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ALKYLATION OF THE LIVER PLASMA MEMBRANE AND INHIBITION OF THE Ca^{2+} ATPase BY ACETAMINOPHEN

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Abstract—Acetaminophen is activated metabolically to yield reactive species that bind covalently to liver cell macromolecules. The extent of covalent binding correlates with the occurrence and severity of hepatic necrosis. We reported previously [J. O. Tsokos-Kuhn, E. L. Todd, J. B. McMillin-Wood and J. R. Mitchell, *Molec. Pharmac.* **28**, 56 (1985)] that active Ca^{2+} accumulation of isolated liver plasma membranes is decreased 60–75% after a hepatotoxic dose of acetaminophen *in vivo*. We now report that the protein of isolated liver plasma membranes was substantially labeled with drug metabolites after administration of [^{3}H]acetaminophen. There was no increase in passive membrane permeability that might cause diminished Ca^{2+} accumulation. Intravesicular volume and relative purity of the vesicle preparations after acetaminophen were not different from controls. However, (Ca^{2+},Mg^{2+})-ATPase, a possible biochemical expression of the Ca^{2+} pump, was decreased 31% ($P < 0.025$) after acetaminophen treatment. ATPase activity in both control and treated groups was enhanced by isolating membranes in the presence of 5 mM reduced glutathione (GSH), but the effects of drug treatment were not reversed. A similar effect of GSH on Ca^{2+} accumulation was observed previously [J. O. Tsokos-Kuhn, E. L. Todd, J. B. McMillin-Wood and J. R. Mitchell, *Molec. Pharmac.* **28**, 56 (1985)]. These data are consistent with a hypothesis wherein alkylation of membrane proteins by reactive acetaminophen metabolites is a factor in the onset of hepatic necrosis after acetaminophen. They are not consistent with an oxidative stress hypothesis where thiol S-thiolation of membrane components is postulated to produce altered membrane permeability or thiol-reversible alterations in membrane protein structure and enzymatic function.

Acute lethal injury of hepatic cells may result from administration of high doses of the common analgesic drug, acetaminophen, in humans or experimental animals [1, 2]. Acetaminophen is metabolically activated to yield an electrophilic species, apparently *N*-acetyl-*p*-benzoquinoneimine, which can bind covalently to liver cell macromolecules, particularly if cellular glutathione is depleted [3]. The correlation between the extent of covalent binding of acetaminophen metabolite to hepatic proteins and the occurrence and severity of hepatic necrosis produced by acetaminophen is well documented [3, 4] and provides a useful working hypothesis as an interaction contributing to the initiating events in hepatic necrosis produced by acetaminophen.

The role of altered Ca^{2+} homeostasis as a possible unifying mechanism of acute lethal cell injury by toxins, ischemia, and other agents has attracted much interest [5]. Many cellular processes are regulated or affected by cytosolic free Ca^{2+} concentrations, which in turn are controlled by membrane transport systems of the plasma membrane, the endoplasmic reticulum, and the mitochondria. Inhibition of microsomal Ca^{2+} uptake following CCl_4, CS_2, and 1,1-dichloroethylene administration has been reported by Moore *et al.* [6–8]. Experiments with substances producing oxidative stress, primarily carried out in isolated hepatocytes, have led Orrenius and colleagues to suggest enhanced release of Ca^{2+} from mitochondria [9], inhibition of microsomal Ca^{2+} sequestration [10], and inhibition of the plasma membrane Ca^{2+} pump [11] as mechanisms of oxidant stress-related cytotoxicity.

Recently, we reported [12] that the ability of isolated liver plasma membrane vesicles to carry out ATP-dependent Ca^{2+} accumulation is strikingly decreased after treatment of rats with acetaminophen, bromobenzene, or CCl_4. Subsequently, we demonstrated an increased permeability of the plasma membranes isolated after CCl_4 treatment [13]. The present paper describes a study aimed at testing the hypothesis that changes in plasma membrane Ca^{2+} metabolism observed in acetaminophen hepatotoxicity [12] occur in conjunction with the alkylation of sensitive membrane protein sites by reactive metabolites of acetaminophen, and not by protein thiol S-thiolation occurring due to oxidative stress.

