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CYTOSOLIC CALCIUM AFTER CARBON TETRACHLORIDE, 1,1-DICHLOROETHYLENE, AND PHENYLEPHRINE EXPOSURE

STUDIES IN RAT HEPATOCYTES WITH PHOSPHORYLASE *a* AND QUIN2*†

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Abstract—Carbon tetrachloride (CCl_4) and 1,1-dichloroethylene (DCE), both hepatotoxins, inhibit sequestration of Ca^{2+} by rat liver endoplasmic reticulum (ER) both *in vivo* and *in vitro*. It is possible that, as a result, cytosolic Ca^{2+} concentrations rise in liver cells. In experiments presented here, isolated hepatocytes were exposed to CCl_4, DCE, and phenylephrine (PE), a non-hepatotoxic alpha$_1$-adrenergic agent that mobilizes Ca^{2+}. Cytoplasmic Ca^{2+} concentrations were evaluated by two methods: (1) indirectly by assaying the activity of glycogen phosphorylase *a*, and (2) directly by monitoring the fluorescence of quin2. In primary hepatocyte cultures, CCl_4, DCE, and PE exposure increased the activity of phosphorylase *a* at 5 min from 39 ± 2 to 130 ± 12, 80 ± 13, and 97 ± 10 nmoles PO_4^{3-}/mg protein/min respectively. In rat hepatocyte suspensions loaded with quin2 and exposed to CCl_4, DCE, or PE, cytosolic Ca^{2+} concentrations were elevated within 20 sec to 0.83 ± 0.13, 0.59 ± 0.06 and 0.99 ± 0.14 μM Ca^{2+} respectively. Basal Ca^{2+} levels in these cells averaged 0.25 ± 0.03 μM. Thus, CCl_4 and PE apparently increased cytosolic Ca^{2+} levels to approximately the same extent, whereas DCE was somewhat less effective. The durations of the effects of CCl_4 and PE were examined further by determining their time courses of elevated phosphorylase *a* activity. In hepatocyte cultures, increased phosphorylase *a* activity persisted through at least 60 min following CCl_4 exposure. In contrast, phosphorylase *a* activity returned to basal levels by 20 min after PE. Increases in cytoplasmic Ca^{2+} levels that are sustained rather than transient may distinguish these hepatotoxic chlorinated aliphatic hydrocarbons from non-toxic hormonal agents.

The hepatotoxins carbon tetrachloride (CCl_4) and 1,1-dichloroethylene (DCE, vinylidene chloride) inhibit Ca^{2+} sequestration by the Ca^{2+}/Mg^{2+} ATPase (Ca^{2+} pump) found at rat liver endoplasmic reticulum (ER) both *in vivo* [1–4] and *in vitro* (microsomes, [5–8]). These chlorinated hydrocarbons are distinguished by the fact that CCl_4 produces lipid peroxidation, whereas DCE does not [3, 9]. The ER is an important organelle for maintenance of normal Ca^{2+} homeostasis in liver cells [10, 11]. Previous work from this laboratory has demonstrated that, in primary cultures of rat hepatocytes exposed to CCl_4, ER Ca^{2+} pump activity is inhibited severely, and the activity of a Ca^{2+}-responsive (via phosphorylase kinase§) cytosolic enzyme, glycogen phosphorylase *a*, is correspondingly increased [7]. In those experiments, elevated cyclic AMP did not accompany increased phosphorylase *a* activity, indicating that phosphorylase activation was indeed Ca^{2+}-mediated [7]. The presence of extracellular Ca^{2+} was not required, confirming that Ca^{2+} was released from intracellular stores [7].

In the present work, the appearance of increased phosphorylase *a* activity in hepatocyte cultures was extended to include DCE, the non-lipid peroxidizing hepatotoxin. Elevated intracellular Ca^{2+} levels were confirmed in hepatocyte suspensions exposed to CCl_4 or DCE using quin2. This fluorescent indicator compound reports cytoplasmic concentrations of ionized calcium (Ca^{2+}, [13]). The results are contrasted with these actions of Ca^{2+}-mobilizing hormones that act through alpha$_1$-adrenergic receptors. Exposure of hepatocytes to CCl_4, in contrast to phenylephrine (PE), produced increased phosphorylase *a* activity that was sustained for 60 min or longer. We conclude that, when halocarbons irreversibly inhibit ER Ca^{2+} sequestration, cytosolic Ca^{2+} concentrations are elevated in a sustained manner and persistent stimu-

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† The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or USUHS. These experiments were conducted according to the principles set forth in the "Guide for Care and Use of Laboratory Animals", Institute of Animal Resources, National Research Council, DHEW Pub. No. (NIH) 78-23.

