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SHORT COMMUNICATIONS

Effects of carbon tetrachloride and 1,1-dichloroethylene on rat hepatic microsomal calcium- and/or magnesium-stimulated ATPase

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Alterations in cellular calcium have been implicated as playing an important role in cytotoxicity [1, 2]. Plasma membrane injury and a resulting increase in intracellular calcium were considered by Farber and his associates [1] as the most likely mechanism in lethal cellular injury by toxic chemicals. This hypothesis is supported by the common finding that hepatic calcium levels increase several-fold following administration of hepatotoxic doses of halogenated hydrocarbons, such as carbon tetrachloride (CCl_4), 1,1-dichloroethylene (1,1-DCE) and chloroform ($CHCl_3$) [3–6]. These hepatotoxins were found by Moore and his colleagues [4, 6, 7] to cause an early inhibition of microsomal calcium transport in the liver. Murphy *et al.* [8] found that microsomes contain approximately 30% of the total calcium content of isolated rat hepatocytes. Furthermore, Becker *et al.* [9] reported that hepatic microsomes have higher affinity for calcium than mitochondria. These findings suggest that microsomal calcium transport in hepatocytes may play an important role in maintaining cytosolic calcium at low, physiological levels.

Experiments conducted on the effects of hepatotoxins (e.g. CCl_4, 1,1-DCE and $CHCl_3$) on microsomal calcium pump activity have involved *in vitro* studies of the uptake of calcium from media into microsomal vesicles. It is important to note that the ability of microsomal vesicles to accumulate calcium is dependent not only on the integrity of their active transport systems but on the passive efflux of calcium down concentration gradients. Thus, hepatotoxins which alter microsomal calcium sequestration may do so by altering the permeability of microsomal membranes to calcium or by directly affecting active calcium transport. Investigations reported to date have not addressed the mechanism(s) whereby hepatotoxins alter microsomal calcium sequestration. Hence, studies were initiated to determine whether CCl_4 and 1,1-DCE directly inhibit active calcium transport. Effects on calcium- and/or magnesium-stimulated ATPase are described in the current report.

Materials and methods

Male Sprague–Dawley rats (200–250 g) were obtained from Timco Breeding Laboratories (Houston, TX). The animals were housed under a 12-hr light/12-hr dark cycle and provided with Ralston Purina Formulab chow and tap water *ad lib.* 1,1-DCE (99% minimum purity, inhibited with 200 ppm hydroquinone monomethyl ether) was purchased from the Aldrich Chemical Co. (Milwaukee, WI), and CCl_4 (reagent grade) was purchased from Matheson, Coleman & Bell (Norwood, OH). Na_2ATP was purchased from Boehringer Manneheim (Indianapolis, IN). All other chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO). Appropriate quantities of 1,1-DCE were diluted in corn oil, such that doses of 500, 750 and 1000 mg 1,1-DCE/kg body wt could be given in a total volume of 1.0 ml. Doses of 1.6 or 4.0 g CCl_4/kg body wt were given undiluted. The solutions were prepared fresh daily and given orally with a ball-tipped gastric intubation needle. Dosing was carried out between 8:00 and 10:00 a.m. or 2–4 hr into the inactive cycle of the animals, in order to avoid diurnal variation in response to the toxic agents. Groups of three rats each were terminated by decapitation 30 min, 2 hr or 24 hr after dosing. One gram of tissue was removed from the left median lobe of the liver of each animal and pooled with 1-g portions from other members of the same treatment group. The pooled livers were homogenized in ice-cold 0.25 M sucrose, and the 12,500 *g* supernatant fraction was centrifuged at 100,000 *g* to obtain a microsomal pellet. The microsomes were resuspended in 3 ml of 0.25 M sucrose and utilized for assay of calcium- and/or magnesium-activated ATPases. The microsomal protein content was determined by the method of Lowry *et al.* [10]. Calcium- and/or magnesium-dependent ATPase activity associated with the microsomes was measured by assay for inorganic phosphate (P_i) liberated from ATP [11]. An incubation mixture, containing 25 μg microsomal protein, 69 mM histidine, 1.5 mM Tris, 2.1 mM Na_2ATP, 1.0 mM ouabain and various concentrations of divalent cation in a total volume of 0.7 ml, was incubated under air for 30 min at 37° in a shaking water bath. The reaction was terminated by addition of 2 vol. of chloroform–methanol (2:1, v:v). Aliquots from the methanol layer were assayed for P_i content by the colorimetric method described by Fiske and Subbarow [12]. Blanks containing either 0.18 mM ethyleneglycolbis (amino-ethylether) tetra-acetate (EGTA) or 0.18 mM EDTA were utilized in the assays for calcium ATPase or magnesium ATPase respectively.