MATERIALS AND METHODS

Animal model and toxin treatment. Male Sprague–Dawley rats weighing 175–275 g (Harlan, Houston, TX) were pretreated, as previously described [12], with 3-methylcholanthrene (20 mg/kg, i.p.) 18 hr

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before acetaminophen administration. Animals were fasted for 18 hr. Acetaminophen was administered (2.5 g/kg, p.o.) in 20% Tween 80 solution, while controls received 20% Tween 80 alone; the animals were killed 2.5 hr post-dose.

Preparation of plasma membrane vesicles. Liver plasma membranes (the "purified fraction") were isolated according to van Amelsvoort *et al.* [14] and suspended in 100 mM KCl, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.5, to a final concentration of 5–10 mg protein/ml. Protein was estimated by the method of Lowry *et al.* [15] using bovine serum albumin as standard.

(Ca^{2+},Mg^{2+})ATPase assay. ATPase was measured as described by Lotersztajn *et al.* [16] except that 70 mM KCl or 140 mM sucrose was added to make the assay medium isotonic. Activity in the presence of ethyleneglycolbis(aminoethylether)-tetra-acetate (EGTA) was subtracted from that observed in the presence of Ca^{2+} to obtain the Ca^{2+}-dependent ATPase activity. Linear activity versus [protein] plots were typically employed to evaluate specific activities by linear regression analysis, but in some cases triplicate assays at a single protein concentration and time were employed.

Marker enzyme assays. Glucose-6-phosphatase, a microsomal marker enzyme, was assayed according to Baginski *et al.* [17]. The mitochondrial marker, azide-sensitive ATPase, was assayed as described by Pitts [18]. 5'-Nucleotidase, a plasmalemmal enzyme, was assayed in a medium containing 90 mM Tris–HCl, pH 8.0, 5 mM 5'-AMP, 10 mM $MgCl_2$ and 0.05% Triton X-100, and inorganic phosphate release was measured according to Baginski *et al.* [17].

Ca^{2+} efflux from plasma membrane vesicles. Ca^{2+} efflux was measured by a Millipore filtration method as described by Campbell *et al.* [19]. Vesicles were loaded by incubating for 18–24 hr at 0° in 100 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES, pH 8.0 (KMH), with 1 mM $^{45}CaCl_2$ (sp. act. 1.5×10^4 cpm/nmol). Efflux of $^{45}Ca^{2+}$ was initiated by dilution of an aliquot of loaded vesicle suspension into 6 ml of ice-cold KMH containing 2 mM EGTA. Samples were removed at timed intervals, filtered, and washed; filters were counted in Bray's solution or Liquiscint for estimation of Ca^{2+} content of the vesicles. After 5 min of efflux in the presence of EGTA, the ionophore A23187 (1.6 μM) was added, and additional samples were obtained. Each experiment was repeated twice with each of five separate control and acetaminophen preparations.

Determination of calcium-loading levels at zero time. Calcium associated with plasma membrane vesicles after 18-hr equilibration with 1 mM $^{45}Ca^{2+}$ was measured in two ways. In the first of these, samples (10, 20, 30, and 40 μl, where 10 μl contained 0.02 to 0.04 mg protein) were removed from the loading suspension, filtered, and washed with 10 ml of ice-cold 100 mM KCl, 10 mM HEPES, pH 7.5, 1 mM $LaCl_3$. Duplicate series of such samples were obtained from each suspension. Filters were counted in Liquiscint. In the second method, samples were taken in the same way but applied to Dowex 50W-X8 ($Tris^+$ form) columns (prepared in Pasteur pipettes) to remove extravesicular Ca^{2+}, and eluted with 1 ml of 175 mM sucrose. Eluted vesicle suspensions were counted in entirety after addition of Liquiscint. Good agreement between the two methods was obtained.

