Purinergic Agonist Induction of Metallothionein (43480)

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Abstract. **Metallothionein (MT) protein is readily induced *in vivo* in rat liver by adenosine and adenosine agonists (2-chloroadenosine, 5-(N-ethyl) carboxamido adenosine, and 5-chloro-5-deoxyadenosine). These presumably operate via AMP/adenosine receptors of the P_1 (A_2) type, which use the cAMP pathway. ATP was ineffective as an inducer for MT. 2-Chloroadenosine was the most effective inducer (7.27-fold at 11 hr). This induction was blockable by the adenosine antagonists, caffeine and theophylline. MT protein induction by 2-chloroadenosine in primary cultured rat hepatocytes was modest (1.55-fold), but this was also blocked by theophylline.**

MT mRNA induction was assessed using dot blot and Northern gel assays. Large inductions by 2-chloroadenosine (5.1- to 41-fold) were seen, and these were detectable as early as 2 hr *in vivo*. Two rat hepatoma cell lines (EC3 and 2M) were studied *in vitro*. Modest inductions of MT mRNA were seen: 2.10-fold for EC3 and 4.12-fold for 2M.

Our studies implicate the potential role of the purinergic system in the modulation of transcription of MT genes in rat liver. The sources of adenosine *in vivo* that might cause induction of MT mRNA and protein are not well defined, but adenosine may be important as a signal in stress response situations involving tissue damage, such as ischemia, hypoxia, and hemorrhagic shock. [P.S.E.B.M. 1992, Vol 201]

Metallothionein (MT) is a zinc-binding protein involved in metal ion homeostasis and detoxification (1, 2). It is an inducible protein in rat liver and in cultured cell lines. Inducers include glucocorticoids, metals, hormones that increase cAMP levels, hormones that act via phospholipase C, and cytokines, such as interleukin 6 (3–6). In recent years, several reports have appeared that document the presence of purinergic receptors on liver cells. AMP/adenosine receptors of the P_1 (A_2) type act via the cAMP pathway to affect metabolism, e.g., a decrease of fructose 2,6-bisphosphate (7) and activation of glycogen phosphorylase (8–10). ATP/ADP receptors of the P_2 type act via the phospholipase C pathway to affect metabolism, e.g., activation of phosphorylase via inositol-trisphosphate-mediated increases in cytosolic calcium (11, 12). Reviews on various aspects of purinergic agonists are available: classification (13), function (14), pharmacology (15), and neurotransmitters (16). In our current work, we have examined the effects of ATP, adenosine, and adenosine analogs on the induction of zinc metallothionein protein and mRNA in rat liver *in vivo* and in cultured rat hepatocytes and two rat hepatoma cell lines (EC3 and 2M) *in vitro*. These results indicate that adenosine agonists are good inducers of MT, presumably via the cAMP-mediated pathway.

Materials and Methods

Animals. Male Sprague-Dawley rats (200–300 g) were obtained from Sasco, Inc., Omaha, NE. They were housed for at least 1 week before use, with Purina lab chow and tap water available *ad libitum*. During *in vivo* experiments, animals had full access to food and water. For hepatocyte experiments, livers were harvested in the morning from fully fed and watered animals using pentobarbital anesthesia (50 mg/kg body wt in 10% ethanol and 10% propylene glycol).

Chemicals. Chemicals and reagents were obtained from Sigma Chemical Co., St. Louis, MO. New England Nuclear, Boston, MA, was the source of radioactive chemicals. BioRad, Inc., Richmond, CA, supplied the

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electrophoresis reagents. BRL, Gaithersburg, MD, supplied molecular biology reagents.

Experimental Treatments. All *in vivo* treatments were intraperitoneal injections in sterile, normal saline (1.0 ml) at 0 hr. Rat livers were harvested individually after guillotine decapitation and frozen on dry ice for later analysis. Dosages and times of exposure are indicated in the legends of the figures and tables. In cell culture experiments, reagents were added in sterile saline (100 μl/3.5-cm dish). Concentrations and times of exposure are indicated in the legends of the Figures and Tables.

Metallothionein Protein Assays. The Sephadex G-75/atomic absorption spectroscopy assay has been described previously (17).