Results and discussion

Initial experiments were carried out to delineate the activation profile of calcium- and/or magnesium-stimulated ATPase of hepatic microsomes. Microsomes were incubated in a wide range of concentrations (0.01 to 1.2 mM) of magnesium or calcium. When only calcium was added, maximal activation of the enzyme (2.4 μmoles P_i released/mg protein/hr) was observed at a calcium concentration of 0.8 mM (Fig. 1A). Higher calcium concentrations (> 1.5 mM) were found to be inhibitory (Fig. 1B). Addition of only magnesium produced a substantially greater stimulation than did calcium. It can be seen in Fig. 1A that maximal activation of the enzyme (4.0 μmoles P_i released/mg protein/hr) occurred at a magnesium concentration of about 0.8 mM.

It is apparent in Fig. 1B that addition of magnesium to calcium-containing medium resulted in further stimulation of ATP hydrolysis. Although 0.025 mM $MgCl_2$ produced only a slight increase in enzymatic activity, the presence of 0.125 mM $MgCl_2$ resulted in a substantial increase in the activation of the enzyme over calcium controls. One cannot be certain from these data, however, whether calcium can activate ATPase in the absence of magnesium. We did not take measures to chelate trace amounts of magnesium present in the incubation medium.

The influence of *in vivo* administration of 1,1-DCE on microsomal ATPase activities, as assessed 2 hr post dosing, is illustrated in Fig. 2. In the absence of any added magnesium (Fig. 2A), the 500 and 750 mg/kg doses of 1,1-DCE produced 27 and 51% decreases from controls, respectively, at the maximal stimulatory concentration of calcium (1 mM). With the lowest calcium concentration used

