcium ionophore in vivo and in vitro

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The calcium ionophore, A23187, can induce rat hepatic metallothionein (MT) when administered in vivo (5.8-fold, 5.0 μM, 11 h) and rat hepatocyte MT when administered in vitro (10.70-fold, 1.0 μM, 24 h). Several rat hepatoma cell lines (2M, 4.55-fold; JM2, 12.29-fold; EC3, 14.12-fold; HTC, 7.99-fold) and a normal rat liver cell line (Clone 9, 39.67-fold) were tested for their inducibility of MT mRNA by Cd^{2+} (10 μM, 8 h). Quantitatively, JM2 and 2M made the most MT mRNA, while HTC made the least. A23187 (0.1–7.0 μM) was studied as an inducer of MT mRNA in these cell lines (except for HTC) and in HeLa. A variety of responses and tolerances were seen with inductions ranging up to 32.11-fold. Quantitatively, the best responding cell lines were EC3 and 2M. A combination induction experiment, using TPA, a protein kinase C activator, and A23187 in EC3 cells revealed an additive effect of the two inducers on MT mRNA levels: TPA (10 nM), 11.71-fold; A23187 (3.0 μM), 6.71-fold; and TPA + A23187, 20.00-fold. These studies have implicated perturbations in cytosolic calcium ion concentrations, caused by the ionophore A23187, as being involved in the complicated signaling systems which can lead to induction of MT mRNA and protein.

1. INTRODUCTION

Induction of metallothionein (MT) protein and mRNA by a variety of agents has been a fruitful system in which to study hormone-second messenger cascades and transcription factor–promoter element interactions [1]. MT's are eucaryotic proteins involved in normal zinc ion homeostasis. They are induced in response to generalized stresses on an animal (including acute phase response agents), as well as by specific hormones and other agents (glucocorticoids, metals, catecholamines, glucagon, interleukin-6, angiotensin-II, dibutyryl cAMP and phorbol esters) [1–4]. The involvement of protein kinase A and protein kinase C in the stimulation of synthesis of MT mRNA is well established. These kinases, glucocorticoids, and metals communicate with transcription factors which interact with elements in the MT promoters [5].

The role of calcium ions in the induction of MT mRNA synthesis has not been studied extensively. Hormones which lead to activation of protein kinase C via release of diacylglycerol also result in the elevation of cytosolic calcium ion concentrations via release by inositol triphosphate from endoplasmic reticulum stores [6]. Subsequent activation of calmodulin and calmodulin-dependent protein kinase may lead to communication with MT-promoter elements. But, calcium can affect the activity of other cellular targets besides this kinase. Protein kinase C [7], cAMP response element-binding protein [8], and heat-shock transcription factor [9] are all reported to require calcium for functional activity.

In this report we have studied the effects of the calcium ionophore, A23187, on the induction of MT protein and mRNA in rat liver, in primary cultures of rat hepatocytes, and in several cell lines in culture. Imbra and Karin [10], as part of another study, have reported a moderate induction of MT mRNA by A23187 (7 μM, 8 h) in Hep G2 cells, but they saw no induction in HeLa cells under the same culture conditions. Buscher, et al. [11] reported that A23187 could inhibit c-*fos* gene activation caused by UV exposure in 3T3 cells and normal human skin fibroblasts, but it did not prevent phorbol ester (TPA) induction of c-*fos* mRNA. Bohm et al. [12] reported that A23187 could induce MT-1 and MT-2

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mRNA in SENCAR mouse epidermis, when it was applied topically.

2. MATERIALS AND METHODS

2.1. *Animals and cells*

Male, Sprague–Dawley rats (200–300 g) were obtained from Sasco, Inc., Omaha, NE. They were housed in groups of four for at least one week before use in experiments. They received Purina lab chow and tap water ad libitum.

Primary cultures of rat hepatocytes were obtained, using a mixture of published methods [13–15]. They were cultured in Waymouth's medium without serum or with 10% fetal bovine serum (Gibco). Rat hepatoma cell lines (2M, HTC, JM2 and EC3) were gifts from Ron Lindahl of this Department [16]. Clone 9 is an epithelial culture from normal adult rat liver [16], and it was also obtained from Lindahl. HeLa cells were purchased from American Type Culture Collection, Rockville, MD. All of these cells were maintained in DME/F-12 medium (Sigma) with 5% newborn calf serum, streptomycin sulfate (75 U/ml), penicillin-G (100 U/ml), and 5% CO_2 at 37°C. Cell cultures were trypsinized weekly with 1 × standard trypsin solution (Sigma). Medium was changed every two days. Cells were used for experiments at 80–90% confluence.