The superfine Sephadex G-75/^{109}Cd MT assay was used with primary cultured hepatocytes. They were harvested with 1.2 ml of 0.05 *M* Tris-HCl (pH 8.6) using a rubber policeman. The suspension was sonicated on ice for 10 sec in a microfuge tube and centrifuged at 1600*g* for 15 min. A 200-μl aliquot was mixed with dithiothreitol to 1 m*M*, and 50 μg of phenol red were added as a marker. ^{109}Cd (12 μCi/μg of Cd) was added at 1.5-fold excess over the zinc content. The mixture was applied to a Sephadex G75-superfine column (0.8 × 29 cm), and the column was eluted with 0.01 *M* Tris-HCl (pH 8.6), 0.1 *M* NaCl, 1 m*M* NaN_3. Fractions (0.5 ml) were collected and counted for radioactivity. The middle of three cadmium peaks was the MT peak. This assay was validated using authentic rat hepatic MT-I and MT-II. Quantitative displacement of MT zinc by cadmium and recovery of MT by this method were observed.

MT mRNA Assays. Standard procedures were used to isolate total RNA (18–20). Glyoxal denaturation was used for Northern gels (21). RNA size was determined with a 0.16–1.77-kb RNA ladder from BRL. Zeta-Probe membranes were used in a BioRad dot blot apparatus (22). After autoradiography with an intensifying screen, x-ray film (Kodak XAR5) was scanned quantitatively with a BioRad video densitometer.

The probe for MT was a 21-mer, complementary to the first seven codons of MT-I and MT-II (23). It was end labeled, using T4 polynucleotide kinase and [γ-^{32}P]ATP (sp act 3000 Ci/mmol) (24). The probe for α-tubulin was contained in a plasmid. It was labeled by nick translation, using DNAase I, DNA polymerase I, and [α-^{32}P]dCTP (sp act 3000 Ci/mmol) (25).

Cell Culture. Rat hepatocytes in primary culture were prepared using standard procedures (26, 27). They were isolated by Percoll gradient centrifugation (28) with yields of 50% and cell viability greater than 95%, using trypan blue exclusion and a hemocytometer. Cells (1×10^6) were plated on collagen-coated, Falcon tissue culture plates (3.5 cm) and were cultured for 2 hr to allow for attachment with Waymouth's medium plus 10% fetal bovine serum (Gibco, Grand Island, NY) at 30°C in a 2% CO_2 atmosphere. Media were changed and culturing was continued for 14 to 24 hr before experiments were conducted. Cells were harvested by washing twice with cold saline and then with 0.6 ml of 0.1 *M* NaCl, 0.01 *M* Tris-HCl (pH 8.6) and 1 m*M* dithiothreitol. Cells were disrupted by sonication, and supernatant was obtained by microfuge centrifugation.

Rat hepatoma cell lines, EC3 (29) and 2M (30), were grown at 37°C in Dulbecco's modified Eagle's/F-

Table I. Effects of Purinergic Agonists on Rat Hepatic Zinc Thionein Levels

Compound[a]	Zn in MT[b]	Fold induction
Control	1.31 ± 0.05	1.00
ATP		
1 Time (0 hr)	1.76 ± 0.36	1.34
3 Times (0, 4, 8 hr)	1.79 ± 0.32	1.37
Adenosine		
1 Time (0 hr)	1.33 ± 0.08	1.02
3 Times (0, 4, 8 hr)	5.38 ± 0.36[c]	4.11
2-Chloroadenosine		
1 Time (0 hr)	9.53 ± 0.45[c]	7.27
NECA		
1 Time (0 hr)	7.14 ± 0.48[c]	5.45
2 Times (0, 5.5 hr)	7.48 ± 1.29[d]	5.71
5-Chloro-5-deoxyadenosine		
1 Time (0 hr)	2.80 ± 0.63[d]	2.14
3 Times (0, 4, 8 hr)	5.17 ± 0.57[d]	3.95

[a] All compounds were administered intraperitoneally in 1.0 ml of saline at 0 hr. Dosages were: ATP (10 μmol/kg), adenosine (100 μmol/kg), 2-chloroadenosine (100 μmol/kg), 5-(*N*-ethyl) carboxamidoadenosine (NECA; 10 μmol/kg), and 5-chloro-5-deoxyadenosine (50 μmol/kg).
[b] Zn in MT (μg/g of liver) was determined by atomic absorption spectroscopy after Sephadex G-75 column chromatography of cytosol. At least four animals were in each group.
[c] Statistically significant differences from control levels at $P < 0.001$ using *t* test.
[d] Statistically significant differences from control levels at $P < 0.01$ using ± test.