^3H_2O and [^{14}C]carboxydextran-accessible volumes. Samples prepared in microcentrifuge tubes were 0.2 to 0.3 mg plasma membrane protein in a total volume of 400 μl containing 100 mM KCl, 10 mM HEPES, pH 7.5, plus approximately 0.4 μCi ^3H_2O and 0.25 μCi [^{14}C]carboxydextran (M_r 5–7×10^4). After 10-min equilibration at room temperature, samples were centrifuged for 15 min at 32,000 *g*. Aliquots of the supernatant fractions were removed for counting, tubes were decanted and briefly rinsed with 100 mM KCl, 10 mM HEPES, pH 7.5, and the pellets were suspended in 50 μl of KCl, HEPES and counted in entirety after addition of Liquiscint. Counting was in a dual channel scintillation counter, and efficiencies of ^{14}C and ^{3}H in the two channels were obtained by counting of commercial quenched standards. The ratio of ^{3}H dpm/mg protein in the pellet to ^{3}H dpm/ml in the supernatant fraction estimates the total pellet volume per mg protein. Similarly, the ratio of ^{14}C dpm/mg protein in the pellet to ^{14}C dpm/ml in the supernatant fraction estimates the carboxydextran-accessible volume per mg protein. The difference between them is the carboxydextran-excluded volume, which equals intravesicular volume plus membrane volume per mg protein.

Covalent binding of acetaminophen. Rats were pretreated with 3-methylcholanthrene and fasted as described above before administration of 2.5 g/kg (p.o.) of [^{3}H]acetaminophen. Plasma membranes were prepared from livers in the standard manner, and microsomes were isolated by centrifuging the post-mitochondrial supernatant fraction at 100,000*g* for 1 hr. Protein was precipitated from plasma membrane and microsomal homogenates by the addition of 4 vol. of methanol. Covalently bound material was determined following extensive solvent extraction by liquid scintillation counting [20].

Materials. Acetaminophen and 3-methylcholanthrene were purchased from the Eastman Kodak Co. (Rochester, NY). Nucleotides, neuraminidase, glutathione, *N*-acetyl neuraminic acid, glucose-6-phosphate, Triton X-100 and HEPES were obtained from the Sigma Chemical Co. (St. Louis, MO). $^{45}CaCl_2$, ^3H_2O, [^{14}C]carboxydextran, and [^{3}H]acetaminophen were purchased from New England Nuclear (Boston, MA). [^{3}H]Acetaminophen was purified prior to use by thin-layer chromatography [3]. Other chemicals were reagent grade or better.

RESULTS

Covalent binding of acetaminophen metabolites to plasma membranes. Acetaminophen metabolites bind covalently to hepatic protein, and the extent of binding correlates with the severity of hepatocellular injury. When rats were administered [^{3}H]acetaminophen and plasma membranes were isolated from those livers after 2.5 hr, substantial labeling of the membrane protein with drug metabolite was observed (Table 1). Membrane preparations were

Table 1. Covalent binding of [^{3}H]acetaminophen to liver plasma membranes and microsomes isolated 2.5 hr after acetaminophen administration *in vivo*

Expt.	Acetaminophen administered (g/kg)	Acetaminophen bound (nmol/mg plasma membrane protein)	(nmol/mg microsomal protein)
1	2.5	0.71	
2	2.2	0.48	0.59
3	2.5	0.41	0.60

extracted and washed extensively to remove radiolabeled metabolites not bound covalently to protein. The conclusion that the binding is to elements of the plasma membrane rather than to contaminating protein of alternate origin, such as the endoplasmic reticulum, is supported by our previous report that in the worst case no more than 40% endoplasmic reticulum contamination of the plasma membranes occurs [13]. Because covalent binding of acetaminophen to endoplasmic reticulum membranes was only slightly greater than to plasma membranes (Table 1), contamination by these membranes at most would account for less than half of the binding in the plasma membrane fraction.

Effect of acetaminophen in vivo *on plasma membrane* (Ca^{2+},Mg^{2+})*ATPase.* In previous work [12], we showed that acetaminophen treatment of rats produces a substantial decrease of ATP-dependent Ca^{2+} accumulation in liver plasma membranes isolated from 1 to 2.5 hr after drug administration. If a decrease in the catalytic activity of the ATP-driven Ca^{2+} pump were the basis of the effect, the Ca^{2+}-stimulated ATPase, a putative expression of the pump activity, should exhibit diminished activity as well. We measured (Ca^{2+},Mg^{2+})ATPase as described by Lotersztajn *et al.* [16], which activity is thought likely to be associated with the Ca^{2+} transport activity in plasma membranes [21–23].

