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ATP-Dependent Calcium Uptake by Rat Liver Plasma Membrane Vesicles

Effect of Alkylating Hepatotoxins *in Vivo*

J. O. TSOKOS-KUHN, E. L. TODD, J. B. MCMILLIN-WOOD, AND J. R. MITCHELL

Institute for Lipid Research and Department of Medicine, Baylor College of Medicine, Houston, Texas 77030

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SUMMARY

Lethal cell injury from hepatotoxic drugs has been postulated to result from an alteration in cell Ca^{2+} homeostasis. ATP-dependent Ca^{2+} uptake by the plasma membrane has a sulfhydryl-dependent functional moiety and, therefore, could be vulnerable to chemically reactive drug intermediates. Thus, alkylating hepatotoxins given *in vivo* were examined for their ability to inhibit Ca^{2+} accumulation by plasma membrane vesicles isolated from livers of adult male rats. ATP-dependent Ca^{2+} accumulation was decreased 62% by bromobenzene, 76% by acetaminophen, and 92% by CCl_4. Mitochondrial Ca^{2+} uptake was minimally affected by the toxins, and only CCl_4 affected Ca^{2+} accumulation by liver microsomes. The effect of acetaminophen on plasma membrane Ca^{2+} uptake was apparent as early as 45 min postdose. Depletion of protective intracellular GSH by diethyl maleate treatment (400 mg/kg) alone minimally decreased control plasma membrane uptake activity, although the GSH depletion markedly potentiated the effect of acetaminophen on the plasma membrane and on necrosis. Alkylation of sites on the plasma membrane may be a key chemical-macromolecule interaction in drug-induced liver necrosis, and inhibition of plasma membrane Ca^{2+} regulation may provide a connecting link between the alkylation hypothesis and the perturbed Ca^{2+} homeostasis hypothesis of lethal cell injury.

INTRODUCTION

Dysfunction of any of three major mechanisms regulating free cytosolic calcium (the sequestration of calcium in mitochondria and endoplasmic reticulum or a disequilibrium of efflux and inflow of calcium across the plasma membrane) may result in a nonphysiologic increase in free cytosolic calcium. Indeed, evidence for the involvement of each of the three mechanisms in cell injury produced by chemically reactive intermediates has been presented for different toxins.

Moore and coworkers (1–3) have shown that the hepatic necrosis produced by CCl_4, carbon disulfide, and 1,1-dichloroethylene is associated with severe inhibition of the capacity of microsomes to sequester calcium. In studies with isolated hepatocytes, Orrenius and colleagues (4–8) have shown that an increase in the mitochondrial NADP:NADPH ratio by metabolism of menadione or *t*-butylhydroperoxide leads to a release of calcium from mitochondria; they further showed that metabolism of some xenobiotics impairs the sequestration of calcium by microsomes by increasing the GSSG:GSH ratio.

Since the technique for measuring mitochondrial and microsomal Ca^{2+} in isolated hepatocytes appears to be dependent on an intact plasma membrane Ca^{2+} pump to translocate intracellular Ca^{2+} outside into the incubation medium where it is measured (7), these investigators subsequently speculated that the resulting redistribution of intracellular calcium with loss of Ca^{2+} from the cell might be the critical event in lethal injury, without the mechanism controlling calcium efflux across the plasma membrane being affected (9). Additional studies by the same group (10) on the effect of three differently acting hepatotoxins (CCl_4, bromobenzene, and ethyl methanesulfonate) on isolated hepatocytes incubated in calcium-free medium also support the conclusion that redistribution of intracellular calcium rather than an uncontrolled influx of calcium plays a critical role in cytotoxicity.

However, evidence has been provided by other investigators (11, 12) under different physiologic conditions that the toxic injury seen in cultured hepatocytes and in the perfused liver is clearly dependent on the extracel-

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lular concentration of calcium, suggesting that an altered flow of calcium through the plasma membrane may be the pivotal event in lethal injury, i.e., either an increased influx or a decreased extrusion by plasma membrane calcium translocation. In support of the latter, we reported a marked inhibition of calcium accumulation by liver plasma membrane vesicles after treatment of rats *in vivo* with acetaminophen or bromobenzene (13), and in recent studies Orrenius and colleagues have shown that *t*-butylhydroperoxide added *in vitro* to liver plasma membrane vesicles inhibits calcium uptake (14) and menadione added to isolated hepatocytes inhibits Ca^{2+},Mg^{2+}-ATPase in plasma membrane fragments (15).