Table II. Dose Response for 2-Chloroadenosine Induction of Metallothionein in Rat Liver

Treatment[a]	MT[b]	Fold induction
Control		
0	1.31 ± 0.05	1.00
2-Chloroadenosine		
2	1.23 ± 0.11	0.94
5	1.35 ± 0.21	1.03
15	3.95 ± 0.52[c]	3.02
25	5.70 ± 0.23[d]	4.35
40	7.26 ± 0.88[d]	5.54
100	9.53 ± 0.45[d]	7.27

[a] Control is the administration of saline only (11 hr of treatment). 2-Chloroadenosine was administered intraperitoneally in saline at the indicated dosages (μmol/kg body wt).
[b] Analysis of MT (μg Zn/g liver) was done with the Sephadex G-75/atomic absorption spectroscopy technique. Data are expressed as the mean ± SE for at least four samples.
[c] $P < 0.01$.
[d] $P < 0.001$.

Table III. Time Course for 2-Chloroadenosine Induction of Metallothionein in Rat Liver

Time[a]	MT[b]	Fold induction	Serum Zn[c]
0	1.31 ± 0.05	1.00	2.26 ± 0.21
4	1.17 ± 0.11	0.89	0.89 ± 0.05[d]
7	3.65 ± 0.15[e]	2.79	0.55 ± 0.03[e]
11	9.53 ± 0.45[e]	7.27	0.68 ± 0.07[e]
18	9.57 ± 0.63[e]	7.31	0.82 ± 0.10[e]
25	9.27 ± 0.21[e]	7.08	0.91 ± 0.08[d]

[a] The 0 hr point is for animals injected with saline and sacrificed immediately. For the other time points (hr), animals received intraperitoneally in saline 100 μmol of 2-chloroadenosine/kg body wt.
[b] Analysis of MT (μg Zn/g liver) was done with the Sephadex G-75/atomic absorption spectroscopy technique. Data are expressed as the mean ± SE for at least four samples.
[c] Serum zinc (μg/ml) was determined by atomic absorption spectroscopy on diluted samples.
[d] $P < 0.01$.
[e] $P < 0.001$.

Table IV. 2-Chloroadenosine Induction of Metallothionein: Blockade by Caffeine and Theophylline

Treatment[a]	MT[b]	Fold induction
Saline	1.31 ± 0.05	1.00
2-Chloroadenosine	5.70 ± 0.23[c]	4.35
Caffeine	0.99 ± 0.28	0.76
Caffeine + 2-chloroadenosine	1.00 ± 0.13	0.76
Theophylline	0.97 ± 0.29	0.74
Theophylline + 2-chloroadenosine	0.64 ± 0.09[c]	0.49

[a] Dosages: 2-chloroadenosine (25 μmol/kg), caffeine (100 μmol/kg), theophylline (100 μmol/kg), all given intraperitoneally in 1.0 ml of sterile saline at Time 0. Animals were sacrificed at 11 hr.
[b] MT (μg Zn/g liver) was analyzed by the Sephadex G-75/atomic absorption spectroscopy technique. Data are presented as the mean ± SE for at least four animals in each group.
[c] $P < 0.001$, using t test.

12 medium (Sigma) with newborn calf serum (5%), streptomycin sulfate (75 units/ml), penicillin G (100 units/ml), and 5% CO_2. Cultures were trypsinized weekly with standard trypsin solution (one time, Sigma). Media were changed at 2-day intervals until cells reached 80–90% confluence and were used in experiments.

Results

Metallothionein Protein Induction. In order to evaluate the potential role of purinergic agonists in inducing rat liver metallothionein, an *in vivo* experiment was conducted in which the natural P_1 agonist, adenosine, several synthetic P_1 agonists, and the natural P_2 agonist, ATP, were administered to rats for 11 hr, and Zn MT was determined (Table I). There were no synthetic P_2 agonists available.

