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Evidence for Increased Membrane Permeability of Plasmalemmal Vesicles from Livers of Phenobarbital-Induced CCl_4-Intoxicated Rats

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SUMMARY

We have observed a marked increase in Ca^{2+} permeability of plasma membranes isolated from rats treated *in vivo* with CCl_4 (2 ml/kg), after phenobarbital induction and overnight fast. Regulation of intracellular free Ca^{2+} is vital to cell viability and function, and the increased plasma membrane permeability, if representative of a change occurring *in vivo*, may be a critical biochemical determinant of CCl_4-induced hepatic necrosis. Permeability to small cations of liver plasma membrane vesicles of control and CCl_4-dosed rats was tested by two independent methods: 1) Ca^{2+} efflux after passive loading in 1 mM Ca^{2+}, and 2) $^{86}Rb^+$ uptake driven by valinomycin-induced K^+ diffusion potential after 100 mM KCl-equilibrated vesicles were stripped of external K^+ by cation exchange. Both indicated markedly increased permeability in plasma membranes after CCl_4 *in vivo*. First order rate constants of biphasic Ca^{2+} efflux were 0.272 and 0.0516 min^{-1} for controls and 1.78 and 0.171 min^{-1} for vesicles from CCl_4-treated animals. $^{86}Rb^+$ uptake by CCl_4 vesicles was 47% of control. Total calcium contents of plasma membranes (prepared in the absence of EGTA) by atomic absorption were 17.4 ± 2.0 (control) and 10.9 ± 1.2 (CCl_4) nmol/mg of protein ($\bar{X}$ ± SE, $p < 0.025$). In correlation with altered biochemical function, we found 4-fold increases in the content of 11-, 12-, and 15-hydroxyeicosatetraenoic acids in plasma membranes of CCl_4-treated rats. Although these specific oxidized fatty acids are unlikely to be ionophores, the ionophoretic properties of certain other oxygenated polyunsaturated fatty acids suggest a mechanism whereby accumulation of lipid oxidation products may be responsible for the altered membrane permeability we have observed after CCl_4, and perhaps ultimately for cell death in CCl_4-induced hepatic necrosis.

Both covalent binding of CCl_4 metabolites to microsomal proteins and lipids (1) and microsomal lipid peroxidation (2) may be important in the chain of events leading to pathological changes in metabolism and eventual cell death. Moore *et al.* (3) demonstrated depressed Ca^{2+} accumulation by liver microsomal vesicles of rats administered CCl_4. Furthermore, incubation of isolated microsomes (EDTA-washed to remove Fe^{2+} which may catalyze excessive amounts of peroxidation *in vitro*) in the presence of small amounts of CCl_4 completely destroyed the Ca^{2+} sequestration activity (4). The time course of CCl_4-dependent depression of microsomal Ca^{2+} transport *in vivo* paralleled the time course of lipid peroxidation in these membranes (5). Thus, the possibility emerged that CCl_4, by profoundly depressing Ca^{2+} sequestration of the endoplasmic reticulum, could produce physiologically unacceptable changes in cytosolic free [Ca^{2+}], with pathological consequences leading to cell death.

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Recently, we reported that plasma membranes isolated from livers of rats administered CCl_4 *in vivo* exhibit a marked decrease in net ATP-dependent Ca^{2+} accumulation (6), which might further exacerbate an alteration in Ca^{2+} homeostasis produced by the microsomal lesion. The amount of Ca^{2+} accumulated into or associated with CCl_4 plasma membrane vesicles was notably less than that observed with membranes of control rats. We hypothesized that the ability of the membrane vesicles to retain intravesicular Ca^{2+}, or the ability of the membranes to bind Ca^{2+}, or both, might be diminished following CCl_4 administration *in vivo*. The present communication reports the results of experiments designed to test these hypotheses.

Materials and Methods

Animal model and toxin treatment. Male Sprague-Dawley rats (175–275 g) (Harlan, Houston, TX) were pretreated as described previously (6) for 3 days with phenobarbital (80 mg/kg). After a 16-hr fast, rats were injected intraperitoneally with CCl_4 (2 ml/kg, in corn oil). Controls were treated with phenobarbital for 3 days, fasted, and

ABBREVIATIONS: EDTA, ethylenediaminetetraacetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; RSOT, right side-out tight; ISOT, inside-out tight.

vehicle injected. Four hr after CCl_4 or vehicle injection, animals were killed by cervical dislocation, livers were excised, and plasma membranes were prepared from two or three pooled control or two or three pooled CCl_4 livers.

Preparation of plasma membrane fractions. Liver plasma membranes (the "purified fraction") were isolated by the procedure of Van Amelsvoort *et al.* (7) and suspended to a final concentration of 5–10 mg/ml in 100 mM KCl, 10 mM HEPES, pH 7.5. Protein was estimated by the Lowry method (8) using bovine serum albumin as standard.

Ca^{2+} efflux from plasma membrane vesicles. Passive calcium efflux from plasma membrane vesicles was measured by a Millipore filtration method as described by Campbell *et al.* (9) for sarcoplasmic reticulum vesicles. Vesicles were loaded passively by incubating for 18 hr at 0–4° in 100 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES, pH 7.5 with 1 mM $^{45}CaCl_2$ (0.7 μCi). Total volume was 2.0 ml, vesicle protein was 2–4 mg/ml. Efflux of $^{45}Ca^{2+}$ was initiated by diluting 200 μl of this mixture into 5.8 ml of ice-cold 100 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES, pH 7.5, containing 2 mM EGTA. Samples (400 μl) were removed at timed intervals, filtered (0.22-μm filters), and washed with 10 ml of ice-cold 100 mM KCl, 10 mM HEPES, pH 7.5, 1 mM $LaCl_3$. Filters were counted in Liquiscint Counting Cocktail (National Diagnostics, Somerville, NJ). Each experiment was repeated twice with each of six separate control and CCl_4 preparations.