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§ Increased cytosolic Ca^{2+} stimulates the activity of glycogen phosphorylase kinase, which converts glycogen phosphorylase *b* to phosphorylase *a* by phosphorylation [12].

lation of the activity of Ca^{2+}-sensitive enzymes can result.

METHODS

Hepatocyte cultures. Liver cells were isolated from male, Sprague–Dawley rats (200–250 g, Taconic Farms, Germantown, NY) by a modification of the two-step perfusion method of Berry and Friend [14]. Hepatocytes were then prepared as primary cultures as described by Bissell and Guzelian [15]. Hepatocytes were diluted into Williams E medium (1.8 mM $CaCl_2$) supplemented with 10% fetal calf serum, 50 μg/ml penicillin-streptomycin, 5 μg/ml glutamine (all from Flow Laboratories, Rockville, MD), 5 μg/ml insulin, 5 μg/ml transferrin, 5 μg/ml selenium (ITS Premix, Collaborative Res., Lexington, MA), 4 ng/ml dexamethasone (Sigma Chemical Co., St. Louis, MO), and 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES). Cells were then plated at 1×10^5 per cm^2 onto dishes that were precoated with collagen (40 μg/ml, Vitrogen, Collaborative Res.), and kept in a humidified, 5% CO_2 37° incubator for 18 hr.

Hepatocyte suspensions. Hepatocytes were isolated as described above. Cells were suspended at 5×10^6/ml in Krebs–Henseleit buffer (1 mM $CaCl_2$, 25 mM $NaHCO_3$, 93.5 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 10 mM glucose, 25 mM HEPES, pH 7.4) fortified with 5 mM glutamate, 5 mM fumarate, 5 mM pyruvate, 2% bovine serum albumin, fraction V (all from the Sigma Chemical Co.), and Eagle's essential amino acids (Flow Laboratories). O_2 was bubbled through this medium for 10 min prior to use. Hepatocytes were preincubated for 20 min at 37° in an orbital shaker bath and then were diluted to 2×10^6/ml in Krebs–Henseleit medium without supplements for loading with quin2.

Determination of Ca^{2+} concentration with quin2. Quin2-AM (cell permeant acetoxymethyl ester form, Calbiochem, La Jolla, CA) was added to cell suspensions to a final concentration of 50 μM. Hepatocytes were incubated at 37° for 5 min for hydrolysis of quin2-AM to free quin2. Cells were collected by a 5-sec centrifugation at 13,000 *g*. Hepatocytes were resuspended in Krebs–Henseleit buffer (without supplements) at a final concentration of 1×10^6/ml and transferred to a 1 cm × 1 cm × 4.5 cm quartz cuvette. Fluorometric determinations were made with a Perkin–Elmer MPF-44 fluorometer (Perkin–Elmer Corp., Norwalk, CT) equipped with a 37° water-jacketed cuvette holder with a magnetic stirrer. Excitation wavelength was 339 nm and emission wavelength was 500 nm; slit widths were 5 nm. To calculate the Ca^{2+} concentration from fluorescence (F), calibration procedures were performed as described by Tsien *et al.* [13]. Maximum quin2 fluorescence (F_{max}) for the dye loading was determined by addition of 20 μM digitonin (Sigma Chemical Co.), and minimum fluorescence (F_{min}) was obtained by addition of 4 mM ethylene glycol-(β-aminoethyl ether)-N,N′-tetraacetic acid (EGTA) (Sigma Chemical Co.). Corrections were made for changes in cell autofluorescence that occurred when these manipulations were performed with non-quin2-loaded hepatocytes. Ca^{2+} concentrations were calculated as follows, assuming a dissociation constant (K_d) for Ca^{2+}-quin2 of 115 nM [13]:

$$[Ca^{2+}] = K_d (F - F_{min})/(F_{max} - F).$$

Exposure of hepatocytes to halocarbons and phenylephrine. CCl_4 (Eastman Kodak, Rochester, NY) and DCE (Aldrich Chemical Co., Milwaukee, WI) were dissolved in ethanol or dimethyl sulfoxide, as indicated, and PE (Sigma Chemical Co.) was dissolved in water. For phosphorylase *a* determinations, compounds were added directly to the 35-mm culture dishes (5 μl addition to 1.5 ml medium). Exposures were terminated at the specified times by aspiration of the medium, and dishes with attached cells were plunged into liquid nitrogen. Samples were stored at −70° until assayed. For quin2 determinations, CCl_4, DCE, and PE were added directly to the suspensions of dye-loaded cells using the same vehicles and volumes. Mixing was accomplished by rapid inversion of the fluorometer cuvette.

Actual media concentrations of halocarbons were determined as follows. Aliquots of the culture medium were extracted into ten (CCl_4) or two (DCE) volumes of heptane. Samples were kept on ice in sealed vials with no head space until analyzed. Microliter volumes were injected into a gas chromatograph (Hewlett–Packard model 5710A) equipped with a 2 mm × 6 ft column (0.1% SP-1000 on 80/100 Carbopack) and an electron capture detector. Temperatures used were: injector, 200°; column, 125°; and detector, 300°.

Determination of glycogen phosphorylase a activity. Glycogen phosphorylase *a* activity was assayed directly in the 35-mm culture dishes essentially as described by Hue *et al.* [16]. Cells were allowed to thaw for 5 min at 37° in the presence of 0.25 ml of a lysing buffer containing 100 mM NaF, 20 mM EDTA, 0.5% glycogen, 50 mM glycylglycine, pH 7.4, and 0.5% Triton X-100 (all from the Sigma Chemical Co.). The assay was initiated by addition of 0.25 ml of a buffer containing 100 mM glucose-1-phosphate, 2% glycogen, and 1 mM caffeine (all from the Sigma Chemical Co.), pH 6.1. Dishes were gently rocked at 37° for 45 min. Assays were stopped by addition of 1.0 ml of ice-cold 5% trichloroacetic acid, and samples were allowed to precipitate on ice for 10 min. Aliquots were centrifuged at 13,000 *g* for 1 min, and inorganic phosphate in the supernatant fraction was quantitated in 100 mM glycine buffer, pH 2.5, by the method of Fiske and Subbarow [17]. Samples were assayed in the appropriate ranges where increases in phosphorylase *a* activity were linear with time and protein.

Determination of protein content. Pellets obtained from trichloroacetic acid precipitates were digested overnight in 0.1 ml of 1 N NaOH. Protein contents were determined by modification of the Lowry method as described by Shatkin [18].

Statistical analysis. Statistical significance was determined at the $P \leq 0.05$ level by a one- or two-way analysis of variance (ANOVA) followed by least significant difference tests, as appropriate [19].

RESULTS

Effects of CCl_4, DCE, and PE on phosphorylase

Table 1. Cytoplasmic Ca^{2+} concentrations estimated from phosphorylase *a* activity in hepatocyte cultures

	Phosphorylase *a** (nmoles PO_4^{3-}/mg protein/min)	Estimated $[Ca^{2+}]$† (μM)
Vehicle	39 ± 2	0.04
CCl_4 (3 mM)	130 ± 12‡	≥3
DCE (4 mM)	80 ± 13‡	0.3
PE (10 μM)	97 ± 10‡	1

Hepatocyte cultures exposed to CCl_4, DCE, PE, or vehicle (ethanol, 0.3% by volume) for 5 min. Phosphorylase *a* activity was quantitated as nmoles inorganic phosphate released from glucose-1-phosphate/mg protein/min. Differences between treatments and vehicle were determined by least significant difference tests following one-way ANOVA. Ca^{2+} concentrations were estimated from a calibration curve for phosphorylase *a* activity as a function of intracellular Ca^{2+} concentrations set with EGTA buffers in cultured hepatocytes made permeable with saponin [7].

* Values represent means ± SEM from three cell preparations.