Intraperitoneal injections of ATP into intact rats were ineffective in inducing MT, even when three injections (0, 4, and 8 hr) were given. Three injections (0, 4, and 8 hr) of adenosine induced MT 4.1-fold. Single injections of 2-chloroadenosine (7.27-fold), 5-(*N*-ethyl)carboxamide adenosine (5.45-fold), and 5-chloro-5-deoxyadenosine (2.14-fold) resulted in statistically significant increases in Zn MT after 11 hr. Three injec-

Table V. 2-Chloroadenosine Induction of MT in Hepatocytes

	MT[a]	Fold induction
2-Chloroadenosine[b]		
0	26.7 ± 1.4	1.00
1	42.9 ± 6.8	1.61
3	41.3 ± 3.8[c]	1.55
10	39.3 ± 5.0	1.47
30	40.2 ± 4.4[c]	1.51
100	31.2 ± 5.8	1.17
Theophylline	29.0 ± 0.1	1.09
10 + Theophylline	24.5 ± 1.0[c]	0.92 (0.84[d]) (0.62[e])
30 + Theophylline	33.6 ± 2.9	1.26 (1.16[d]) (0.84[f])

[a] MT protein (pmol Cd in MT/mg protein) was assayed using the Sephadex G-75 superfine/^{109}Cd method.
[b] Hepatocytes were cultured for 24 hr before treatment. Cells were incubated with the indicated concentrations of 2-chloroadenosine (*μM*) for 16 hr. The adenosine antagonist theophylline (200 *μM*) was used to block 2-chloroadenosine effects at 10 *μM* and 30 *μM* of agonist. Data are presented as the mean ± SE for at least three samples processed individually.
[c] Statistically significantly different from saline control or 10 *μM* 2-chloroadenosine for theophylline at $P < 0.05$, using t test.
[d] Fold induction, as compared with theophylline alone.
[e] Fold induction, as compared with 10 *μM* 2-Cl-adenosine.
[f] Fold induction, as compared with 30 *μM* 2-Cl-adenosine.

Table VI. 2-Chloroadenosine Induction of Metallothionein mRNA in Rat Liver[a]

Time (hr)	Dot blot assay[b]			Northern gel assay[c]
	MT mRNA	Tubulin mRNA	Adjusted MT mRNA	Adjusted MT mRNA
0	1.00 ± 0.11	1.00 ± 0.18	1.00	1.0
2	4.08 ± 0.57[d]	0.80 ± 0.03	5.10	21.9
4	6.88 ± 0.08[e]	1.49 ± 0.09[d]	4.62	26.0
6	6.96 ± 0.54[e]	0.79 ± 0.12	8.81	41.0
8	4.58 ± 1.79	1.78 ± 0.35	2.57	28.4
10	4.67 ± 1.72	1.10 ± 0.34	4.25	21.5

[a] 2-Chloroadenosine was administered at 100 μmol/kg body wt for the indicated times (hr).
[b] Data (from Fig. 1) are presented for fold induction as compared with 0-hr time points. Results are expressed as the mean ± SE for three samples at 2.5 μg of total RNA/dot.
[c] Total RNA (10 μg) was applied to each lane by mixing 3.33 μg of RNA from each of three rats in an experimental group. Data (from Fig. 2) are presented for fold induction as compared with 0-hr time point after adjustment for variation in tubulin mRNA levels.
[d] $P < 0.01$.
[e] $P < 0.001$.