In some experiments, Ca^{2+} ionophore A23187 (1.6 μM final concentration) was added after 5 min to bring efflux to completion. The Ca^{2+} content of plasma membranes 8 min after A23187 addition was taken to represent Ca^{2+} bound tightly to the membranes. In certain experiments Ca^{2+}-loaded plasma membranes, still in 1 mM Ca^{2+}-loading medium, were exposed to concentrations of Triton X-100 (0.005–0.15%) for 20 min at 0–4° prior to initiation of Ca^{2+} efflux.

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 0.02–0.04 mg of 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 50-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. Less than 0.05% of total ^{45}Ca appeared in the eluate in the absence of vesicles. Good agreement between the filtration and ion exchange methods was obtained.

^3H_2O and ^{14}C-carboxydextran-accessible volumes. Samples prepared in microcentrifuge tubes were 0.2–0.3 mg of 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 of ^3H_2O and 0.25 μCi of ^{14}C-carboxydextran (M_r 50,000–70,000). After 10-min equilibration at room temperature, samples were centrifuged 15 min at 32,000 × *g*. Aliquots of the supernatants 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 ^3H in the two channels were obtained by counting of commercial standards. The ratio of ^3H dpm/mg of protein in the pellet to ^3H dpm/ml in the supernatant estimates the total pellet volume/mg of protein. Similarly, the ratio of ^{14}C dpm/mg of protein in the pellet to ^{14}C dpm/ml in the supernatant estimates the carboxydextran-accessible volume/mg of protein. The difference between them is the carboxydextran-excluded volume which equals intravesicular volume + membrane volume/mg of protein.

In some experiments, 0.1% Triton X-100 was added and samples were incubated at 37° for 10 min before centrifuging the samples and carrying out the remainder of the procedure. Under these conditions, the carboxydextran-excluded space estimates the membrane volume/mg of protein. Subtracting this value from the volume obtained in the absence of Triton X-100 gives the intravesicular volume/mg of protein.

Estimation of vesicle membrane permeability for K^+ and other ions. The procedure used is a modification of a method described by Garty *et al.* (10). Plasma membrane vesicles prepared as described were already equilibrated with 100 mM KCl. Just before assay, samples of vesicles (200 μg of protein) were applied to Dowex 50W-X8 ($Tris^+$ form) columns made in Pasteur pipettes and eluted with 1 ml of 175 mM sucrose under light vacuum, to remove extravesicular K^+. Then, 1-ml portions of the eluates were added to incubation vials that already contained 1 ml of 175 mM sucrose, 175 mM sucrose + 4 μM valinomycin, or 100 mM KCl at 25°. After 30 sec, $^{86}RbCl$ (2 μCi) was added and 100-μl samples were removed at intervals up to 10 min. Extravesicular ^{86}Rb was removed by passage of samples through Dowex 50-X8 columns as above, eluting with 1 ml of 175 mM sucrose, and the eluted samples were counted in entirety. ^{86}Rb uptake has been expressed in cpm/100 μg of protein, corrected for differences in initial radioactivity where necessary.

Marker enzyme assays. Glucose-6-phosphatase, a microsomal marker enzyme, was assayed according to the method of Baginski *et al.* (11). The mitochondrial marker enzyme, azide-sensitive ATPase, was assayed as described by Pitts (12). 5′-Nucleotidase was selected as a plasma membrane marker and assayed in a medium containing 90 mM TrisHCl, pH 8.0, 5 mM adenosine-5′-monophosphate, 10 mM $MgCl_2$ and 0.05% Triton X-100. Release of inorganic phosphate was monitored as described by Baginski *et al.* (11).

Plasma membrane calcium content. Calcium content of plasma membranes was measured in the supernatant fluid remaining after precipitation and sedimentation of the protein with 3% trichloroacetic acid, in the presence of 0.5% $LaCl_3$ and 5% trichloroacetic acid, in a Perkin-Elmer Atomic Absorption Spectrometer model 403, using a standard addition procedure. The membranes were not treated with EGTA prior to analysis.

Materials. CCl_4 was a product of Eastman Kodak Co. (Rochester, NY); phenobarbital was obtained from Mallinckrodt, Inc. (St. Louis, MO); $^{45}CaCl_2$, $^{86}RbCl$, ^{14}C-carboxydextran, and ^3H_2O were purchased from New England Nuclear (Boston, MA). Na_2ATP, 5′-AMP, Dowex 50W-X8 (50–100 mesh), valinomycin, ionophore A23187, Triton X-100, and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). Other chemicals were reagent grade or better.

Results

Passive Ca^{2+} permeability of liver plasma membrane vesicles from control and CCl_4-intoxicated rats. The results of experiments to test the ability of plasma membrane vesicles to retain intravesicular calcium ions, by a method previously described by Campbell *et al.* (9), are shown in Fig. 1. Zero time values plotted on the *ordinate* were obtained by filtration and washing of samples taken directly from the loading suspension. The ion exchange method gave values similar to those obtained by filtration for both control and CCl_4 membranes (Table 1).

Efflux of Ca^{2+} was biphasic, and efflux from CCl_4 vesicles took place much more rapidly and more completely than was the case with control vesicles (Fig. 1A). For phenobarbital control vesicles, the first order rate constants of calcium efflux were 0.272 and 0.0516 min^{-1} for the rapid and slow phases, respectively (Fig. 1B). For CCl_4 vesicles, the corresponding rate constants were 1.78 and 0.171 min^{-1}, representing a more than 6-fold increase in the rapid phase and a more than 3-fold increase in the slow phase of Ca^{2+} efflux.