Accordingly, additional studies were carried out under *in vivo* conditions to examine further the hypothesis that inhibition of plasma membrane calcium regulation may be a critical element in the acute toxic cell injury produced *in vivo* by acetaminophen, bromobenzene, and CCl_4. The effects of these alkylating toxins on ATP-dependent calcium accumulation by liver plasma membrane vesicles were contrasted with their effects on mitochondrial and microsomal calcium uptake.

MATERIALS AND METHODS

Animals and toxin administration. Male Sprague-Dawley rats (150–300 g) were obtained from TIMCO (Houston, TX) and maintained in a 12-hr light-dark cycle. They were provided lab chow and water *ad libitum* until 18 hr prior to sacrifice, when fasting was begun. Animals to be treated with bromobenzene or CCl_4 and their controls were pretreated 3 days with phenobarbital (80 mg/kg IP[1]) to induce cytochrome P-450 monooxygenases and hence ensure cytotoxicity of the xenobiotics. Bromobenzene was administered at a dose of 1.5 mmol/kg IP in corn oil (controls were vehicle-injected) and animals were killed by cervical dislocation 4 hr postdose. CCl_4 was administered at a dose of 2 ml/kg IP in corn oil (controls were vehicle-injected); animals were killed at 4 hr postdose. Acetaminophen was administered orally (2.5 g/kg) in 20% Tween 80 solution (controls received Tween 80 only); animals were sacrificed 2.5 hr postdose unless otherwise indicated. Both controls and acetaminophen animals were pretreated 1 day with 3-methylcholanthrene (20 mg/kg IP) to induce monooxygenase. In some experiments, diethyl maleate (400 mg/kg in corn oil) was given to controls and experimental animals 1 hr before dosing with acetaminophen.

Preparation of plasma membrane vesicles. In the usual preparation, the livers from three animals were pooled to comprise a control or a toxin-treated group. Livers were excised, weighed, and finely minced with scissors in ice-cold medium containing 250 mM sucrose, 10 mM HEPES, pH 7.5, and 0.2 mM $CaCl_2$ (to stabilize the nuclear envelope and minimize nuclear contamination).

Following homogenization, EGTA was added to a final concentration of 0.5 mM. Isolation of the "purified fraction" of plasma membrane vesicles was carried out essentially as described by Van Amelsvoort *et al.* (16). Final yields of plasma membrane vesicles were typically about 0.75 mg of protein/g of liver wet weight. In certain experiments, six livers were minced together; then the mince was divided into two portions, one of which was carried through the plasma membrane isolation in the presence of 5 mM reduced glutathione and the other of which was treated as described above. Protein concentrations were measured by the Lowry method (16a). Plasma membrane vesicles prepared as described comprise a mixed population of right-side-out and inside-out vesicles (Ref. 17 and see Results below). Only the inside-out vesicles are capable of ATP-supported calcium accumulation, since the ATP and calcium sites of the pump normally face the cytoplasm of the cell.

Evaluation of vesicle sidedness. Neuraminidase was used to cleave sialic acid residues from intact and Triton X-100-treated plasma membrane vesicles to assess the sidedness of the vesicles as described by Resch *et al.* (18). The amount of sialic acid released was measured by the colorimetric method of Warren (19). The ratio of sialic acid released in the absence of detergent to that released in the presence of detergent estimates the proportion of right-side-out vesicles in the preparation. The ratio may also include an unknown amount of inside-out vesicles exhibiting permeability to neuraminidase.

Calcium uptake assay. ^{45}Ca uptake was measured as described by Kraus-Friedmann *et al.* (17) in a medium containing 100 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES, pH 8.0, 1 mM NaN_3, 20 μM digitoxigenin, 2 μM ruthenium red, 1 mM ATP (when present), and nominally 28.5 ± .95 μM $^{45}CaCl_2$ (specific activity, approximately 7×10^3 cpm/nmol). Calcium concentration in the uptake medium was measured by atomic absorption spectroscopy for each experiment, and actual calcium concentrations were utilized in specific activity computations. The final volume was 3 ml and the temperature was 37°. The reaction was initiated by addition of plasma membranes (100 μg of protein/ml). Timed samples (400 μl) were filtered through Millipore filters (0.45 μm), and washed with 2 ml of 100 mM KCl, 10 mM HEPES, pH 8.0. Zero time incubations were held on ice and samples were taken immediately after protein addition. Filters were placed in Bray's solution for liquid scintillation counting. Counting efficiency was approximately 98%.