When (Ca^{2+},Mg^{2+})ATPase activity was measured in liver plasma membranes obtained from acetaminophen-intoxicated rats, the activity was decreased substantially in comparison to controls (Fig. 1A). The average inhibition was 32 ± 3% (mean ± SEM), and represented a highly significant decrease ($P < 0.025$) (Table 2). ATP hydrolysis was proportional to plasma membrane protein concentration (Fig. 1A) and was linear for at least 10 min under the assay conditions; in different experiments 5-, 6-, or 10-min incubations were carried out. Because added Mg^{2+} markedly enhances an interfering Mg^{2+}-ATPase activity that is present in native plasma membranes, and obscures the activation of ATP hydrolysis by Ca^{2+}, as described previously [16], assays contained only Mg^{2+} introduced by reagents and membrane fractions alone. Ca^{2+} was buffered with EGTA (400 μM total Ca^{2+} plus 400 μM EGTA

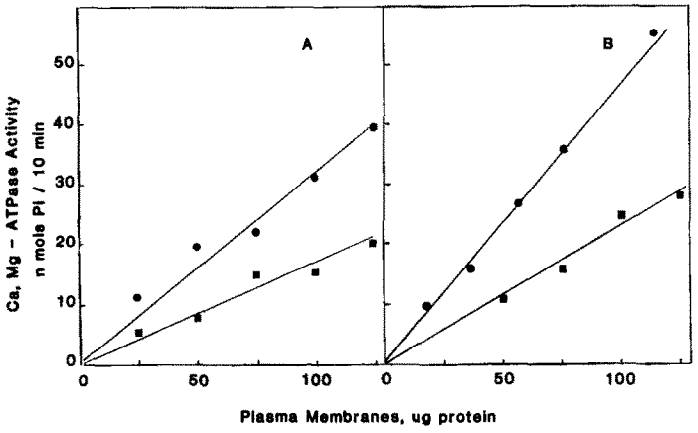

Fig. 1. (Ca^{2+},Mg^{2+})ATPase activity and effect of the presence of GSH during isolation. Control and acetaminophen-dosed animals were treated as described in Materials and Methods. Experiments were carried out using six pooled control livers and six pooled acetaminophen livers, and in each case the liver tissue was divided into two equal amounts after mincing. One portion was carried through the plasma membrane isolation procedure as usual, the other in the presence of 5 mM added reduced glutathione throughout. (Ca^{2+},Mg^{2+})ATPase was measured as described [16]. (A) Control (●) and acetaminophen (■) plasma membranes were isolated in the absence of added GSH, and ATPase activity was determined as a function of the amount of plasma membrane protein in the assay. (B) Plasma membranes were isolated in the presence of 5 mM GSH, and ATPase was measured as in A (symbols also as in A). The specific activities of the enzyme (slopes of the lines) in both control and acetaminophen membranes increased about 50% after isolation in GSH: 31.5 to 47.1 nmol/min/mg protein for control; 17.5 to 27.5 nmol/min/mg protein in acetaminophen membranes. Data shown are representative of those obtained in three similar sets of experiments.

Table 2. (Ca^{2+},Mg^{2+})ATPase activity of liver plasma membranes from control and acetaminophen-treated rats

Expt.	(Ca^{2+},Mg^{2+})ATPase activity (nmol P_i/min/mg protein)		% Inhibition
	Control	Acetaminophen	
1	42.0	32.0	24
2	72.9	49.7	32
3	41.9	40.7	3*
4	41.4	32.3	22
5	29.2	15.1	48
6	45.5	32.5	29
7	45.0	32.3	28
8	50.9	35.1	31
9	48.1	28.7	40
Mean ± SEM	46.9 ± 4.4	32.3 ± 3.3†	32 ± 3

(Ca^{2+},Mg^{2+})ATPase was measured by the method described [16] in liver plasma membranes isolated from controls or acetaminopmen-treated rats 2.5 hr after gavage with vehicle or drug. In each experiment, livers of two or three rats were pooled for each membrane preparation. Ca^{2+} uptake was also measured in the same preparations (data not shown), and inhibition of uptake correlated well with inhibition of the ATPase. Rates of inorganic phosphate release in the presence of excess EGTA were subtracted from the rates measured in the presence of 1 μM free Ca^{2+} to obtain the Ca^{2+}-dependent ATPase activity.