2.2. *Chemicals*

All chemicals were reagent grade quality or better. They were usually obtained from Sigma, St. Louis, MO. Radioactive chemicals were obtained from New England Nuclear, Boston, MA. Electrophoresis reagents were obtained from Bio-Rad, Richmond, CA. Molecular biology reagents were obtained from BRL, Gaithersburg, MD.

2.3. *Metallothionein protein assays*

The Sephadex G-75/Atomic Absorption Spectroscopy (AAS) assay has been described previously [17]. The Superfine Sephadex G-75/[^{109}Cd]MT assay is a metal displacement assay used with small samples. An aliquot of hepatocyte supernatant (200 μl) was mixed with dithiothreitol to make the solution 1 mM and with 50 μg of Phenol red as a marker. The sample was doped with ^{109}Cd (12 μCi/μg Cd), giving 1.5 × as much cadmium as there was zinc in the sample. The mixtures were applied to Sephadex G-75 Superfine columns (0.8 × 29 cm), and the columns were developed with 0.01 M Tris-HCl, pH 8.6, 0.1 M NaCl, 1 mM NaN_3. Fractions were collected and counted. Three peaks were obtained with the middle one being the MT peak. This method was validated for metal displacement and MT recovery using authentic rat hepatic MT-I and MT-II.

2.4. *Metallothionein mRNA assay*

Total RNA was isolated by standard methods [18–20] from cultured cells. Northern gels were run as previously described, using glyoxal denaturation [21]. Size of RNA was determined using a 0.16–1.77 kb RNA ladder from BRL. Numerical results were obtained by scanning the developed X-ray film (XAR5, Kodak) with a Bio-Rad video densitometer after autoradiography with an intensifying screen.

Two probes were used for hybridization: a 21-mer, complementary to the first seven codons of MT-I and MT-II [22], and a plasmid, containing the α-tubulin cDNA. Dr. Mark Martzen, U. of Rochester, supplied the oligomer, and Dr. Robert Chiu, UCLA, supplied the tubulin plasmid. The 21-mer was end labeled, using bacteriophage T4 polynucleotide kinase and [γ-^{32}P]ATP (spec.act. 3000 Ci/mmol) [23]. The tubulin probe was labeled by nick translation [24], using DNAase I, DNA polymerase I, and [α-^{32}P]dCTP (spec.act. 3000 Ci/mmol).