To monitor vesicular sequestration of Ca^{2+}, 2 μM A23187 was added after 5 min of uptake, and samples were taken at various times following the addition.

Microsomal and mitochondrial Ca^{2+} transport. Liver microsomes were isolated from control and toxin-treated animals according to Moore *et al.* (1). Calcium uptake was measured in a medium containing 100 mM KCl, 30 mM HEPES, pH 6.8, 5 mM NaN_3, 5 mM $MgCl_2$, 7.5 mM ammonium oxalate, 5 mM ATP, and 20 μM $^{45}CaCl_2$ (3.2 μCi/mmol of Ca^{2+}). The reaction was stopped by rapid Millipore fitration; the filters were washed with 2 ml of 100 mM KCl and 10 mM HEPES, pH 6.8, and then counted in Bray's solution.

Liver mitochondria were isolated as previously described (20). Substrate-supported calcium uptake was measured in a medium containing 110 mM sucrose, 70 mM KCl, 5 mM succinate, 5 μg of rotenone, 1 mM potassium phosphate, pH 7.4, and 50 μM antipyrylazo III in an Aminco DW 2 dual-wavelength spectrophotometer using the wavelength pair 620-642 nm. Ca^{2+} uptake was initiated by addition of $CaCl_2$ (6.7–67 μM) to cuvettes containing 3 ml of medium and 2 mg of mitochondrial protein at 30°.

Materials. Ruthenium red, HEPES, Na_2ATP, diethyl maleate, glutathione (reduced form), digitoxigenin, and A23187 were obtained from Sigma Chemical Co. (St. Louis, MO). Acetaminophen, 3-methylcholanthrene, and CCl_4 were products of Eastman Kodak Co. (Rochester, NY); bromobenzene was purchased from Fisher Scientific (Pittsburgh, PA); and phenobarbital was obtained from Mallinckrodt, Inc. (St. Louis, MO). $^{45}CaCl_2$ was purchased from New England Nuclear (Boston, MA). Other chemicals were reagent grade or better.

Statistical analyses. When appropriate, Student's *t* test (21) was used for statistical analyses with $p < 0.05$ established for significance.

RESULTS

ATP-dependent calcium uptake of liver plasma membrane vesicles. Plasma membrane vesicles isolated from normal rat livers catalyze an ATP-dependent calcium sequestration (Fig. 1) that initially proceeds rapidly but reaches steady state level by about 5 min. This level is maintained for at least 20 min (not shown). The complete release of calcium upon addition of the ionophore A23187 (Fig. 1) indicates that the calcium associated with the

[1] The abbreviations used are: IP, intraperitoneal; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid.

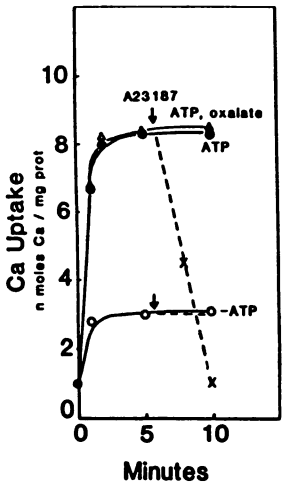

FIG. 1. *Ca^{2+} uptake by rat liver plasma membrane vesicles*

Plasma membrane vesicles (100 μg of protein/ml) were incubated in the presence or absence of 1 mM ATP as described in Materials and Methods. Samples were removed and filtered at indicated times for estimation of ^{45}Ca content. Zero time measurements were also made. The data are representative of three separate determinations in which similar results were obtained. 1 mM ATP (●); 1 mM ATP and 5 mM ammonium oxalate (△); −ATP (○) with A23187 (2 μM) added at 5.5 min (---); 1 mM ATP with A23187 added at 5.5 min (×).