* Ca^{2+} transport was also not inhibited in Expt. 3, and these values were excluded from the means.

† Significantly different from control as assessed by Student's unpaired *t*-test ($P < 0.025$).

corresponding to a free Ca^{2+} concentration of 1 μM); 1 μM free Ca^{2+} is more than sufficient to saturate the (Ca^{2+},Mg^{2+})ATPase, which exhibits $[S]_{0.5}$ for calcium of about 12 nM [16].

We reported previously [12] that both control and acetaminophen plasma membranes exhibit enhanced Ca^{2+} accumulation when the isolation of membrane vesicles is carried out in the presence of 5 mM reduced glutathione. (Ca^{2+},Mg^{2+})ATPase activity was similarly increased by glutathione in such preparations (Fig. 1B). However, the inhibition of (Ca^{2+},Mg^{2+})ATPase produced by acetaminophen treatment was not reversed by exposure to glutathione. That might be the result expected if a shift of the intracellular disulfide:thiol equilibrium were responsible for altered plasma membrane Ca^{2+} pump activity, as postulated by Bellomo *et al.* [11]. On the other hand, the observation that both activities were affected similarly by GSH exposure in these preparations suggests that at least a portion of the Ca^{2+}-dependent ATPase activity measured as described by Lotersztajn *et al.* [16] is associated with the plasma membrane Ca^{2+} pump.

Effect of acetaminophen treatment on purity and vesicle polarity of plasma membrane preparations. Acetaminophen treatment had no effect on the specific activities of marker enzymes of contaminating organellar membranes in the liver plasma membrane preparations (Fig. 2). Glucose-6-phosphatase, an endoplasmic reticulum marker, azide-sensitive ATPase, a mitochondrial inner membrane marker, and 5′-nucleotidase, a plasma membrane marker, were present at similar specific activities in control and acetaminophen preparations. Thus, decreased Ca^{2+} accumulation [12] and (Ca^{2+},Mg^{2+})ATPase activity observed in plasma membranes of acetaminophen-intoxicated livers are not attributable to increased contamination by mitochondrial or endoplasmic reticulum protein. The plasma membrane vesicle preparations used in this study contain a mixed population of inside-out, right-side-out, intact, and leaky vesicles [12, 13]. Vesicles of control and acetaminophen-treated rats release similar amounts of sialic acid when treated with

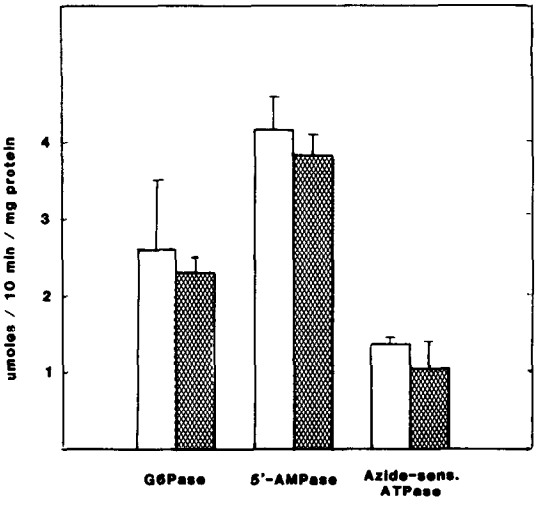

Fig. 2. Activities of glucose-6-phosphatase, 5′-nucleotidase, and azide-sensitive ATPase, markers for endoplasmic reticulum, plasma and mitochondrial membranes, respectively, in isolated plasma membrane vesicles. Open bars (mean ± SEM, N = 6) represent control membranes; cross-hatched bars, acetaminophen membranes (N = 6). There was no significant difference in any of the three activities when acetaminophen and control membranes were compared.

neuraminidase in the presence and the absence of detergent [12], indicating that control and acetaminophen preparations contain similar amounts of intact inside-out plasma membrane vesicles that should be capable of Ca^{2+} accumulation.