When the ionophore A23187 (1.6 μM) was added to control vesicles after 5 min of efflux in EGTA medium, a considerable additional efflux of Ca^{2+} was elicited (Fig. 1A). In contrast,

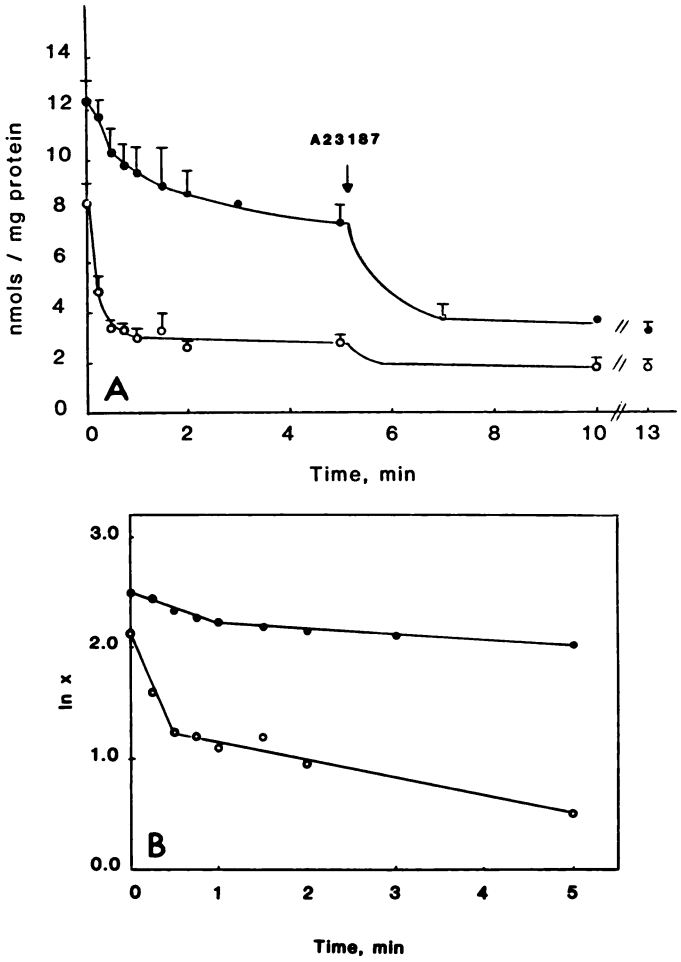

Fig. 1. Passive calcium efflux from phenobarbital control and CCl_4 plasma membrane vesicles. A. The results of efflux experiments with six separate preparations in each group were averaged and the nmol of calcium associated with the vesicles was plotted versus time after 30-fold dilution of calcium-loaded vesicles into medium containing 2 mM EGTA. After 5 min, ionophore A23187 (1.6 μM) was added. *Standard error bars* are shown except at two time points where only two measurements were made. ●, phenobarbital control vesicles; ○, CCl_4 vesicles. B. Semilogarithmic plots of the same data. The first order rate constants of the initial rapid phase and the subsequent slower phase were calculated by taking the least squares fit of each linear portion. For phenobarbital control vesicles, the first order rates of calcium efflux were 0.272 and 0.0516 min^{-1} for the rapid and slow phases, respectively. For CCl_4 vesicles, the corresponding values were 1.78 and 0.171 min^{-1}, respectively.

TABLE 1

Calcium content of control and CCl_4 plasma membrane vesicles after passive loading as measured by filtration and ion exchange methods

Four samples of 10–40 μl were taken from the loading suspensions, which contained 2–4 mg of protein/ml, and either filtered and washed or applied to Dowex 50W-X8 columns and eluted with 175 mM sucrose. Filters and eluates were counted to determine calcium content. Calcium/mg of protein was calculated by taking the least squares fit of the nmol of calcium vs. mg of protein plot. Results are the means ± standard errors.

	Zero time Ca^{2+} content after passive loading	
	Millipore	Ion exchange
	nmol/mg protein	
Control	12.3 ± 0.8 (5)[a]	11.2 ± 1.8 (3)
CCl_4	8.3 ± 0.8 (5)	8.1 ± 0.4 (3)

[a] Numbers in parentheses, numbers of separate preparations tested.

A23187 addition to CCl_4 vesicles elicited a relatively small additional loss of Ca^{2+}. Table 2 summarizes vesicle Ca^{2+} content after efflux in EGTA and after A23187 addition. Values obtained when EGTA or A23187 was added after loading with Ca^{2+} for 5 min in the presence of ATP at 37° are included for comparison. The major difference is between control vesicles loaded passively and actively, since only inside out vesicles can accumulate calcium actively but all vesicles can load calcium passively. A much smaller difference in calcium loading occurs between CCl_4 vesicles loaded actively and passively.

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. CCl_4 membranes retained significantly less Ca^{2+} after A23187, but the difference was not so great as after EGTA alone in the time intervals studied. Control vesicles lost 45.3–51.9% of the Ca^{2+} present at zero time (using ion exchange and filtration zero time measurements, respectively) without A23187 addition, whereas CCl_4 vesicles lost 81.0–81.7% without A23187 addition.

The average difference in the amount of Ca^{2+} bound by the control and CCl_4 membranes (1.69 nmol/mg of protein) accounts for approximately half the difference in average zero time values, which are 4 nmol/mg measured by filtration, and 3.1 nmol/mg measured by the ion exchange method (Table 1). The source of the remaining difference may be decreased low affinity binding as well as decreased tight binding capacity in CCl_4 membranes (see below). Zero time samples were not exposed to EGTA, and control vesicles may contain an additional increment of loosely bound Ca that CCl_4 vesicles do not have. Alternatively, CCl_4 vesicles might have a different intravesicular volume and load less Ca^{2+}/mg of protein.