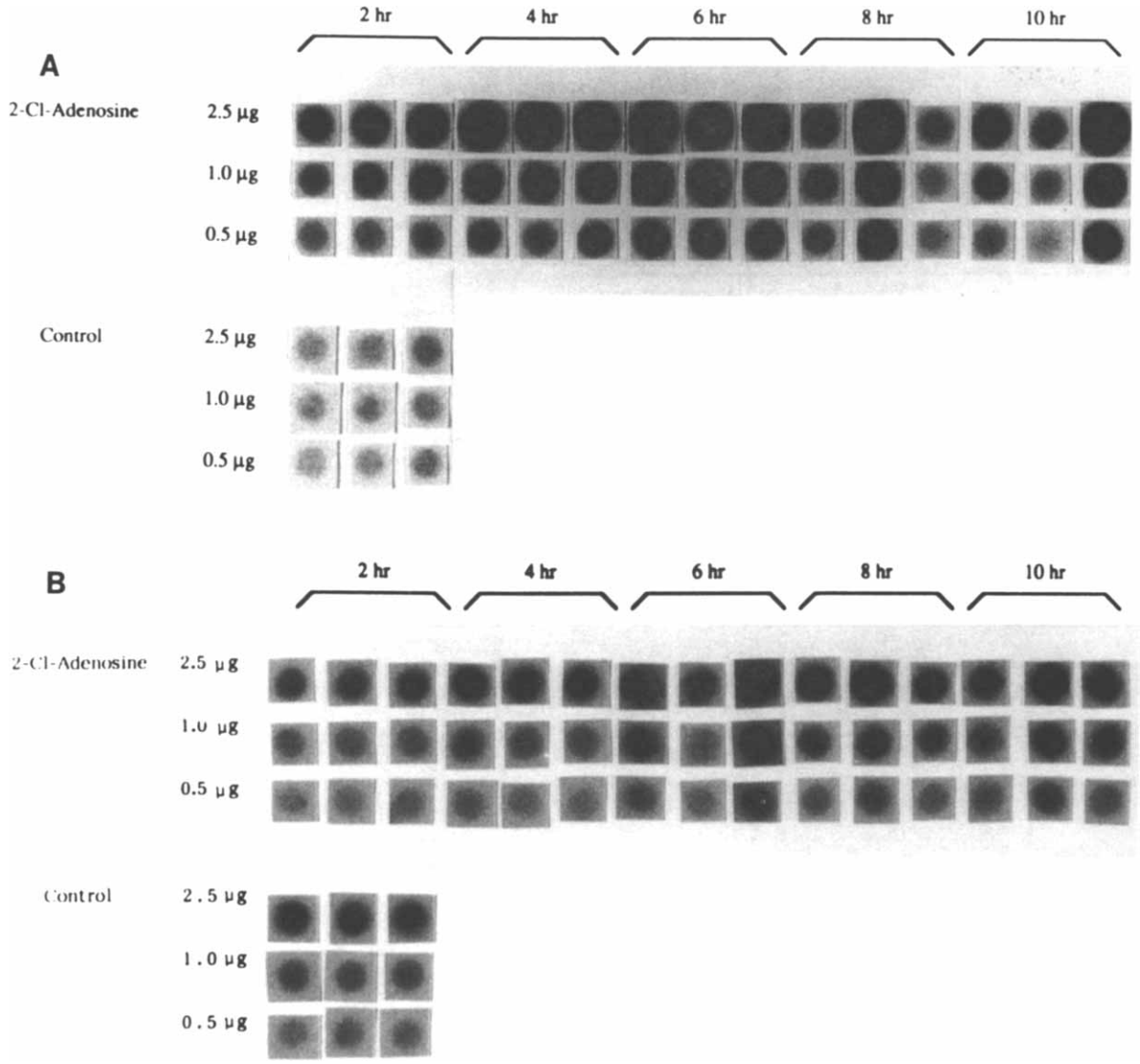

Figure 1. Dot blot analysis of rat hepatic (A) MT mRNA and (B) tubulin mRNA after 2-chloroadenosine induction *in vivo*. 2-Chloroadenosine was given intraperitoneally at 100 μmol/kg body wt. Each dot contained 2.5 μg of total RNA.

tions (0, 4, and 8 hr) of the last agonist induced Zn MT 3.95-fold after 11 hr.

A dose response (11 hr) and a time course (100 μmol/kg body wt) for *in vivo* induction of hepatic Zn MT by 2-chloroadenosine (intraperitoneally) are shown in Tables II and III. The induction reached a maximum at 100 μmol/kg body wt with an effective dose for a half maximal increase at 22 μmol/kg body wt. One group of animals was given a dose of 200 μmol/kg body wt, but most of these died before 11 hr. The animals in the other groups showed little behavioral effects of administration of these compounds. In the time course experiment, severe and persistent hypozincemia occurred, which is usually an *in vivo* correlate for strong metallothionein inducers.