Efflux of passively loaded Ca^{2+} from plasma membrane vesicles of control and acetaminophen-treated rats. A possible basis of the acetaminophen-induced decrease in Ca^{2+} accumulation [12], at least in part, is that the Ca^{2+} transporting vesicles of such preparations might be more permeable to accumulated calcium ions than controls. To test this, vesicles were passively loaded with $^{45}Ca^{2+}$ at 0°, and efflux was measured as a function of time after dilution of aliquots of loaded vesicles into EGTA-containing medium. Both inside-out and right-side-out vesicles are present but the proportions are the same in both control and acetaminophen vesicles, as indicated in the previous section, so passive Ca^{2+} efflux from these vesicle preparations can be compared directly. Because the Ca^{2+}-loaded vesicles are diluted into a large excess of EGTA and Ca^{2+} lost from vesicles is immediately chelated, essentially unidirectional efflux of Ca^{2+} from the vesicles is measured. Similar Ca^{2+} efflux profiles were obtained with both control and acetaminophen vesicle preparations (Fig. 3). Zero time values plotted on the ordinate were obtained by sampling the loading suspension directly, filtering, and washing. Zero time Ca^{2+} contents were: control, 13.5 ± 0.8 nmol/mg protein; acetaminophen, 12.9 ± 0.6 nmol/g protein (mean ± SEM, N = 5). The first-order rate constants

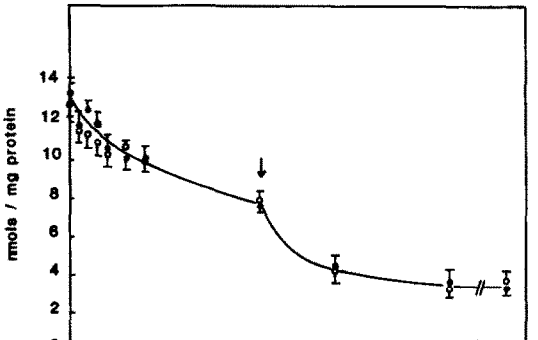

Fig. 3. Passive calcium efflux from control and acetaminophen plasma membrane vesicles. The results of efflux experiments with five separate preparations in each group were averaged and the nanomoles of calcium associated with the vesicles plotted versus time after 30-fold dilution of calcium-loaded vesicles into medium containing 2 mM EGTA. After 5 min (see discontinuity in the progress curve), ionophore A23187 (1.6 μM) was added (arrow). Data are mean ± SE. Key: 3-methylcholanthrene control vesicles (●); acetaminophen vesicles (○). Semilogarithmic plots of the same data (not shown) were used to evaluate first-order rate constants of the initial rapid phase (0–1 min) and the subsequent slower phase (1–5 min) of efflux by taking the least squares fit of each linear portion. For control vesicles, the first-order rates of calcium efflux were 0.19 and 0.081 min^{-1} for the rapid and slow phases respectively. For acetaminophen vesicles, the corresponding values were 0.20 and 0.073 min^{-1} respectively.

for the initial phase of efflux (up to 1 min) did not differ (control, 0.19 min^{-1}; acetaminophen, 0.20 min^{-1}). Thus, acetaminophen membranes were no more leaky to Ca^{2+} than controls, providing evidence that inhibition of Ca^{2+} accumulation [12] may arise from altered function of the plasma membrane Ca^{2+} pump *per se*. These data contrast with those obtained in similar experiments with liver plasma membranes from CCl_4-treated rats [13], where markedly increased permeability accompanied the decrease in Ca^{2+} accumulation.

When the ionophore A23187 (1.6 μM) was added to control vesicles after 5 min of efflux in EGTA medium, a considerable additional effux of Ca^{2+} was elicited. A23187 addition to acetaminophen vesicles produced a virtually identical response (Fig. 3). Control vesicle Ca^{2+} content after efflux in 2 mM EGTA for 5 min was 7.71 ± 0.41 nmol/mg protein versus 7.95 ± 0.50 for acetaminophen (mean ± SE, N = 5 each), i.e. no significant difference was found. The amount of Ca^{2+} associated with the vesicles 8 min after addition of ionophore A23187 may be taken to represent Ca^{2+} tightly bound to the membranes. There was no significant difference in Ca^{2+} bound by control and acetaminophen membranes. Values obtained were: control, 3.40 ± 0.35 nmol/mg protein; acetaminophen, 3.65 ± 0.49 nmol/mg protein.