Estimation of intravesicular volume of plasma membrane vesicles. In order to determine whether control and CCl_4 membrane vesicles were of comparable internal volumes, total pellet volume and carboxydextran-accessible volume/mg of protein were measured. The difference between these gives the carboxydextran-excluded volume/mg of protein, equal to the intravesicular volume plus the volume occupied by the membranes. Table 3 summarizes total pellet volume and carboxydextran-excluded volume/mg of protein of several preparations of control and CCl_4 vesicles. The carboxydextran-excluded volume/mg of protein was significantly greater in CCl_4 vesicles than in control vesicles—6.1 versus 4.1 μl/mg of protein. However, a larger volume/mg of protein would be expected

TABLE 2

Calcium content of control and CCl_4 plasma membrane vesicles after efflux in the presence of EGTA and ionophore A23187

Passive loading conditions were those described in Materials and Methods and in Fig. 1. Calcium contents (passive loading) in the table are those 5 min after induction of efflux by dilution into EGTA-containing medium, and those 8 min after A23187 addition. These data are compared to the calcium contents after active uptake of calcium for 5 min in the presence of ATP, as described previously (6), followed by addition of 2 mM EGTA or 1.6 μM A23187; calcium contents 5 min after addition of EGTA or ionophore are shown. Results are means ± standard errors; four to seven separate preparations were tested in each case. Significance was evaluated with the *t* test.

	Passive loading		Active loading:
	Ca^{2+} remaining bound after EGTA	Ca^{2+} remaining bound after A23187	Ca^{2+} remaining bound after EGTA or A23187
	nmol/mg protein		
Control	7.62 ± 0.67	3.29 ± 0.27	2.45 ± 0.42
CCl_4	2.82 ± 0.28	1.60 ± 0.27	1.25 ± 0.07
Significance	$p < 0.005$	$p < 0.005$	$p < 0.025$

TABLE 3

Control and CCl_4 plasma membrane total pellet water volumes, and carboxydextran-excluded volumes

Total pellet water space and carboxydextran-accessible space were determined as described in Materials and Methods, with ^3H_2O and ^{14}C-carboxydextran, and 0.15–0.3 mg of plasma membrane protein per sample. Three to six replicate samples were prepared for each of the separate preparations tested. Averaged carboxydextran-accessible volumes were subtracted from averaged total pellet water values to obtain the carboxydextran-excluded spaces given in the table. Data are means ± standard errors, and the *t* test was employed to evaluate statistical significance.

	Total pellet ^3H_2O	^{14}C-Carboxydextran-excluded volume
	μl/mg protein	
Control	12.2 ± 0.9 (6)[a]	4.1 ± 0.5 (3)
CCl_4	14.3 ± 0.8 (6)	6.1 ± 0.1 (3)
Significance	NS[b]	$p < 0.025$

[a] Numbers in parentheses, number of separate preparations.
[b] NS, not significant.

to produce an increased zero time Ca^{2+} content/mg of protein, not the reverse, as observed in CCl_4 membranes.

The estimated intravesicular volumes obtained here may be compared to those reported by Duggan and Martonosi (13) for sarcoplasmic reticulum vesicles isolated from rabbit skeletal muscle, where intravesicular volume was 4–5 μl/mg of protein after subtracting 1–1.5 μl/mg of membrane volume from the carboxydextran-excluded space. In the present study, control plasma membrane carboxydextran-excluded volume (Table 3) was 4.1 μl/mg, and for CCl_4 it was 6.1 μl/mg. Subtracting the value of 1 μl/mg of protein, the carboxydextran-excluded volume we obtained in the presence of 0.1% Triton X-100, the resulting plasma membrane intravesicular volumes are control, 3.1, and CCl_4, 5.1 μl/mg of protein.

Vesicles equilibrated in medium containing 1 mM calcium may be assumed to have an internal calcium concentration equal to that of the medium. The intravesicular calcium content of control vesicles was then 1 nmol of Ca/μl (i.e., 1 mM) × 3.1 μl/mg of protein = 3.1 nmol/mg of protein; and for CCl_4 vesicles, it was 5.1 nmol/mg of protein. Furthermore, the zero time calcium content, less the intravesicular content, represents the total bound calcium (after passive loading under the conditions described). For control vesicles, 12.3 nmol/mg of protein (Table 1) *minus* 3.1 nmol/mg of protein = 9.2 nmol of calcium bound/mg of protein. For CCl_4 vesicles, 8.29 − 5.1 = 3.19 nmol of calcium bound/mg of protein. Thus, CCl_4 plasma membranes bound 65% less total calcium than did controls.

If the A23187-insensitive calcium pool is subtracted from the total bound Ca, the remaining fraction may be taken to represent a more loosely bound component. The resulting values are 5.91 (control) and 1.59 (CCl_4) nmol of calcium/mg of protein. Clearly, the more loosely bound pool, as well as the A23187-insensitive pool, is diminished markedly in CCl_4 vesicles.

Calcium content of the control and CCl_4 plasma membrane vesicles was measured by atomic absorption spectroscopy, and CCl_4 membrane vesicles had significantly decreased calcium content by this measure as well: 10.9 ± 1.2 versus 17.4 ± 2.0 nmol/mg of protein (mean ± SE) for five preparations in each group ($p < 0.025$). These values include total bound and intravesicular calcium in plasma membranes freshly prepared in the absence of EGTA; the plasma membranes were not filtered and washed in the presence of $LaCl_3$ as in zero time determinations after calcium loading.

Further permeability studies based on $^{86}Rb^+$ uptake driven by K^+ diffusion potentials. Additional evidence for altered permeability of plasma membranes from livers of CCl_4-dosed rats was obtained from experiments based on the method of Garty *et al.* (10). Permeability of membrane vesicles or liposomes may be assessed by measuring their ability to maintain a K^+ ion gradient when KCl-loaded vesicles are treated to replace extravesicular K^+ with a relatively impermeable cation such as $Tris^+$. As a consequence of the K^+ gradient produced across the membrane in this way, a K^+ diffusion potential, interior negative, is generated, and if a tracer quantity of $^{86}Rb^+$ is present in the exterior solution, $^{86}Rb^+$ will tend to equilibrate with the diffusion potential without itself affecting the potential. Vesicles having a relatively low K^+ conductance (and, therefore, a low diffusion potential) will slowly accumulate $^{86}Rb^+$ over a period of several min. However, if the ionophore valinomycin is present to increase K^+ conductance, more rapid and extensive $^{86}Rb^+$ uptake will occur. The greater the K^+ gradient maintained by the native vesicles, the greater the diffusion potential after the addition of valinomycin and the more rapid and extensive the $^{86}Rb^+$ uptake. As the gradient dissipates, $^{86}Rb^+$ efflux will also be rapid. More permeable vesicles will accumulate $^{86}Rb^+$ to a lesser extent, having a lesser K^+ gradient and diffusion potential, and lose it more slowly, because the movement of other ions also can compensate for K^+ backflow into the vesicles. Furthermore, in either case, if K^+ is added to the external medium to match the internal concentration, the diffusion potential is abolished, and $^{86}Rb^+$ will only equilibrate passively across the membrane, allowing an estimate of vesicle volume to be made. For a more rigorous discussion of the rationale of this method for evaluating vesicle ion permeability, the reader is referred to Garty *et al.* (10) and to Glynn and Warner (14), on which the formulation in the first mentioned work was based.