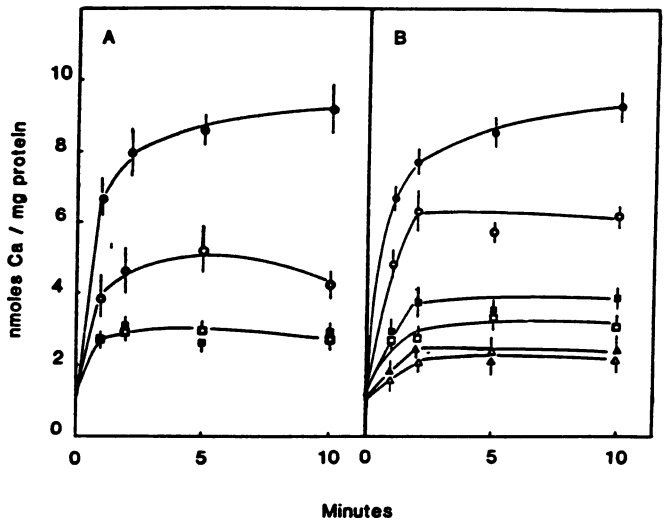

FIG. 2. *Ca^{2+} uptake by liver plasma membrane vesicles of control and toxin-treated animals*

Ca^{2+} uptake was measured as described in Materials and Methods. Data points and bars represent the means ± standard error of determinations from three to six experiments with separate plasma membrane preparations. Doses and times for toxin treatment are given in the text. A, 3-methylcholanthene-treated control + ATP (●); 3-methylcholanthrene control − ATP (■); acetaminophen-treated + ATP (○); acetaminophen-treated − ATP (□). B, phenobarbital-treated control + ATP (●); phenobarbital-treated control − ATP (■); bromobenzene-treated + ATP (○); bromobenzene-treated − ATP (□). CCl_4-treated + ATP (▲); CCl_4-treated − ATP (△). Differences between controls (+ATP) and treated (+ATP) vesicles are significant at $p < 0.05$ in all cases (analysis performed using the *t* test).

vesicles has been accumulated into the vesicular lumen. The Ca^{2+} associated with the vesicles in the absence of ATP is not released into the medium upon addition of A23187 (Fig. 1), suggesting that this Ca^{2+} may be bound to specific membrane sites, but not translocated.

The addition of 5 mM oxalate does not further enhance ATP-supported calcium uptake (Fig. 1). This finding confirms other work (17) and suggests the absence of a mechanism (channel or carrier) for oxalate entry into the vesicles. Further, microsomal calcium accumulation is known to be enhanced by oxalate (1). The effect of oxalate on Ca^{2+} uptake by isolated rat liver microsomes at pH 8 is less pronounced than at pH 6.8, but under incubation conditions like those used for plasma membrane Ca^{2+} transport measurements, microsomal Ca^{2+} uptake is stimulated 50% by oxalate (data not shown). The lack of effect of oxalate on measurements of plasma membrane Ca^{2+} uptake suggests that the microsomal calcium pump does not make a major contribution to these measurements although the plasma membrane preparation may contain up to 40% contamination by membranes of the endoplasmic reticulum as indicated by marker enzyme analysis (data not shown).

Effects of in vivo toxins on plasma membrane calcium transport. When plasma membrane vesicles are obtained from livers of rats treated *in vivo* with the model toxins CCl_4, bromobenzene, or acetaminophen, their ATP-dependent calcium uptake is considerably depressed in comparison to control (Fig. 2, A and B). Uptake at early time points is decreased, and at 10 min, membrane vesicles from acetaminophen-, bromobenzene-, or CCl_4-treated animals have accumulated markedly less Ca^{2+} than their respective controls. ATP-independent Ca^{2+} uptake is not affected significantly by treatment with acetaminophen or bromobenzene. In contrast, membranes from CCl_4-treated animals may accumulate less Ca^{2+} than control membranes in the absence of ATP.

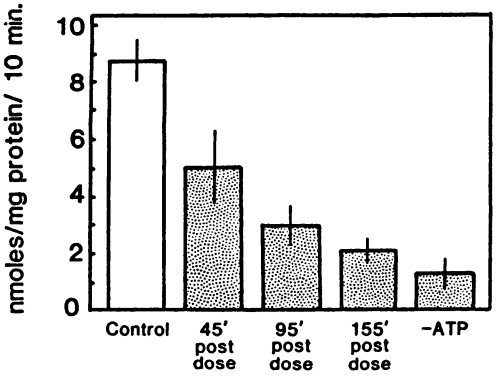

FIG. 3. *Plasma membrane Ca^{2+} transport activity as a function of time of exposure of the animals to acetaminophen*

Rats were pretreated with 3-methylcholanthrene, dosed with acetaminophen, and then sacrificed at 45, 95, or 155 min after treatment for isolation of membranes. Ca^{2+} transport of the plasma membrane vesicles of drug-treated animals and their 3-methylcholanthrene-treated controls was measured as described in Materials and Methods. All bars in the graph represent uptake in the presence of ATP except as noted. Data are the means ± standard deviation of three separate plasma membrane preparations.