† Estimated by use of a calibration curve, presented elsewhere [7].

‡ Significantly different from vehicle by one-way ANOVA at $P \leq 0.05$.

a *activity*. Basal phosphorylase *a* activity in cultured hepatocytes averaged 39 ± 2 nmoles PO_4^{3-}/mg protein/min. When CCl_4 was added to the medium to an initial concentration of 3 mM, phosphorylase *a* activity increased to 3.3-fold of control levels after 5 min (Table 1). In hepatocyte cultures made 4 mM in DCE, phosphorylase *a* activity increased to 2.0-fold basal levels at 5 min (Table 1). In cultured cells exposed to 10 μM PE, phosphorylase *a* activity increased to 2.5-fold control activity at 5 min (Table 1). Treatment with the corresponding vehicles did not affect phosphorylase *a* activity in control cultures. All increases in phosphorylase *a* activity were independent of changes in cyclic AMP concentrations, indicating that enzyme activation was mediated by Ca^{2+} ([7] and data not shown). Media levels of halocarbons were quantitated by gas chromatography with electron capture detection. It was determined that CCl_4 and DCE concentrations had decreased approximately 10-fold after 5 min at 37° (data not shown). This suggested that effects of these chlorinated hydrocarbons developed at early times and were not reversed as CCl_4 and DCE vaporized out of the culture dishes.

Cytosolic Ca^{2+} concentrations were estimated from phosphorylase *a* activities, using a calibration curve that has been presented elsewhere [7]. Briefly, phosphorylase *a* activity was determined in detergent-permeabilized cultured hepatocytes that had intracellular Ca^{2+} concentrations set with Ca^{2+}/EGTA buffers, as described by Burgess *et al.* [20]. Phosphorylase *a* activity increases as a linear function of the logarithm of the Ca^{2+} concentration from 0.01 to 3 μM Ca^{2+} [7]. Using this calibration curve, cytoplasmic Ca^{2+} concentrations were estimated from phosphorylase *a* activities determined here to be: 0.04 μM in control cultures, ≥3 μM in hepatocytes exposed to CCl_4, 0.3 μM in cells exposed to DCE, and 1 μM in cells exposed to PE (Table 1).

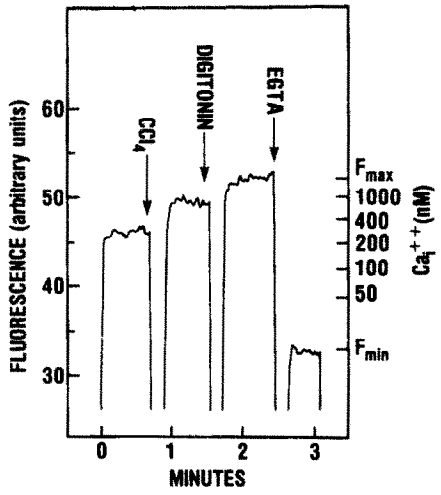

Fig. 1. Quin2-loaded hepatocyte suspensions exposed to carbon tetrachloride. CCl_4 (2 mM) was added to quin2-loaded hepatocyte suspensions. Fluorescence was monitored at excitation wavelength 339 nm and emission wavelength 500 nm; slit widths were 5 nm. Maximum and minimum fluorescence were determined by successive additions of digitonin (20 μM) and EDTA (4 mM). Calcium concentrations were calculated from fluorescence as described in Methods. Vertical lines indicate opening and closing of the sample compartment door for additions.

Effects of CCl_4, *DCE, and PE on* Ca^{2+} *concentrations determined with quin2*. A complete record of an experiment with a suspension of freshly isolated hepatocytes exposed to 2 mM CCl_4 and then subjected to calibration procedures as described in Methods is shown in Fig. 1. Slight changes in cell "autofluorescence" produced by additions (notably digitonin) to non-quin2 loaded cells are not shown. Calculated resting intracellular Ca^{2+} concentrations averaged 0.25 ± 0.03 μM. This value is in close agreement with the cytoplasmic Ca^{2+} concentrations estimated by others using a variety of techniques with hepatocyte suspensions.* Figure 2 depicts fluorescence increases in aliquots of cells from a

* Charest *et al.* [21] estimated cytosolic Ca^{2+} concentrations in suspensions of rat hepatocytes to be 0.2 μM with quin2. Murphy *et al.* [22] estimated intracellular Ca^{2+} to be 0.19 μM in rat liver cell suspensions by null point titration with arsenazo III. Burgess *et al.* [20] estimated cytoplasmic Ca^{2+} to be 0.18 μM from phosphorylase *a* activity in guinea pig hepatocyte suspensions.