Fig. 2 illustrates data from experiments performed with three different pairs of control and CCl_4 plasma membrane preparations. It is clear from Fig. 2 that the rate and extent of $^{86}Rb^+$ movement into the vesicles in the presence of valinomycin were significantly less in the CCl_4 membranes than in controls. The similar levels of intravesicular $^{86}Rb^+$ attained by passive equilibration in the control and CCl_4 membranes suggest that the vesicles have similar Rb^+-accessible volumes. Volumes estimated on the basis of these measurements were 2.6 ± 0.1 μg/mg of protein (control) and 2.8 ± 0.5 μl/mg of protein (CCl_4). These values are lower than our estimates by other techniques (see previous section); the reason for this difference is not known.

Effect of Triton X-100 on passive efflux of Ca^{2+} from plasma membrane vesicles. Experiments described in the preceding sections suggest that plasma membrane vesicles isolated from livers of CCl_4-intoxicated rats have more permeable membranes than do controls. Exposure of control membranes to concentrations of detergent sufficient to increase permeability without solubilizing the membranes (15) is another way to alter membrane permeability, and would be predicted to produce Ca^{2+} efflux patterns resembling those observed with CCl_4 membranes. Fig. 3 shows data obtained from experiments with Triton X-100 concentrations varied from 0.005% to 0.15%. Protein/detergent ratios were comparable to those used by McIntosh and Davidson (15). These authors reported that 0.02% Triton X-100 abolished completely the Ca^{2+} transport of sarcoplasmic reticulum vesicles, probably through an in-

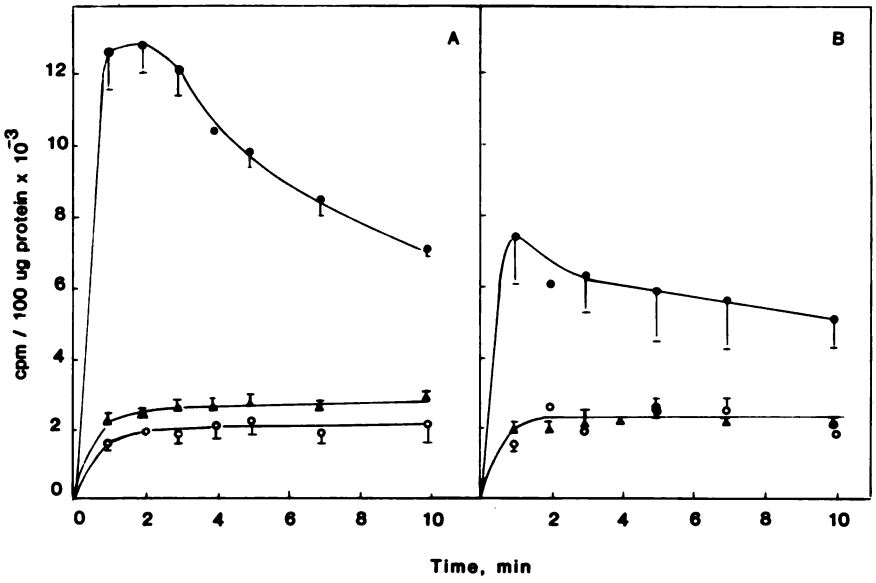

Fig. 2. Uptake of $^{86}Rb^+$ by KCl-loaded plasma membrane vesicles of control and CCl_4-dosed rats. A, control membranes; B, CCl_4 membranes. Vesicles were in 175 mM sucrose (▲), 100 mM KCl (○), or sucrose + 2 μM valinomycin (●). Data are mean ± standard error, $n = 3$ separate preparations in each group. Only two measurements were made at time points without *standard error bars.*