A time course of the effect of acetaminophen treatment on calcium uptake activity (Fig. 3) shows a significant decrease in activity as early as 45 min after acetamino-

phen. Activity is further depressed as the time of exposure lengthens to 95 and 155 min.

Effect of toxins on plasma membrane vesicle sidedness. Incubation of plasma membrane vesicles with neuraminidase in the presence and absence of detergent and subsequent analysis of the sialic acid released permit estimation of the sidedness of the vesicles (18). Liver plasma membrane vesicles from phenobarbital- or 3-methylcholanthrene-treated control rats were about 67% right-side-out (Table 1). After bromobenzene and acetaminophen treatment, vesicle sidedness was changed minimally (72.6 and 75% right-side-out). In contrast, after CCl_4 treatment, the proportion of right-side-out vesicles, or of vesicles permeable to neuraminidase, increased to nearly 100%.

Effects of toxins in vivo on microsomal and mitochondrial calcium transport. Microsomes were isolated from the livers of animals treated with bromobenzene, acetaminophen, or CCl_4, and microsomal calcium uptake activities were measured. Microsomes from bromobenzene- and acetaminophen-dosed livers were identical to controls in calcium uptake activity (Fig. 4). CCl_4 treatment produced a marked decrease in activity, as previously reported by Moore and colleagues (1). Mitochondrial calcium uptake was minimally affected by acetaminophen, bromobenzene, or CCl_4 treatment (Fig. 4), although bromobenzene treatment may have produced minor inhibition of mitochondrial calcium accumulation at high Ca^{2+} concentrations (20–67 μM).

Effects of depletion of GSH by diethyl maleate. Experiments were designed to test the hypothesis that changes in the GSH concentration might be responsible for the decrease in plasma membrane calcium uptake activity observed after toxin treatment. Calcium uptake by plasma membranes from control or acetaminophen-dosed animals was examined with and without pretreatment of the animals with diethyl maleate. Although diethyl maleate pretreatment markedly diminishes intracellular glutathione concentrations and is known to increase acetaminophen-induced liver necrosis (22), it has relatively little effect alone on control plasma membrane calcium uptake (Fig. 5). On the other hand, hepatic necrosis (22) as well as inhibition of calcium uptake by membranes from acetaminophen-treated animals (Fig. 5) is potentiated with diethyl maleate pretreatment. Thus, decreased uptake activity correlates with increased necrosis but not with glutathione depletion alone.

TABLE 1

Proportions of right-side-out vesicles in preparations from toxin-treated or control rats

The proportion of right-side-out vesicles is estimated as the ratio of sialic acid released by neuraminidase from vesicles incubated in the absence of detergent to that released in the presence of detergent (18). Data are the mean ± standard error for three separate preparations.

	Right-side-out vesicles		
	Control	Toxin-treated	Significance
	%		
Acetaminophen	67.8 ± 6.9	72.6 ± 9.2	NS
Bromobenzene	71.2 ± 8.5	75.0 ± 6.7	NS
CCl_4	62.6 ± 4.5	97.1 ± 7.2	$p < 0.005$

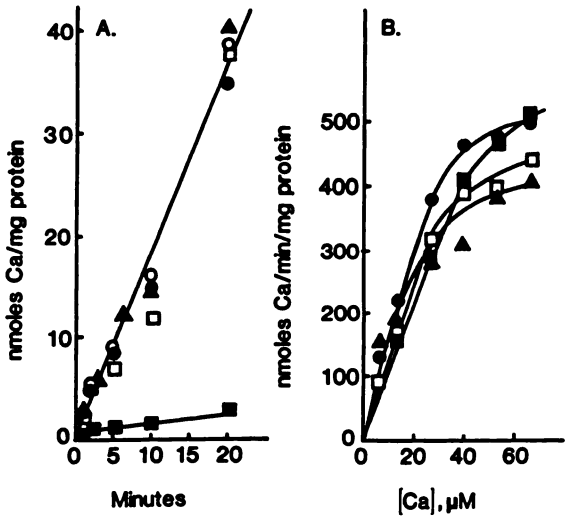

FIG. 4. *Ca^{2+} uptake by liver microsomes and mitochondria from control and toxin-treated rats*

A, liver microsomes were isolated from pooled livers of two animals for each treatment group and Ca^{2+} uptake was measured as described in Materials and Methods. Data points are means of determinations from two separate microsomal preparations. Phenobarbital control (●); 3-methylcholanthrene control (○); bromobenzene, 4 hr (▲); acetaminophen, 2.5 hr (□); CCl_4, 4 hr (■). B, mitochondria were isolated from pooled livers of two animals for each treatment group, and Ca^{2+} uptake was measured as described in the text. Data are means of determinations from two separate experiments. Symbols are as in A except phenobarbital and 3-methylcholanthrene controls were combined (●) to simplify the figure.