3. RESULTS

3.1. *A23187 induction of MT protein*

Our initial experiment with the calcium ionophore,

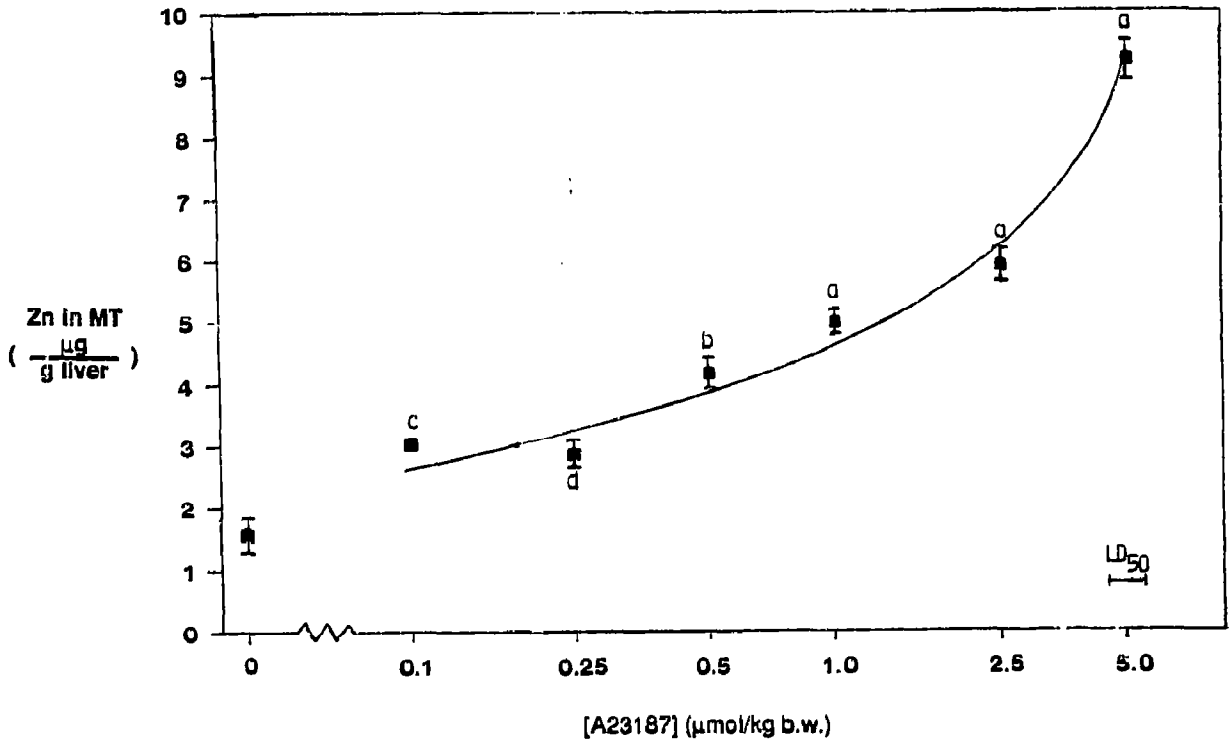

Fig. 1. A23187 dose–response for MT induction in rat liver. A23187 was administered at the indicated dosages i.p. at time 0. Animals were killed at 11 h, and MT (μg Zn/g liver) was determined by the Sephadex G-75/AAS method. The data are presented as the mean ± S.E. for at least four animals in a group. Statistically significantly different from controls (vehicle only) at $^{a}P<0.001$; $^{b}P<0.01$; $^{c}P<0.02$, using t-test. LD_{50}, lethal dose that kills 50% of the animals by 24 h, as reported by the manufacturer. No animal in these groups died during the 11 h treatment period.