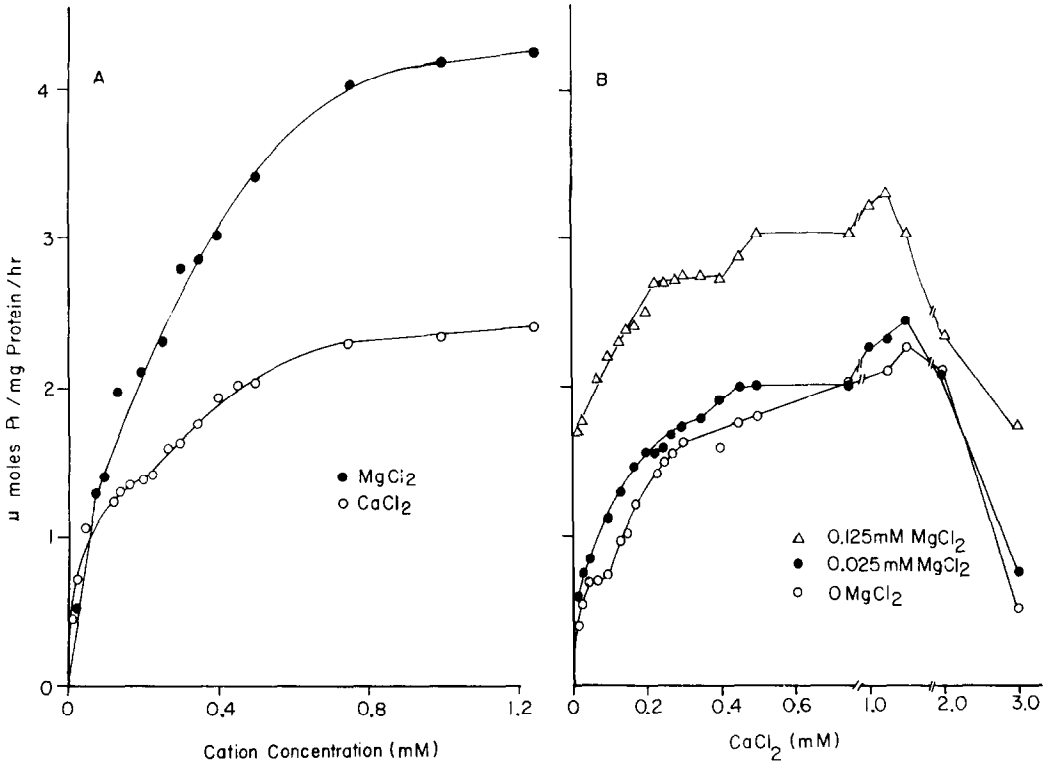

Fig. 1. Activation profiles of hepatic microsomal ATPase by calcium and/or magnesium. Stimulation of ATPase by calcium or magnesium concentrations of 0.01 to 1.2 mM is illustrated in panel A. This influence of calcium (0.01 to 3.0 mM), in the absence and the presence of 0.025 and 0.125 mM magnesium, is shown in panel B. The incubation conditions were as described in Materials and Methods.