To test the specificity of the effects of these agonists on purinergic receptors, commercially available antagonists for the adenosine receptor were utilized *in vivo*. Both caffeine and theophylline were able to block Zn MT induction by 2-chloroadenosine (25 μmol/kg body wt; 11 hr) (Table IV). This supports pharmacologically that the induction of Zn MT by 2-chloroadenosine is mediated by adenosine receptors.

Zn MT induction by 2-chloroadenosine was also studied in cultured hepatocytes. The inductions seen were modest (1.5-fold), but they were blockable by treatment with the adenosine antagonist theophylline (Table V).

Metallothionein mRNA Induction. To determine whether the Zn MT induction by purinergic agonists was occurring because of increased transcription, metallothionein mRNA levels were measured using dot blot and Northern gel assays. Inductions were done *in*

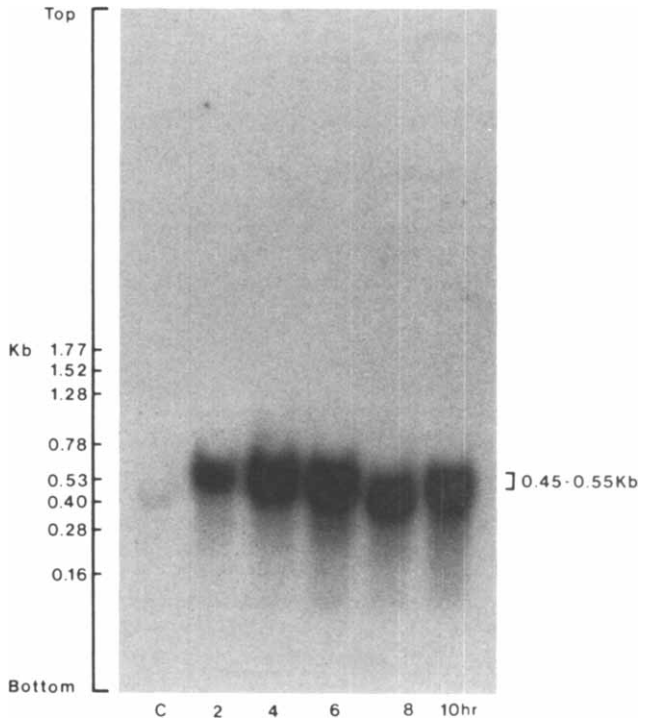

Figure 2. Northern gel analysis of rat hepatic MT mRNA after 2-chloroadenosine induction *in vivo*. 2-Chloroadenosine was administered at 100 μmol/kg body wt. Ten micrograms of total RNA were applied to each lane, which was a mix of 3.33 μg of RNA from each of three rats in an experimental group.

vivo and in two rat hepatoma cell lines (EC3 and 2M) in culture.

2-Chloroadenosine induction of MT mRNA *in vivo* in rat liver is shown in Table VI and Figure 1 for the dot blot assay and in Table VI and Figure 2 for the Northern gel assay. Statistically significant increases in MT mRNA were seen as early as 2 hr after treatment with the purinergic agonist. The higher relative inductions seen in the Northern gel assays as compared with the dot blot assays is probably due to the separation of background RNA on the Northern gels, e.g., the control levels are more sensitively measured on the Northern gels than in the dot blot assays.

To confirm that the induction of MT mRNA by 2-chloroadenosine is a direct effect on rat liver, we used two rat hepatoma cell lines, EC3 and 2M, to study purinergic induction *in vitro*. Figure 3 presents the results, using Northern gel assays, of a time course (50 μM 2-chloroadenosine) of induction of MT mRNA by 2-chloroadenosine in these cell lines. Good inductions for cells in culture were seen with both of these cell lines, confirming a direct effect of a purinergic agonist on a rat liver cell in the induction of MT mRNA. For the EC3 cell line, a maximal induction of 2.10-fold was seen at 10 hr. For the 2M cell line, a maximal induction of 4.12-fold was seen at 10 hr. Consistently, two distinct bands light up in the 2M cell line, the lower one at 0.45 kb and the higher one at 0.70 kb. We do not see this in hepatocytes or in EC3 and other cell lines (Clone 9, JM2, and HeLa; data not shown). Perhaps, in 2M cells, the mRNA for MT-I and MT-II are of sufficiently different size to be revealed by our oligo-probe in Northern gel assays.