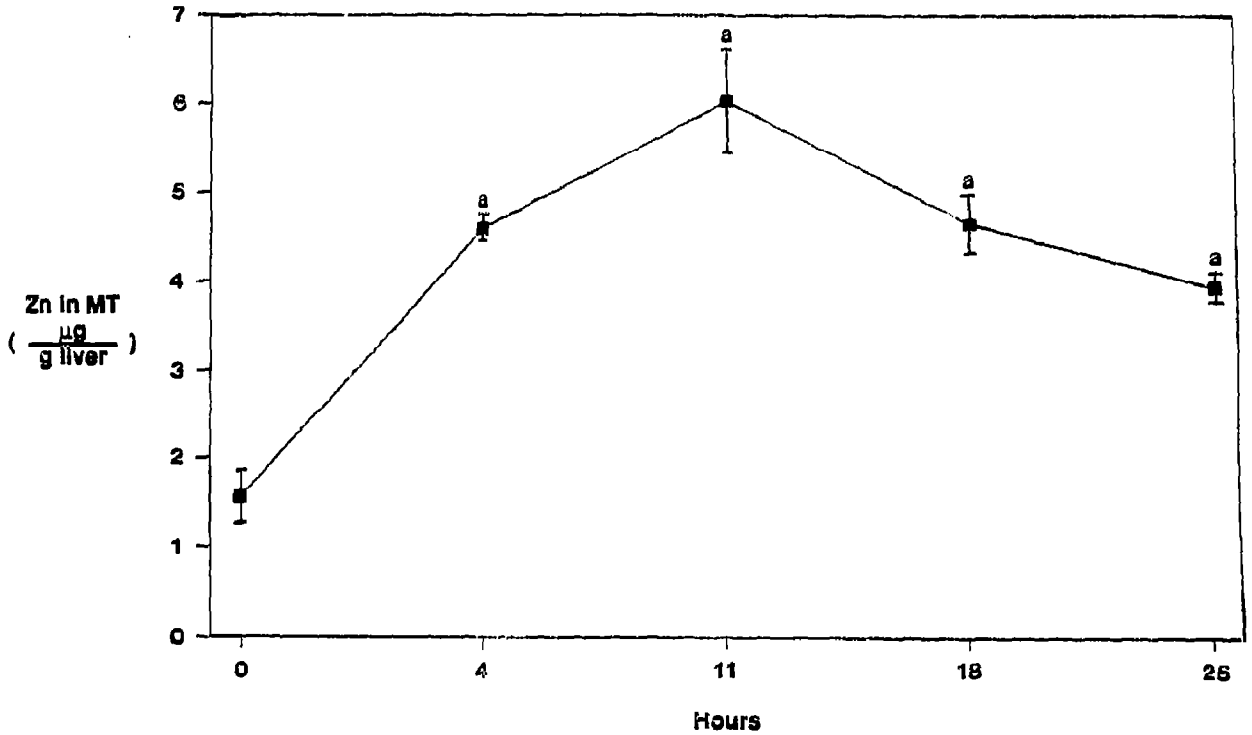

Fig. 2. A23187 time course for MT induction in rat liver. A23187 was administered i.p. at 2.5 µmol/kg b.w., and animals were sacrificed at the indicated times. MT (µg Zn/g liver) was determined by the Sephadex G-75/AAS method. The data are presented as the mean ± S.E. for at least four animals in a group. Statistically significantly different from controls (0 h) at $^{a}P<0.001$, using *t*-test.

A23187, involved in vivo treatment of rats with the compound and subsequent determination of ZnMT levels in liver. A dose–response (11 h) is shown in Fig. 1 and a time course is shown in Fig. 2. Statistically significant increases in hepatic MT are seen at all dose levels and time points with maximal inductions of 5.8-fold in the dose–response and 3.7-fold in the time course experiments. These results indicate that A23187 has an inductive effect on hepatic ZnMT protein levels in vivo, but whether or not this is a direct effect on liver required further studies with isolated hepatocytes. The results of such a study are presented in Table I. Strong induction of ZnMT protein is seen at 0.1 and 1.0 µM ionophore, indicating a direct effect of A23187 on hepatocyte MT synthesis. At 3.0 µM A23187 the induction decreases probably due to toxicity, as significant cell death was observed when assessed with the trypan blue exclusion test.

3.2. *A23187 induction of MT nRNA*

In order to confirm that A23187 alteration of cytosolic calcium levels was responsible for the induction of MT, we turned our attention to studies of the induction of MT mRNA in cultured cells. Fig. 3 presents the results of Northern gel analyses of MT mRNA levels in five cell lines in culture ±c cadmium ion treatment (10 µM, 8 h). Cadmium is a classic inducer of MT. HTC, 2M, EC3, and JM2 are rat hepatoma cell lines. Clone 9 is an established cell line from normal, adult rat liver. Among these lines Clone 9 showed the highest fold induction by Cd^{2+} of MT mRNA. 2M had the highest level of MT mRNA, followed by EC3. We are currently using these two cell lines extensively in further studies because of the quantitatively higher levels of MT mRNA. HTC showed a 7.99-fold induction in response to Cd^{2+}, but the basal and induced levels of MT mRNA were extremely low. It was not studied further.

The effects of A23187 on MT mRNA levels were

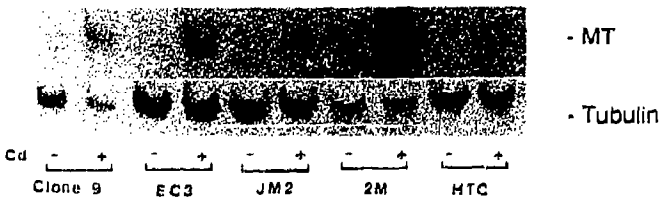

Fig. 3. Northern gel analysis of MT mRNA and α-tubulin mRNA in five cell lines following cadmium induction. Each cell line was grown in culture in triplicate to 80–90% confluence. Controls (−) received no addition. Cadmium (+) received 10 µM $CdCl_2$. Total RNA was harvested by pooling the three plates from each cell line after 8 h exposure ± cadmium. Northern gels were run with 10 µg total RNA and were probed sequentially for MT mRNA with a 21-mer and for α-tubulin with a cDNA. Autoradiographs of the gels are presented. MT mRNA is 0.55–0.59 kb, and α-tubulin mRNA is 1.73–1.77 kb in size. The inductions seen for each group after adjusting for α-tubulin mRNA variation were: clone 9, 39.67-fold; EC3, 14.12-fold; JM2, 12.29-fold; 2M, 4.55-fold; and HTC, 7.99-fold.