Estimation of intravesicular volume of acetaminophen plasma membrane vesicles. To ensure that control and acetaminophen membrane vesicles were of comparable internal volumes, total pellet volume and carboxydextran-accessible volume per mg protein were measured. The difference between these gives the carboxydextran-excluded volume per mg protein, equal to the intravesicular volume plus the volume occupied by the membranes. Table 3 summarizes total pellet volume and carboxydextran-excluded volume per mg protein of several preparations of control and acetaminophen vesicles. The acetaminophen vesicles did not differ from control vesicles in carboxydextran-excluded volume per mg protein or in total pellet water. These data compare well to those obtained with controls in the CCl_4 study [13], where we measured a carboxydextran-excluded volume of 4.1 ± 0.5 μl/mg protein, but contrast with the values obtained with CCl_4 membranes, which were significantly larger, 6.1 ± 0.1 μl/mg protein.

DISCUSSION

We report here evidence that acetaminophen treatment of rats, in doses known to engender covalent binding of acetaminophen metabolites to hepatic tissue and cause hepatocellular necrosis [2–4], produced covalent binding to plasma membranes and decreased (Ca^{2+},Mg^{2+})ATPase activity of the membranes. We demonstrated earlier a 60–75% decrease in ATP-dependent Ca^{2+} accumulation in these liver plasma membrane vesicles [12].

Plasma membrane vesicles isolated from acetaminophen-intoxicated livers were not significantly different from controls in sidedness or permeability, in amounts of contaminating organellar membranes present, intravesicular volumes, or passive Ca^{2+} binding capacity. The decrease in active

Table 3. Total pellet water volume, [^{14}C]carboxydextran-accessible and -inaccessible volumes, and estimated intravesicular volumes for plasma membranes of control and acetaminophen-treated rats

	^3H_2O	[^{14}C]Carboxydextran	[^{14}C]Carboxydextran-excluded	Estimated intravesicular volume
		(μl/mg protein)		
Control	13.8 ± 1.2 (5)	9.85 ± 1.54 (5)	3.95 ± 0.59 (5)	2.95
Acetaminophen	13.2 ± 0.4 (4)	9.12 ± 1.08 (4)	4.03 ± 0.69 (4)	3.03

Total pellet water space and carboxydextran-accessible space were determined as described in Materials and Methods, with ^3H_2O and [^{14}C]carboxydextran, and 0.15 to 0.3 mg plasma membrane protein per sample. Three to six replicate samples were prepared for each of the separate preparations tested. Data are mean ± SEM (N). Averaged carboxydextran-accessible volumes were subtracted from averaged total pellet water values to obtain the carboxydextran-excluded spaces given in the table. Intravesicular volumes were estimated by subtracting the value (previously determined [13]) of 1 μl/mg of membrane volume from the carboxydextran-excluded space.

Ca^{2+} transport with no evidence for altered membrane permeability or other changes in vesicle properties suggests that at least one molecular lesion produced by acetaminophen *in vivo* involves a direct effect on the liver plasma membrane Ca^{2+} pump normally responsible for extrusion of excess cytosolic Ca^{2+} from the cell. Digitoxigenin-sensitive (Na^+,K^+)ATPase, another plasma membrane ion pump, was largely unaffected after acetaminophen (J. O. Tsokos-Kuhn, unpublished work), further suggesting that the observed decreases in Ca^{2+} transport [12] and Ca^{2+}-dependent ATPase activity may be a relatively specific phenomenon.