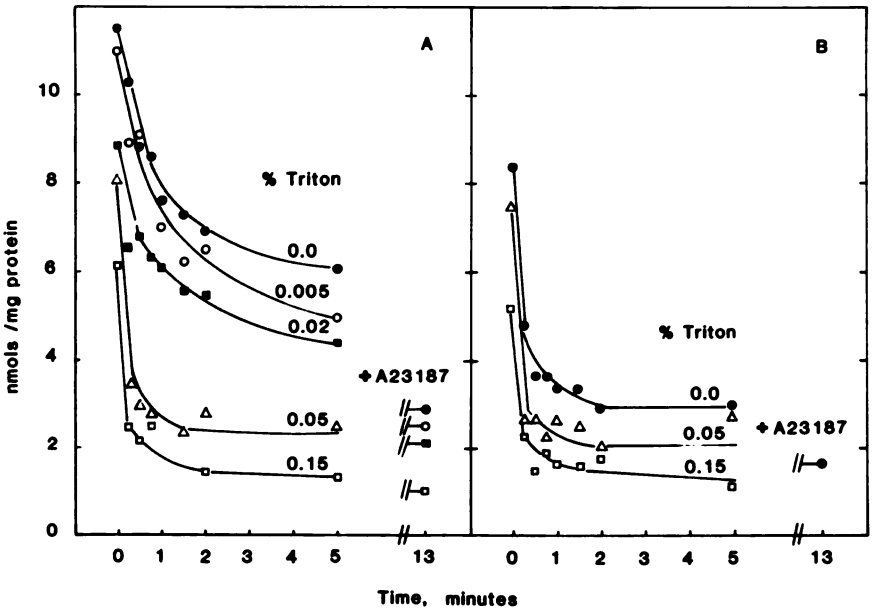

Fig. 3. Passive calcium efflux from control and CCl_4 plasma membrane vesicles and control vesicles preincubated in varying concentrations of Triton X-100. Ca^{2+}-loaded vesicles, still in 1 mM calcium loading medium, were exposed to Triton X-100 for 20 min at 0–4° prior to initiation of calcium efflux. A23187 (1.6 μM) was added 5 min after efflux was initiated by dilution into EGTA-containing medium. Data shown are a composite of three experiments with separate control and CCl_4 preparations. A. Control vesicles (●), and control vesicles preincubated with Triton X-100 at 0.005% (○), 0.02% (■), 0.05% (△), and 0.15% (□). B. CCl_4 vesicles exposed to 0.05% (△) or 0.15% (□) Triton X-100 exhibited efflux curves identical to those of control vesicles exposed to these concentrations.

creased permeability of the membranes, but that under their conditions, membrane solubilization did not begin until the Triton X-100 concentration reached 0.15%.

The rate and extent of passive Ca^{2+} efflux from control plasma membrane vesicles increased with detergent concentration. With 0.005% and 0.02% Triton X-100, the control efflux profiles decreased in the direction of the CCl_4 efflux profile. At 0.05% and 0.15% Triton X-100, control and CCl_4 efflux curves were similar and both were depressed below the CCl_4 profile obtained in the absence of detergent. Ca^{2+} associated with the vesicles at 10 min was similar in both conditions to that seen with CCl_4 membranes after A23187, suggesting that no intravesicular free Ca^{2+} remained under these conditions. In other experiments, the effect of Triton on ATP-dependent Ca^{2+} uptake was tested; 0.02% Triton X-100 was sufficient to abolish control Ca^{2+} uptake in the presence of ATP (data not shown) as reported for sarcoplasmic reticulum by McIntosh and Davidson (15). Furthermore, Ca^{2+} uptake ± ATP was tested on every preparation used for efflux studies, and the rate and extent of uptake were strongly negatively correlated with rate and extent of efflux in given control and CCl_4 preparations (data not shown).

Contamination of plasma membrane vesicle preparations by endoplasmic reticulum and mitochondria. Liver plasma membrane vesicles prepared as described contained significant endoplasmic reticulum contamination, estimated to be 25–40% by experiments in which recovery of total glucose-6-phosphatase activity and specific activity enrichment were followed in all fractions throughout the preparations (Fig. 4). The ratio of specific activities in plasma membrane and microsomal fractions = 0.12 μmol/min/mg ÷ 0.313 μmol/min/mg = 0.38, in this particular preparation. However, the specific activities of microsomal and mitochondrial marker enzymes in the plasma membrane fraction were not increased by CCl_4 treatment; rather, they decreased in CCl_4 plasma membrane preparations (Fig. 5), as they did also in homogenates (not shown). This may be indicative of general membrane damage, because the plasma membrane marker 5′-nucleotidase decreased in

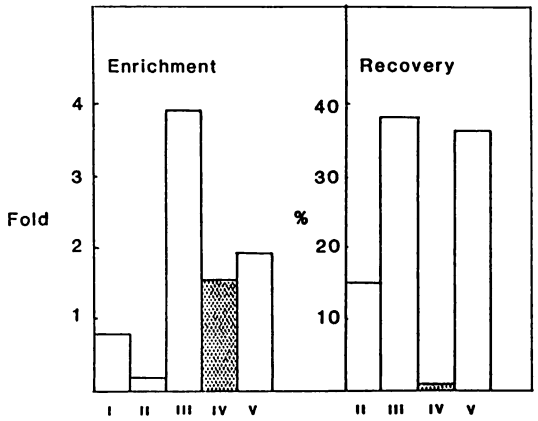

Fig. 4. Glucose-6-phosphatase activity is subcellular fractions obtained during isolation of liver plasma membrane vesicles. -Fold enrichment and percentage of recovery of activity in the fractions are shown. Fraction *I*, post-mitochondrial supernatant; fraction *II*, 100,000 × *g* supernatant, fraction *III*, microsomes; fraction *IV*, plasma membranes; fraction *V*, material found just below the 39.5–20% sucrose interface in the sucrose density gradient step of plama membrane isolation according to the method of Van Amelsvoort *et al.* (7). Enrichment over the specific activity in the initial homogenate is shown. Percentage of recovery of fraction *I* activity in the subsequent fractions is presented.

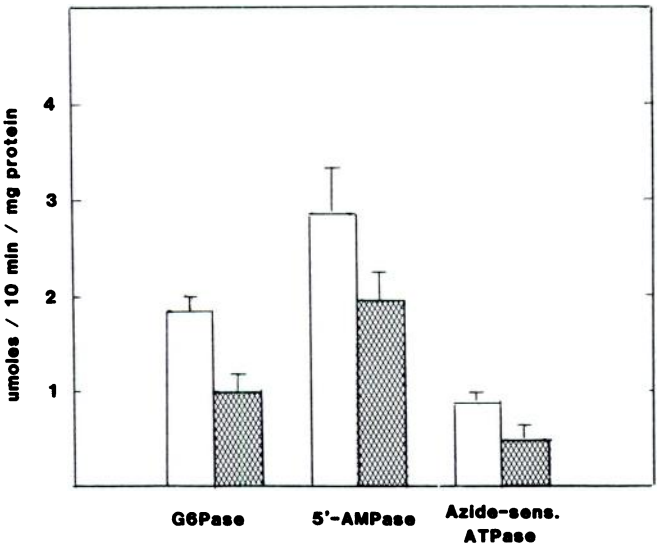

Fig. 5. Activities of glucose-6-phosphatase, 5′-nucleotidase, and azide-sensitive ATPase, markers for endoplasmic reticulum, plasma, and mitochondrial membranes, respectively, in isolated plasma membrane vesicles. □ (mean ± standard error, $n = 6$), control membranes; ■, CCl_4 membranes ($n = 6$). Decreases in these activities in plasma membrane fractions are proportionate to. corresponding decreases observed in crude homogenates of livers of CCl_4-dosed rats.

these preparations as well (Fig. 5). Microsomal glucose-6-phosphatase is also diminished following CCl_4 administration (16).