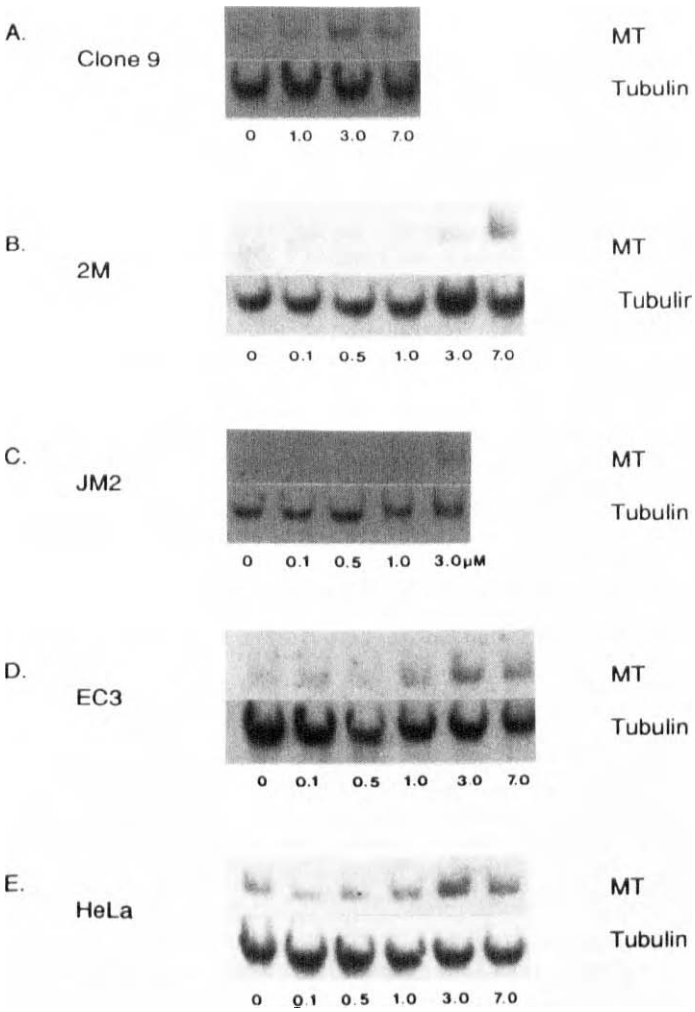

Fig. 4. Northern gel analysis of MT mRNA and α-tubulin mRNA in five cell lines after exposure to increasing amounts of A23187. Experiments were conducted as described in Fig. 3 with the indicated amounts of A23187 (0.1–7.0 μM) with 6 h exposure. Maximal inductions seen were: Clone 9, 7.59-fold (7.0 μM); 2M, 4.49-fold (7.0 μM); JM2, 2.09-fold (3.0 μM); EC3, 5.31-fold (3.0 μM); and HeLa, 2.32-fold (3.0 μM).

examined in the remaining four cell lines and in the human cell line, HeLa. The results are presented in Fig. 4. A variety of responses were seen. Clone 9 cells responded only at high levels of ionophore (7 μM). JM2 cells gave modest inductions at 0.5–3.0 μM, but they did not tolerate higher dosages. 2M cells responded modestly at 7 μM ionophore. In a separate time course experiment at 7.0 μM ionophore 2M cells showed a more dramatic response (2 h, 5.97-fold; 4 h, 25.57-fold; 6 h, 27.01-fold; 8 h, 27.07-fold; 10 h, 32.11-fold). EC3 cells responded well at all doses of ionophore that were tried. HeLa cells responded at 3.0 and 7.0 μM ionophore but only with modest inductions.

Inducers of MT which operate via the phosphoinositide cascade result in activation of both protein kinase C by diacylglycerol and calmodulin-dependent protein kinase by the calcium ion released from the endoplasmic