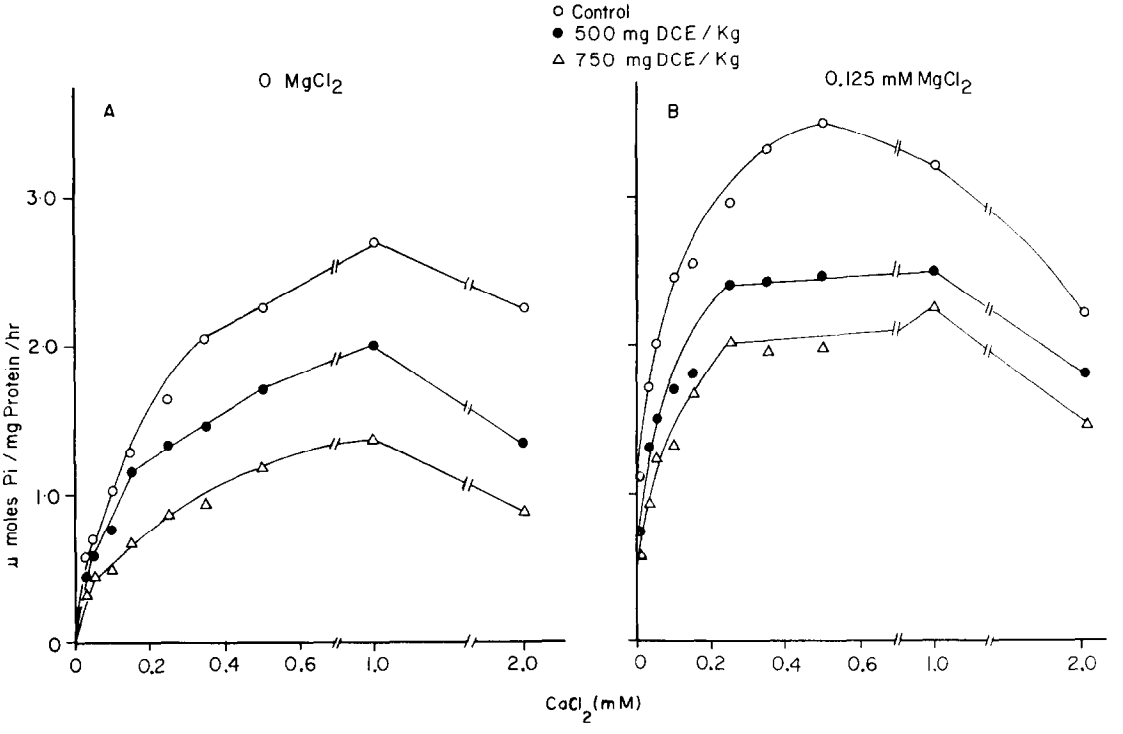

Fig. 2. Inhibition of hepatic microsomal calcium- and/or magnesium stimulated ATPase by 1,1-DCE. Microsomes were isolated from the livers of rats killed 2 hr following oral administration of 0, 500 or 750 mg 1,1-DCE/kg. ATPase activity in pooled samples was assayed over a range of calcium concentrations (0.012 to 2.0 mM) in the absence (panel A) or the presence of 0.125 mM magnesium (panel B).

(0.025 mM), the 500 and 750 mg/kg doses of 1,1-DCE produced 23 and 42% decreases from controls respectively. The degree of 1,1-DCE-induced inhibition of ATPase (relative to controls) was approximately the same at each concentration of calcium utilized. Dose-dependent inhibition of ATPase activity by 1,1-DCE also occurred over a wide range of calcium concentrations when 0.125 mM magnesium was present in the incubation mixture (Fig. 2B). Although the degree of enzyme inhibition varied somewhat over the range of calcium concentrations employed, percentage decreases from controls were comparable at the lowest (0.01 mM) and the maximal stimulatory (0.5 mM) calcium levels (Fig. 2B). It should be recognized that these *in vitro* concentrations of calcium and magnesium are considerably higher than usual cytosolic concentrations of the cations.

The effects of CCl_4 on hepatic microsomal ATPase activity, 2 hr following oral dosing, are illustrated in Fig. 3. There were very marked decreases from the control level of enzyme activity when the incubation mixture contained 0.012 mM calcium. The 1.6 and 4.0 g CCl_4/kg oral doses caused 80 and 90% decreases from controls, respectively, at 0.012 mM calcium. CCl_4 also produced dose-dependent inhibition of ATPase activity when higher levels of calcium were present in the incubation media, but the decreases from controls were not so pronounced. A similar pattern of enzyme inhibition by CCl_4 was seen when 0.125 mM magnesium was included in the media (data not shown).

High, hepatotoxic doses of CCl_4 and 1,1-DCE were utilized in order to compare previously reported effects of the chemicals on microsomal calcium sequestration with our own enzymatic data. Toxin-induced inhibition of microsomal ATPase activity and reported decreases in microsomal calcium sequestration proved to be quite similar, both in magnitude and in time of onset. Moore *et al.* [4] observed significant depression in calcium uptake by liver microsomes isolated 30 min after rats were administered 4 g CCl_4/kg by gavage. Uptake was reduced by more than 80% by 2 hr. We also observed depression in hepatic microsomal ATPase activity 30 min after giving 4 g CCl_4/kg, and even more marked depression 2 hr post dosing (Table 1). ATPase activity returned towards normal by 24 hr after CCl_4. The pattern of response to 1,1-DCE was similar, though 1,1-DCE appeared to be a more potent ATPase inhibitor than CCl_4. Moore [6] found 1,1-DCE to be a potent inhibitor of calcium uptake by rat hepatic microsomes. Recently, Moore [7] observed that microsomal calcium uptake is inhibited 45% within 20 min of a 1.8 g/kg i.p. dose of 1,1-DCE. Maximal (65–70%) inhibition of uptake was seen for 1–4 hr after dosing with the 1,1-DCE. Despite some differences in the incubation conditions used by Moore and colleagues [4, 6, 7] and those employed in the current investigation, the inhibitory effects of 1,1-DCE and CCl_4 on microsomal calcium uptake and on microsomal ATPase activity are remarkably parallel. This suggests that chemically-induced inhibition of microsomal calcium uptake results from inhibition of microsomal calcium- and/or magnesium-dependent ATPase. Moore *et al.* [13] have previously demonstrated microsomal calcium uptake to be ATP dependent. Although it is not clear whether calcium- and/or magnesium-dependent ATPase is in fact a calcium translocase, the microsomal ATPase is required for calcium transport.