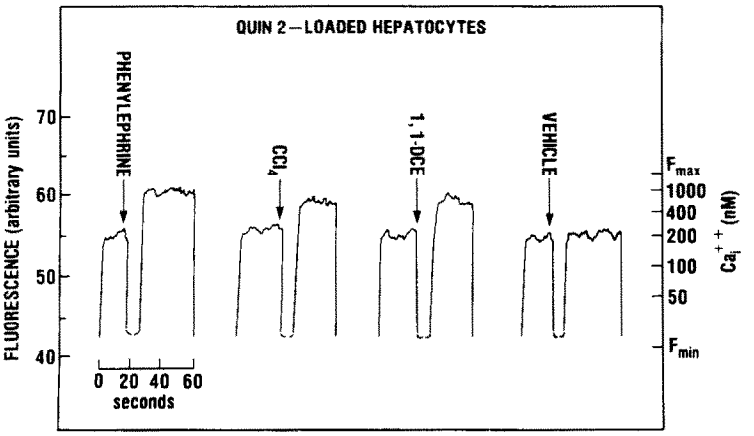

Fig. 2. Quin2-loaded hepatocyte suspensions exposed to phenylephrine, carbon tetrachloride and 1,1-dichloroethylene. PE (10 μM), CCl_4 (2 mM), 1,1-DCE (4 mM), or vehicle (ethanol, 0.3% by volume) was added to different samples from a single preparation of quin2-loaded hepatocytes. Fluorescence was monitored at excitation wavelength 339 nm and emission wavelength 500 nm; slit widths were 5 nm. Maximum and minimum fluorescence were determined (not shown), and calcium concentrations were calculated from fluorescence as described in Methods. Vertical lines indicate opening and closing of the sample compartment door for additions.

Table 2. Cytoplasmic Ca^{2+} concentrations determined with quin2 in hepatocyte suspensions

	Ca^{2+} (μM)	
	Before treatment	After treatment
CCl_4 (2 mM)	0.25 ± 0.03	0.83 ± 0.13*
DCE (4 mM)	0.26 ± 0.05	0.59 ± 0.06*
PE (10 μM)	0.24 ± 0.01	0.99 ± 0.14*

CCl_4, DCE, or PE was added to quin2-loaded hepatocyte suspensions, and fluorescence was monitored for 20–40 sec as depicted in Fig. 1. Calcium concentrations were calculated from fluorescence changes after calibration procedures were performed, as described in Methods. Differences between before and after treatments were determined by least significant difference tests following two-way ANOVA. Values represent means ± SEM from different cell preparations; N was as follows: CCl_4 = 3, DCE = 2, and PE = 5.

* Different than "before treatment" by two-way ANOVA at $P \leq 0.05$.

representative hepatocyte preparation after addition of PE (10 μM), CCl_4 (2 mM), DCE (4 mM), or vehicle. After calibration procedures were performed and results were averaged over all cell preparations examined, cytosolic Ca^{2+} concentrations rose to 0.83 ± 0.13 μM after CCl_4, 0.59 ± 0.06 μM after DCE, and 0.99 ± 0.14 μM after PE (Table 2). Fluorescence changes were complete within 20 sec, confirming that intracellular Ca^{2+} release caused by these agents occurred very rapidly. Fluorescence was unaffected by the vehicles.