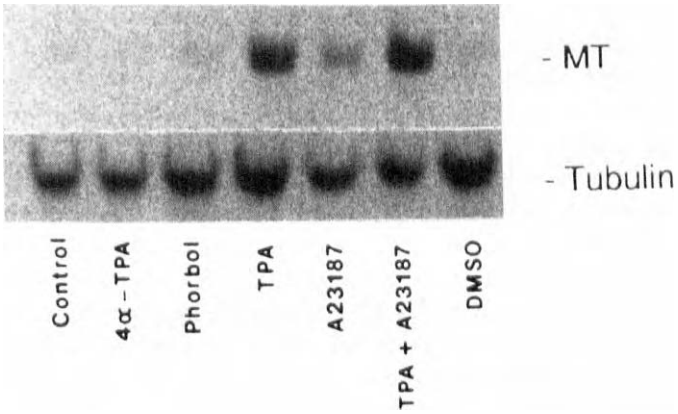

Fig. 5. Northern gel analysis of MT mRNA and α-tubulin mRNA in EC3 cells after exposure to phorbol esters and A23187. Experiments were conducted as described in Fig. 3 for 6 h with the indicated additions (control, none; 4α-TPA, 10 nM; 4β-phorbol, 10 nM; TPA, 10 nM; A23187, 3 μM; TPA, 10 nM + A23187, 3 μM; and DMSO, 73.5 μl). DMSO is the vehicle used to dissolve these agents. Inductions seen were: 4α-TPA, 0.25-fold; 4β-phorbol, 2.77-fold; TPA, 11.71-fold; A23187, 6.71-fold; TPA + A23187, 20.00-fold and DMSO, 1.70-fold.

reticulum by IP_3. TPA (tetradecanoyl phorbol acetate) is a direct activator of protein kinase C, and A23187 can alter cytosolic calcium levels. Fig. 5 shows the results of inducing MT mRNA in EC3 cells with TPA alone (11.71-fold), A23187 alone (6.71-fold) and the two inducers together (20.00-fold). The two inducers together gave an additive effect on the level of MT mRNA attained. This implies that activation of both protein kinase C and calmodulin dependent protein kinase leads to a signal which can interact with responsive elements in the MT gene's promoter to stimulate synthesis of MT mRNA.

4. DISCUSSION

Our studies have implicated perturbations in cytosolic calcium ion concentrations, caused by the io-

Table I

A23187 induction of MT in hepatocytes*

A23187†	MT‡	Fold induction
0	26.7 ± 4.0	1.10
DMSO	24.3 ± 2.1	1.00
0.1	71.4 ± 2.0[a]	2.94
1.0	260.0 ± 35.0[a]	10.70
3.0	78.5 ± 20.3[d]	3.23

*Hepatocytes were cultured for 24 h prior to treatment
†Cells were incubated with the indicated concentrations of A23187 (μM) for 20 h. Data is presented as the mean ± S.E. for three plates (3 cm) processed individually. DMSO is equivalent to the amount present in the added A23187 (3.0 μM).
‡MT (pmol Cd/mg protein) was assayed using the G-75 Superfine/^{109}Cd method. The [Ca^{2+}] in the media is 0.82 mM. Statistically significantly different from DMSO (50 μl) control at [a]$P<0.001$; [d]$P<0.05$, using t-test.

nophore A23187, as being involved in the complicated signaling systems which can lead to the induction of MT mRNA and protein. A23187 is a carboxylic antibiotic which is isolated from broths of *Streptomyces chartreusensis*. It was identified as a divalent cation ionophore by Reed and Lardy [25,16]. Their laboratory characterized the affinity of this ionophore for divalent cations as $Mn^{2+} \gg Ca^{2+} \approx Mg^{2+} \gg Sr^{2+} > Ba^{2+}$ [27]. The stoichiometry of binding is 2:1 for ionophore/divalent cation. Ion size [28] seems to be important in ionophore binding with smaller ions (Mn, Ca, Mg) binding better than larger ions (Sr, Ba). In biological systems, such as the ones we have studied, A23187 is considered to be a Ca^{2+} specific ionophore, probably because of the large gradients that are maintained between extracellular and cytoplasmic spaces and between intracellular compartments (endoplasmic reticulum, mitochondria) and the cytoplasm.

However, since we are studying the effects of the calcium ionophore on MT mRNA and protein induction, it should be recognized that zinc ions have a sufficiently small ionic radius (comparable to Mn^{2+}) that they might readily be transported across the plasma membrane by A23187, leading to a zinc induction of MT. In cell culture this is probably not so important, as the zinc concentration in media is usually about 10 μM or less, while calcium concentration is near mM. In vivo plasma zinc concentrations are in the range of 200–400 μM, so zinc transport by A23187 into liver in vivo might become more significant in the mechanism of A23187 induction of MT. Total [Zn] in the liver increases in response to in vivo A23187 administration, and 90–100% of this is associated with increased levels of MT (data not shown).

Our tendency is to conclude that increased calcium levels in the cytosol are modulating induction of MT mRNA synthesis because of the results of the in vitro studies. In order to prove a direct involvement of calcium we are planning further studies in which we will use calcium chelators, calcium channel blockers, and calmodulin antagonists to dissect the specific role of calcium in MT mRNA induction.

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