The relationship of these findings to the observed inhibition of (Ca^{2+},Mg^{2+})ATPase has been clouded by a controversy regarding the identity of the ATPase. Lin [24, 25] recently reported that the liver plasma membrane (Ca^{2+},Mg^{2+})ATPase activity, for which a successful assay was first introduced by Lotersztajn and coworkers [16], is not identical with the plasma membrane Ca^{2+} pump as was generally thought to be the case [21, 22]. Rather, Lin [25] attributed the activity to a separate ectoenzyme with different nucleotide specificity, inhibitor sensitivity, and K_ms values for Ca^{2+} and Mg^{2+} than the Ca^{2+} pump. Lin's ATPase studies [24, 25] were carried out with the same plasma membrane preparation and assay reported by Lotersztajn *et al.* [16]; her Ca^{2+} pump studies were carried out in a reconstituted system [24]. More recent work by Pecker and coworkers [23], however, indicates that the two activities are the expression of the same protein, since the purified ATPase reconstituted into soybean phospholipid vesicles catalyzes Ca^{2+} uptake with characteristics similar to those observed in native membranes. Similarly, Lin [25] indicates that the molecular weight of the ecto ATPase is approximately 70 kD. In contrast, the phosphorylated form of the Ca^{2+} pump protein was shown to be 105–110 kD [22, 23].

Difficulty arises in comparing the Ca^{2+}-ATPase and Ca^{2+} transport activities in native plasma membranes, such as the Van Amelsvoort *et al.* [14] preparation we have employed, because of the sizeable difference in their maximal reaction velocities, as previously noted by others [21]. These rates vary with individual preparations, but 10–12 nmol Ca^{2+}/mg protein/min and 40–50 nmol phosphate/mg protein/min are typical for normal Ca^{2+} uptake and Ca^{2+}-dependent ATPase, respectively, in our hands. Therefore, the apparent ATP:Ca^{2+} stoichiometry is 4 to 5, too large for the ATP hydrolysis to be the result of the action of the Ca^{2+} pump alone. Ca^{2+} pumps in some other membrane systems exhibit a stoichiometric ratio of 0.5 to 1 ATP per Ca^{2+} transported. On the other hand, if the ATPase inhibition observed here were the result of inhibition of an ecto ATPase, it would be necessary to postulate alkylation of an external membrane site by the reactive acetaminophen metabolites generated intracellularly. Furthermore, if the activity we measure is a composite of two activities, then the measured inhibition of total ATPase may represent a substantial underestimation of the actual inhibition of the Ca^{2+} pump.

In any event, these data are consistent with a hypothesis involving alkylation of plasma membrane protein(s), possibly the plasma membrane Ca^{2+} pump enzyme or related regulatory protein(s), by reactive species derived from metabolic activation of acetaminophen. These data do not support an oxidative stress hypothesis, wherein decreased GSH:GSSG ratios are postulated to lead to oxidation of membrane lipid and/or protein components with resulting altered membrane permeability and/or thiol-reversible protein structural alterations [11, 26, 27].

Acetaminophen toxicity correlates with covalent binding, and specifically to protein, since covalent binding to lipids by acetaminophen does not occur *in vivo* [28]. Acetaminophen hepatotoxicity *in vivo* does not correlate with lipid peroxidation in liver, and we found no evidence for the presence of increased lipid oxidation products in plasma membranes isolated from acetaminophen-treated rats (H. Hughes *et al.*, unpublished results). Our recent study [29] of acetaminophen hepatoxicity in which pretreatment of Fischer-344 rats with bis(2-chloroethyl)-*N*-nitrosourea inhibited glutathione reductase by 73%, but did not potentiate acetaminophen hepatotoxicity, or produce increased biliary efflux of GSSG or measurable depletion of hepatic protein thiol content in response to a hepatotoxic dose of acetaminophen, similarly demonstrated that oxi-

dative stress mechanisms are unlikely to play a major role in acetaminophen hepatotoxicity. In contrast, in CCl_4 intoxication, a marked increase occurs in plasma membrane content of 11-, 12-, and 15-hydroxyeicosatetraenoic acids [13] concomitant with markedly decreased Ca^{2+} accumulation [12] and evidence of increased membrane permeability in the plasma membrane [13].

Thus, data reported here and elsewhere indicate that these two alkylating toxins both seem to be capable of producing alterations in hepatocellular Ca^{2+} regulation, as well as liver necrosis, but by quite different mechanisms.

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