Time-dependent effects of CCl_4 *and PE on phosphorylase a activity.* Cultured hepatocytes were exposed to CCl_4 (3 mM), PE (10 μM), or vehicles, and phosphorylase *a* activity was determined at 2.5 through 60 min. In cells exposed to PE, phosphorylase stimulation was transient. Phosphorylase *a* activity increased maximally to 3.5-fold control levels at 2.5 min and then returned to levels statistically indistinguishable from controls by 20 min (Fig. 3). PE did not produce greater stimulation of phosphorylase *a* activity at earlier times (1 or 2 min) or at higher doses (>10 μM) than were used here (data not shown). In contrast, phosphorylase stimulation was sustained in cells exposed to CCl_4, even as media levels of CCl_4 declined greatly (see first section of Results, above). Phosphorylase *a* activity increased to a greater extent (5-fold), and activity remained elevated (3-fold) through at least 60 min, the latest timepoint examined. Differences from basal levels were statistically significant at 2.5, 5, and 10 min for PE, and at 2.5 through 60 min for CCl_4.

DISCUSSION

This report presents evidence that the hepatotoxins CCl_4 and DCE increase Ca^{2+} levels in the cytosol of rat liver cells. Cytoplasmic Ca^{2+} reflects the net effect of injury (or lack thereof) at all organelles that regulate intracellular Ca^{2+} homeostasis. Elevated cytoplasmic Ca^{2+} could result from inhibition of ER Ca^{2+} pumping and concomitant release of Ca^{2+} sequestered by that organelle. ATP-dependent accumulation of Ca^{2+} by ER is crucial to the maintenance of low cytosolic Ca^{2+} (on the order of 10^{-7} M) in liver cells [10, 23]. Extensive inhibition of the ER Ca^{+} pump has been described after both CCl_4 and DCE *in vivo* [1–4] and *in vitro* [5–8]. There are other Ca^{2+}-sequestering systems in liver that are also important regulators of cytoplasmic Ca^{2+} concentrations. Likewise, they may also be adversely affected by these chlorinated hydrocarbons. Plasma membrane aids ER in the control of intracellular Ca^{2+} levels by both active and exchange (for Na^{+}) processes [24, 25]. CCl_4 is known to inhibit Ca^{2+} pumping by plasma membrane in liver tissue [26]. The effects of DCE on Ca^{2+} extrusion by plasma membrane are unknown at this time. Mitochondria contribute to liver cell Ca^{2+} homeostasis by sequestering Ca^{2+} at relatively higher cytoplasmic Ca^{2+}

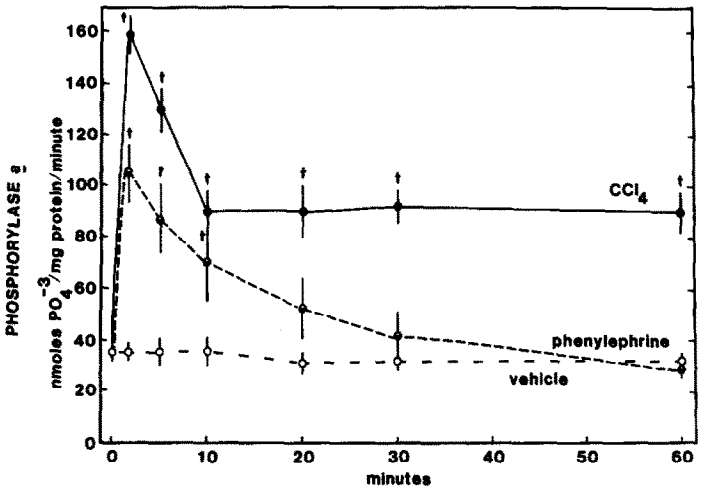

Fig. 3. Time-dependent effects of carbon tetrachloride or phenylephrine on phosphorylase *a* activity in hepatocyte cultures. CCl_4 (3 mM), PE (10 μM), or vehicle (dimethyl sulfoxide or water respectively) was added to hepatocyte cultures at 0 min. Exposures were terminated at 2.5 through 30 min. Phosphorylase *a* activity was quantitated as nmoles inorganic phosphate released from glucose-1-phosphate/mg protein/min. Data represent means ± SEM from three cell preparations. Differences between treatments and vehicle were determined to be significant at $P \leq 0.05$ (†) by the least significant difference test following two-way ANOVA.

concentrations ($\geq 5 \times 10^{-7}$ M) [23, 27]. *In vivo* exposure to CCl_4 does not appear to alter the ability of mitochondria to accumulate Ca^{2+}; in fact, mitochondrial Ca^{2+} content increases several-fold [28, 29]. When isolated hepatocytes are exposed to CCl_4, however, it has been reported that mitochondrial Ca^{2+} content decreases [30], and thus mitochondrial functions may be inhibited by CCl_4 in experiments described here. The effects of DCE on mitochondrial Ca^{2+} sequestration are not known. Further work will be necessary to determine which sites are specifically affected by this halocarbon.