It is widely recognized that CCl_4 and 1,1-DCE exert their toxic effects on the liver by different mechanisms. CCl_4 is generally believed to be metabolically activated to a trichloromethyl free radical, which initiates a series of lipoperoxidative reactions which culminate in disruption of the structural and functional integrity of membranes [14]. CCl_4 selectively affects microsomal membranes, producing ultrastructural and functional changes as early as 15–30 min after dosing. 1,1-DCE has been shown to be a more potent hepatotoxin than CCl_4 in rats [15]. Although it is also

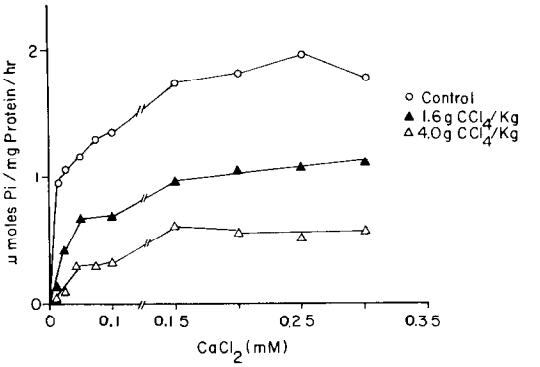

Fig. 3. Inhibition of hepatic microsomal calcium- and/or magnesium-stimulated ATPase by CCl_4. Microsomes were isolated from the livers of rats killed 2 hr following oral administration of 0, 1.6 or 4.0 g CCl_4/kg. ATPase activity in pooled samples was assayed over a range of calcium concentrations (0.01 to 0.3 mM) without any added magnesium.

believed that 1,1-DCE requires metabolic activation [16], 1,1-DCE does not cause lipoperoxidation in the liver [6, 17] nor does it selectively damage the endoplasmic reticulum. Ultrastructural [18] and most functional [5] changes in the endoplasmic reticulum occur relatively late in the course of injury by 1,1-DCE, at a time when hemorrhagic centrolobular necrosis has become manifest. As previously related, 1,1-DCE-induced inhibition of the microsomal calcium pump occurs within minutes of dosing. Thus, it would appear that this very early effect of 1,1-DCE is not associated with chemically-induced disruption of the microsomal membranes. Rather, findings in the current study suggest that the inhibition of microsomal calcium pump activity is mediated via direct inhibition of magnesium- and/or calcium-dependent ATPase by 1,1-DCE. We have also demonstrated that CCl_4 inhibited ATPase, although it is possible that apparent decreases seen in calcium uptake by microsomal vesicles may be due in part to increased vesicular membrane permeability. Lowrey *et al.* [19] have reported inhibition of hepatic microsomal calcium uptake within 3–5 min of oral dosing of fasted rats with 4 g CCl_4/kg. The inhibition occurred at the same time as the appearance of microsomal lipid diene conjugates and slightly after the maximal incorporation of ^{14}C from $^{14}CCl_4$ into microsomal lipids, as previously reported by the same laboratory [20, 21]. Recently, it has been shown *in vitro* that CCl_4 can inhibit microsomal calcium sequestration in the absence of lipoperoxidation, so long as the CCl_4 is

Table 1. Percent inhibition of hepatic microsomal calcium-dependent ATPase activity by 1,1-DCE and CCl_4*