Discussion

Our studies illustrate that the control of metallothionein levels in rat liver cells may be connected to the purinergic system. However, it is not known to what extent adenosine has an effect on MT levels under "normal" situations in the rat, e.g., conditions when tissue damage or insult is not occurring. Sources of extracellular adenosine that may affect liver are not well delineated. Hypoxic and ischemic tissues, including liver, have been shown to release adenosine (15, 31). Purinergic receptors are known to exist on liver cells, and these may help mediate stress responses. Some studies have suggested that during hemorrhagic shock, increases in plasma levels of adenosine mediate the hypotensive response to this stress (32). Rat hepatic membranes have been shown to have adenosine receptors that communicate with adenylate cyclase via G_s and G_i, depending upon GTP concentrations (33).

The failure of ATP to induce MT *in vivo* was probably due to the instability of extracellular ATP. Cellular uptake and degradation by hydrolases and deaminases are potential ways in which extracellular ATP could be eliminated. The induction by adenosine and its synthetic analogs was quite good. 2-Chloroadenosine is a persistent agonist because it is resistant to

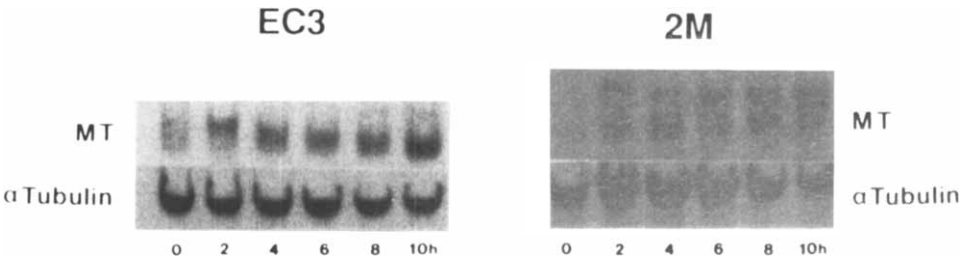

Figure 3. Northern gel analysis of MT mRNA and tubulin mRNA in EC3 and 2M rat hepatoma cells after 2-chloroadenosine induction *in vitro*. Time course after exposure to 50 μM 2-chloroadenosine. Ten micrograms of total RNA was applied to each lane.

deamination, and the amino group in Position 6 is necessary for agonist effects (34).

Metallothionein has been shown over the last 10–12 years to be a protein that is inducible in rat liver parenchymal cells by a variety of external and internal agents (1–6). Many of these stimuli are part of a stress response, and, indeed, MT has been tentatively designated as an acute phase-response protein. Induction of MT has been demonstrated with metals and glucocorticoids that have a direct effect on MT gene transcription, and with other agents (catecholamines, phorbol esters, and polypeptide hormones) (3–5) that operate via plasma membrane receptors and cytosolic second messengers, which in turn communicate with MT gene promoter regions via activation of several transcription factors (35). Adenosine agonists fit into the latter category. Their action in inducing MT mRNA and protein is most likely due in liver to their ability to increase cytosolic cAMP levels with subsequent activation of protein kinase A. This would be similar to the actions of epinephrine and glucagon on MT induction.

Many of the agents known to induce rat hepatic MT are toxic organic chemicals and metal ions. A common theme in their mechanisms of action leading to induction of MT may be the damage that they impose on the liver and other tissues. It is just such damage that may result in the release of purinergic agonists, such as adenosine, from impaired cells with consequent interaction with purinergic receptors on neighboring competent cells. This could allow for a rapid response by the liver to combat the toxic insult. Induction of zinc metallothionein may be an early response by the liver to mobilize zinc for use with various proteins in the *de novo* nucleic acid and protein synthesis which would be necessary for the hepatic response to the toxic insult (acute phase response). In fact, a recent note has illustrated the ability of apometallothionein to affect the DNA binding activity of a zinc finger containing transcription factor Sp 1 (36). The use of the rat hepatoma cell lines EC3 and 2M has allowed us to begin studies of direct actions of these agents on MT induction in an *in vitro* system.

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