Effect of GSH during plasma membrane isolation. Since homogenization and preparation of plasma membranes occur in a depleted glutathione environment following acetaminophen and bromobenzene administration, possible effects of exogenous reduced glutathione in the isolation medium on protection of Ca^{2+} uptake activities were tested. Some stimulation of uptake is observed in the membranes prepared in the presence of glutathione (Fig. 6). However, a similar enhancement of activity is observed when plasma membranes were prepared from control liver under the same conditions (Fig. 6), confirming the report of Bellomo *et al.* (14). However, reversal of the inhibition produced by toxin treatment, which would be expected if a simple shift of the disulfide:thiol equilibrium were responsible for uptake inhibition, is not observed.

DISCUSSION

As previously shown by Kraus-Friedmann *et al.* (17), liver plasma membrane vesicles are capable of an ATP-dependent accumulation of calcium, the calcium being released completely by the ionophore A23187. We have confirmed other observations that the calcium uptake is not influenced by oxalate (17), which, if transported, complexes calcium ions and enhances the retention of calcium within the vesicles. Since oxalate markedly enhances microsomal calcium uptake and no such enhancement is observed here, we believe that contaminating endoplasmic reticulum membranes do not substantially contribute to the measured calcium uptake activities.

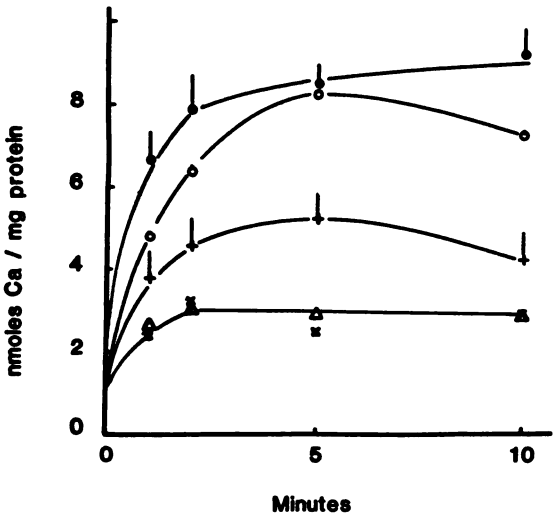

FIG. 5. *Effect of diethyl maleate treatment on Ca^{2+} uptake of plasma membrane vesicles from 3-methylcholanthrene-treated controls and acetaminophen-treated rats.*

3-Methylcholanthrene-treated control + ATP (●); 3-methylcholanthrene- and diethyl maleate-treated control + ATP (○); acetaminophen-treated + ATP (+); acetaminophen, after diethyl maleate + ATP (×); Ca^{2+} uptake − ATP (△). The two progress curves with standard error bars are identical to the +ATP curves in Fig. 2A. The curves for diethyl maleate treatment, both control and acetaminophen groups, were obtained by averaging data from experiments with two separate preparations each obtained from three pooled livers. The −ATP curve (△) is the mean from all four treatment groups; the standard errors were of magnitude similar to the symbols and therefore not demonstrated.

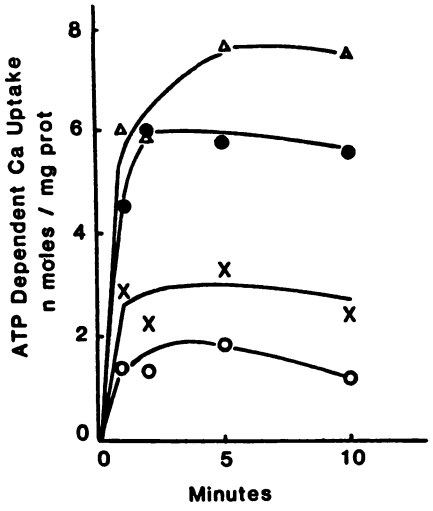

FIG. 6. *Effect of reduced glutathione on control and acetaminophen-depressed Ca^{2+} transport activity of plasma membrane vesicles*