	% Inhibition of ATPase activity		
	Hours post-dosage		
	0.5	2	24
1,1-DCE	27	65	8
CCl_4	30	70	10

* Groups of three rats received 1 g of 1,1-DCE/kg or 4 g of CCl_4/kg orally. Pooled hepatic microsomes were incubated in medium containing 1.0 mM calcium (no added magnesium) for 30 min, as described in Materials and Methods. Mean control value was 2.6 μmoles P_i liberated/mg protein/hr.

metabolically activated [22]. This finding discounts the role of altered membrane permeability and lends support to our proposal that CCl_4 (as well as 1,1-DCE) inhibits calcium pump activity via inhibition of microsomal ATPase. Studies to determine whether CCl_4 or 1,1-DCE enhances back diffusion of calcium from microsomal vesicles would provide additional information on the mechanism by which hepatotoxins alter microsomal sequestration.

In summary, we have shown that hepatotoxic doses of CCl_4 and 1,1-DCE promptly cause pronounced inhibition of hepatic microsomal ATPase activity. Microsomal calcium-dependent ATPase activity can be measured satisfactorily *in vitro* in either the presence or absence of added magnesium. Inhibition of ATPase activity appears to be a common mechanism whereby hepatotoxins diminish the ability of microsomes to sequester calcium. As the microsomal calcium pump is believed to play an active role in maintaining calcium homeostasis in hepatocytes, toxin-induced inhibition of calcium- and/or magnesium-dependent ATPase may be an initial biochemical lesion which triggers a sequence of events which may culminate in cell death.

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Role of intracellular dTTP levels in fluorodeoxyuridine toxicity

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A growing concern in cancer chemotherapy is the apparent heterogeneity among primary tumors in the response to chemotherapeutic agents and the appearance of secondary tumors which are no longer sensitive to the chemotherapeutic agent. Resistance to chemotherapeutic agents has often been linked to changes in enzyme activities which either are required for drug activation or represent the ultimate target of the drug [1]. On the other hand, drug activation may be also regulated by intracellular metabolites and thus variations in intracellular levels of these metabolites may largely affect drug toxicity [2].

5-Fluoroauracil (5-FU) and 5-fluorodeoxyuridine (FdUrd) are used in the treatment of various kinds of cancer [3]. The block in DNA synthesis that results from treatment of cells with FdUrd may be accounted for by inhibition of thymidylate synthase by the intracellular phosphorylation product FdUMP (4). In addition, DNA synthesis may be impaired due to the misincorporation of FdUrd into the DNA [5]. The phosphorylation of FdUrd is catalyzed by thymidine kinase, an enzyme which is inhibited by dTTP in extracts from various cells [6–8]. If this feedback inhibition also occurs in intact cells, the intracellular dTTP pools may influence the rate of FdUrd phosphorylation and, as a result, may also affect FdUrd toxicity. To test this hypothesis we exploited S49 mutants with genetically altered intracellular dTTP pools.

Materials and methods

Materials. [6-^{3}H]FdUrd (20 Ci/mmole), [6-^{3}H]FdUMP (20 Ci/mmole) and [^{3}H(G)]nitrobenzyl-6-thioinosine (16 Ci/mmole) were purchased from Moravek Biochemicals (Brea, CA). [8-^{3}H]dATP (17 Ci/mmole), [*methyl*-^{3}H]dTTP (44 Ci/mmole) and [5-^{3}H]dCTP (25 Ci/mmole) were purchased from ICN (Irvine, CA). [*Methyl*-^{3}H]Thymidine (5 Ci/mmole) was purchased from the New England Nuclear Corp. (Boston, MA). Purine and pyrimidine nucleotides were purchased from the Sigma Chemical Co. (St. Louis, MO). Poly[d(A,T)], poly[d(G,C)] and *Escherichia coli* DNA polymerase 1 were purchased from the Miles Chemical Co. (Elkhart, IN).