Discussion

The present work was designed to investigate the possibility that the greatly decreased Ca^{2+} accumulation of liver plasma membrane vesicles isolated from rats administered CCl_4 *in vivo* (6) might be associated with increased Ca^{2+} permeability and/or a decrease in Ca^{2+}-binding capacity of the membranes. Evidence of two independent types suggests that an increase in cation permeability does occur in these membranes, and that Ca^{2+} binding capacity is decreased also.

Table 4 lists the four possible segments of the population of vesicles in these preparations, i.e., right side-out and tight, inside-out and leaky, etc., along with the characteristics predicted in the experimental situations described. Given the data presented here, along with some data from our previous report (Ref. 6, Table 1), the percentage of the control and CCl_4 vesicle populations falling into these categories can be estimated. In the previous report, control plasma membrane vesicles averaged approximately 67% right side-out, based on the release of sialic acid from intact and detergent-treated vesicles by neuraminidase. The 67% includes right side-out tight (RSOT) and right side-out leaky plus any inside-out leaky vesicles that might become permeable to neuraminidase during the incubation of the vesicles with the enzyme. Therefore, 33% are ISOT plus inside-out leaky not permeable to neuraminidase, and thus ≤ 33% are ISOT vesicles capable of active Ca^{2+} accumulation.

When control vesicles were passively loaded with Ca^{2+} and diluted into EGTA medium, about 55% of the Ca^{2+} initially present was released only after A23187 addition (where ion exchange zero time Ca^{2+} content *minus* Ca^{2+} content after A23187 = total intravesicular Ca^{2+}, and Ca^{2+} content before A23187 *minus* Ca^{2+} content after A23187 = Ca released by A23187). If 55% of passively loaded Ca^{2+} was only released by A23187, then 55% of the vesicles were tight (including both ISOT and RSOT). Probably, then, only 55% of the control vesicles maintained a K^+ gradient.

CCl_4 vesicles from the same preparations with which the passive efflux studies were carried out typically exhibited 85% decreased Ca^{2+} accumulation (6), and 81% of passively loaded Ca^{2+} was lost without A23187 addition (Tables 1 and 2). Also, maximum $^{86}Rb^+$ uptake in CCl_4 vesicles was less than 50% of that observed in control vesicles (Fig. 2).

Only ISOT vesicles actively accumulate Ca^{2+}, and ATP-dependent accumulation was decreased 85% from control, thus, ≤33% ISOT × (1 − 0.85) = ≤5% of ISOT vesicles remaining. Likewise, if 81% of the (non-bound Ca^{2+} was easily lost, then

TABLE 4

Possible vesicle types and predicted properties

Vesicle type	Predicted properties
Inside-out, tight (ISOT)	Accumulate Ca^{2+} actively Maintain K^+ gradient Lose passively loaded Ca^{2+} slowly Exclude ^{14}C-carboxydextran
Inside-out, leaky (ISOL)	Do not accumulate Ca actively Do not maintain K^+ gradient Lose passively loaded Ca^{2+} rapidly Exclude ^{14}C-carboxydextran
Right side-out, tight (RSOT)	Do not accumulate Ca^{2+} actively Maintain K^+ gradient Lose passively loaded Ca^{2+} slowly Exclude ^{14}C-carboxydextran
Right side-out, leaky (RSOL)	Do not accumulate Ca^{2+} actively Do not maintain K^+ gradient Lose passively loaded Ca^{2+} rapidly Exclude ^{14}C-carboxydextran

only 19% of vesicles were tight, and it appears that in CCl_4 preparations, the proportion of tight vesicles was decreased from 55% in control to 19% of the total. Since ≤5% are ISOT, then ≥14% are RSOT.

However, the apparent K^+ gradient (as indicated by maximum $^{86}Rb^+$ uptake, Fig. 2) was 47% of the control or (0.47 ± 55%) = 26% of that to be expected if all vesicles were tight, suggesting only 26% of CCl_4 vesicles were tight. This value is not quite as low as the 19% estimated from the passive Ca^{2+} efflux data. It may be that a fraction of vesicles that are permeable to Ca^{2+} are not permeable to K^+. More likely, K^+ loss from the CCl_4 vesicles prior to exposure to valinomycin may be slow enough that the extent of K^+ gradient loss in the 30 sec after removal of extravesicular K^+ cannot be compared directly to the extent of Ca^{2+} efflux in the presence of EGTA after 5 min.

Summarizing, the total tight vesicle fraction of the CCl_4 vesicle population was decreased from 55% (control) to 26%, or from 55 to 19%, depending on which measure of passive permeability is employed. ISOT vesicles were decreased from ≤33 to ≤5%, based on the decrease in Ca^{2+} accumulation activity of CCl_4 vesicles. The decrease in ISOT vesicles and/or in Ca^{2+} accumulation (the former having been estimated from the latter) was proportionally greater than the apparent increase in permeability—85% versus 53% for the K^+ or nonspecific permeability measurements and 65% for the Ca^{2+} permeability measurements. The additional increment might be contributed by decreased activity of the Ca^{2+} pump in the active Ca^{2+} accumulation experiments.