Control and acetaminophen-dosed animals were treated as described in Materials and Methods, with animals killed at 2.5 hr postdose. These experiments were carried out using pooled control livers (six animals) and pooled acetaminophen-treated liver (six animals). Plasma membrane isolations and Ca^{2+} transport were measured as described in the text. Each curve represents data from experiments with two separate plasma membrane preparations. Control (●), control prepared in the presence of GSH (△), acetaminophen-treated (○), acetaminophen-treated prepared in the presence of GSH (×). Note that the uptakes measured in the absence of ATP have been subtracted; net ATP-dependent Ca^{2+} uptake is shown here.

Moreover, since the assay medium contains both azide and ruthenium red to abolish any contribution to calcium uptake by mitochondrial membranes, we believe we are measuring activity specific to the liver plasma membrane.

When plasma membrane vesicles are isolated from livers of animals that have been dosed with one of the model toxins acetaminophen, bromobenzene, or CCl_4, the ATP-dependent calcium uptake is considerably decreased in comparison to controls, most severely by CCl_4 and acetaminophen but also by bromobenzene treatment.

The decreased Ca^{2+} accumulation of plasma membrane vesicles isolated from livers of acetaminophen- or bromobenzene-treated rats does not appear to be caused by substantial alterations in vesicle sidedness or permeability. An increased proportion of right-side-out vesicles or increased vesicle permeability may be responsible, however, for the marked depression of Ca^{2+} accumulation observed in vesicles from livers of CCl_4-dosed rats. Indeed, whether measured in the presence or absence of ATP, Ca^{2+} accumulation by these vesicles is less than observed in any other case tested (Fig. 2). Increased permeability of these membranes would be consistent with the well known peroxidation of microsomal membrane lipids that occurs in CCl_4-poisoned livers (23). Furthermore, we recently have found that CCl_4 treatment *in vivo* produces a marked peroxidation in plasma membrane vesicles, as determined by specific increases in membrane content of 11-, 12-, and 15-hydroxyeicosatetraenoic acids (24).

Mitochondrial Ca^{2+} uptake is not markedly affected by any of the three toxins administered *in vivo*. Effects of CCl_4 (and related simple haloalkanes) on microsomal calcium uptake are well documented (1–3), and more recent studies in the isolated hepatocyte system (9) suggest a microsomal lesion in the *in vitro* cytotoxicity of hydroperoxides. From our data, however, acetaminophen and bromobenzene treatment *in vivo* does not affect the activity of the microsomal calcium pump. Thus, microsomal Ca^{2+} pump inhibition may not represent a common pathway of lethal cell injury for hepatic toxins in general.

In further studies focusing on acetaminophen toxicity, a marked decrease in plasma membrane calcium uptake activity is observed as early as 45 min after acetaminophen, and becomes progressively more profound at 95 and 155 min after treatment. An effect established so early may well be a critical event in the progression from reversible to irreversible injury in the acetaminophen-poisoned liver.

Although glutathione depletion by diethyl maleate pretreatment *in vivo* exacerbates the deleterious effect of acetaminophen treatment on necrosis and on plasma membrane calcium uptake activity, there is only a slight effect on calcium uptake activity in diethyl maleate-treated controls. If alteration in GSH concentration alone were sufficient to produce the loss of plasma membrane calcium pump activity, then controls should be affected by diethyl maleate pretreatment, which depletes

liver intracellular glutathione similarly to acetaminophen (22). It is clear that glutathione depletion by itself is not requisite for inhibition of plasma membrane Ca^{2+} regulation by hepatic toxins.

In conclusion, we have shown that administration *in vivo* of three model toxins that produce liver necrosis after conversion to alkylating metabolites markedly impairs ATP-dependent calcium uptake by hepatic plasma membrane vesicles. None of the three toxins appears to have a physiologically relevant effect on mitochondrial calcium accumulation and only carbon tetrachloride inhibits microsomal calcium uptake. Thus, the inhibition of plasma membrane calcium regulation may be a distinct biochemical lesion providing a connecting link between two major hypotheses of chemical-induced liver necrosis, i.e., alkylation and altered calcium homeostasis.

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Send reprint requests to: Dr. Janice O. Tsokos-Kuhn, Room 826E, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.