Two potentially complicating factors were not factored into this analysis. These are: 1) the contamination of the plasma membrane vesicle fraction with vesicles derived from the endoplasmic reticulum, and 2) the possibility that, particularly in control membranes, a considerable fraction of the zero time Ca^{2+} content may be loosely bound, rather than intravesicular, Ca^{2+}. Contaminating microsomes contribute little to ATP-dependent Ca^{2+} uptake measured in plasma membranes (6), so that their effect on this aspect of the analysis would be small. Control microsomes passively load Ca^{2+} to a level similar to that of control plasma membrane fractions, and efflux measured under the same conditions is similarly biphasic but more rapid (average first order rate constant = 0.45 min^{-1} for two preparations). Some of the increase in rate of Ca^{2+} efflux observed in CCl_4 plasma membrane fractions could be due to a change in Ca^{2+} permeability of microsomes as well as of plasma membrane vesicles. Although the calculated percentages of plasma membrane vesicles that are tight or permeable would be altered by taking the microsomal contamination into account, control and CCl_4 membranes appear to have similar amounts of contamination and the overall result would not be different. A similar argument may be made for calculations based on ^{86}Rb upake experiments.

The second factor, that loosely bound Ca^{2+} may comprise a significant fraction of the zero time Ca^{2+} content, would operate to increase the estimated percentage of tight vesicles in the preparations. If, for example, only the A23187-releasable fraction is intravesicular, then the proportion of tight vesicles in control preparations may approach 100%. The effect would be to increase the estimate of tight CCl_4 vesicles from the $^{86}Rb^+$ experiments to as much as 47% (0.47 × 100% tight vesicles in control), whereas the estimate based on Ca^{2+} efflux after A23187 would be 19% or greater, depending on the fraction of zero time Ca^{2+} that is loosely bound in CCl_4 vesicles.

Therefore, the conclusions will be unaltered by including these factors, i.e., more of the CCl_4 than control vesicles are permeable by either assay, and neither gives an estimate of increased permeability of magnitude similar to the decrease in Ca^{2+} accumulation activity, suggesting again that Ca^{2+} pump activity may also be decreased in CCl_4 membrane vesicles.

Kagan *et al.* [Ref. 17; reviewed in Meerson *et al.* (18)] reported that lipid peroxidation in sarcoplasmic reticulum vesicles *in vitro* produced, first, an increased permeability of the membranes and, subsequently, inhibition of the Ca^{2+}-dependent ATPase. We have shown (19) that CCl_4 administration *in vivo* leads to accumulation of 11-, 12-, and 15-hydroxyeicosatetraenoic acids, products of membrane lipid peroxidation, not only in the microsomes but in plasma membranes as well. These specific lipid oxidation products represent a small fraction of the many such products formed under peroxidizing conditions. Several types of oxygenated polyunsaturated fatty acids have been shown to possess Ca^{2+} ionophoretic activity, e.g., prostaglandins B_2, E_2, and $F_{2\alpha}$ (20), leukotriene B_4 (21), thromboxane A_2 (22), and products of oxidation of linolenic, 8,11,14-eicosatrienoic, linoleic, 11,14,17-eicosatrienoic, and 11,17-eicosadienoic acids (23). In the latter study, the ionophoretic activity of the oxidized derivatives was abolished by their reduction. Induction of ionophoresis by lipid peroxidation products might provide a mechanism for the altered permeability observed in membranes in CCl_4 intoxication.

Altered structure of the membrane lipid bilayer may also be associated with the decreased Ca^{2+} content and Ca^{2+} binding of the plasma membrane vesicles isolated from CCl_4-treated animals, since phospholipid phosphate and carboxylate groups are major Ca^{2+}-binding sites. In turn, decreased Ca^{2+} content of the plasma membranes may be related to altered permeability. Duggan and Martonosi (13) were able to produce sarcoplasmic reticulum vesicles permeable to inulin (M_r = 5000) by exposing vesicles to EGTA at pH 8–9, and showed that decreased membrane Ca^{2+} content correlated with the increased permeability to inulin.

Both detergents at low concentrations (15) and lipid peroxidation *in vitro* (17) have been shown to produce increased membrane permeability and subsequent Ca^{2+}-ATPase inhibition in isolated sarcoplasmic reticulum vesicles. We cannot rule out an inhibition of the calcium pump in plasma membranes isolated after CCl_4 administration *in vivo* that might occur in conjunction with, or following, the increase in membrane permeability. Measurements of plasma membrane (Ca^{2+} + Mg^{2+}) ATPase by the method of Lotersztajn *et al.* (24) show a minor (0–20%) inhibition of the enzyme in CCl_4 membranes (25). However, recently, Lin (26, 27) presented compelling evidence that the ATPase activity measured by this assay is that of a separate ectoenzyme unrelated to the Ca^{2+} pump of liver plasma membranes.

The animal model employed in this study is the same used in our previous study (6) in which the effects of CCl_4, bromobenzene, and acetaminophen (administered to induced and fasted rats) on plasma membrane, microsomal, and mitochondrial Ca^{2+} transport were compared. The combination of high CCl_4 dose, phenobarbital induction, and fasting comprise more severe conditions than are necessary to demonstrate increased plasma membrane lipid peroxidation, increased permeability to

Ca^{2+}, and decreased ATP-dependent Ca^{2+} accumulation after CCl_4.[1] The present work extends the results of the earlier report (6), and a change in animal model was not appropriate.

In summary, liver plasma membrane vesicles isolated after CCl_4 administration *in vivo* are more permeable to Ca^{2+} and K^+ than are controls. These membrane structural changes can be postulated to produce functional changes *in vivo*, including a loss of the permeability barrier property of the plasma membrane. Membranes of the endoplasmic reticulum may be affected similarly to the plasma membranes, or even more profoundly, and the powerful effect of CCl_4 administration on microsomal Ca^{2+} transport reported by Moore *et al.* (3) may be in part attributable to this phenomenon. This seems worth investigating with methods similar to those developed here.

It is interesting to note that acetaminophen and bromobenzene, toxins that inhibit plasma membrane Ca^{2+} transport when administered *in vivo* (6) without apparent increase in membrane permeability or lipid peroxidation (19), have no effect on microsomal Ca^{2+} transport (6), whereas CCl_4, which alters membrane permeability and induces lipid peroxidation, virtually abolishes Ca^{2+} accumulation in both membrane systems.

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