CCl_4 and DCE differ in their mechanisms of injury at the cellular level. Unlike CCl_4, DCE does not produce evidence of lipid peroxidation in rat liver [3, 4, 9] or in microsomes [8, 9]. Furthermore, DCE does not produce the early and dramatic changes in ER morphology that are characteristic of CCl_4-induced liver injury [31–33]. Data presented here indicate that the effects of DCE on both Ca^{2+} levels (measured with quin2, Table 2) and on Ca^{2+}-dependent enzyme activation (phosphorylase *a*, Table 1) were less pronounced than those changes seen after CCl_4. DCE-induced injury to the ER Ca^{2+} pump can be less severe and takes somewhat longer to develop [3, 4, 8], which may explain why DCE mobilized Ca^{2+} to a lesser extent than CCl_4 did in hepatocytes. Alternatively, DCE and CCl_4 may differentially affect the other organelles that regulate cytoplasmic Ca^{2+} concentrations. For example, if the mitochondria and/or plasma membrane were/was selectively spared from injury following DCE exposure, either could perhaps partially compensate for the ER Ca^{2+} pump inhibition these agents have in common. Results obtained here do suggest that neither lipid peroxidation nor marked changes in ER morphology are essential prerequisites for the release of Ca^{2+} into cytosol and resultant stimulation of Ca^{2+}-sensitive enzyme activity.

The direct and indirect methods as employed here for determination of cytoplasmic Ca^{2+} concentrations represent complementary techniques. Phosphorylase *a* activity has been used extensively to estimate intracellular Ca^{2+} levels in isolated liver cells exposed to hormonal agents [20, 22] and, more recently, with hepatocytes exposed to toxicants [7, 34, 35]. However, few investigators have calibrated the phosphorylase *a* response [7, 20] in order to assign actual Ca^{2+} concentrations. Quin2, on the other hand, readily provides numerical Ca^{2+} levels in stimulated cell preparations [13, 21] but is susceptible to artifactual results as a consequence of fluorescence quenching produced by NADPH oxidation, contaminant heavy metals, leakage of dye from cells, etc. [36]. In this study, quantitative comparison can be made between cytosolic Ca^{2+} concentrations predicted by phosphorylase *a* and quin2 determinations in the different hepatocyte preparations. In cultured hepatocytes, elevated phosphorylase *a* activity indicated that intracellular Ca^{2+} rose to ≥ 3 μM following CCl_4 exposure, to approximately 0.3 μM after DCE exposure, and to about 1 μM after PE addition (Table 1). In suspensions of hepatocytes, quin2 fluorescence indicated that cytoplasmic Ca^{2+} rose to about 0.8 μM after CCl_4 exposure, to approximately 0.6 μM following DCE addition, and to about 1 μM after PE (Table 2). Thus, the two techniques yielded similar but not identical estimates of the concentrations of cytosolic Ca^{2+} produced by CCl_4, DCE, and PE exposure. Quantitatively different Ca^{2+} levels could have resulted from the use of different hepatocyte preparations (cultures vs suspensions). It was extremely difficult to obtain sufficient fluorescence signal to analyze quin2 responses in hepatocyte monolayers (data not shown). Likewise, although phosphorylase *a* activity increases could be demonstrated following exposure of hepatocyte suspensions to CCl_4, DCE, and PE (data not shown), low basal phosphorylase *a* levels were more reproducible in hepatocyte

cultures. Interestingly, we report for the first time that the resting Ca^{2+} concentration estimated in primary hepatocyte cultures from phosphorylase *a* activity (0.04 μM, Table 1) was considerably lower than that found in freshly isolated hepatocyte suspensions by quin2 fluorescence (0.25 μM, Table 2). This is believed to be a true difference in the cell preparations and not a methodological artifact, because we found in hepatocyte suspensions that phosphorylase *a* activity predicted a basal intracellular Ca^{2+} concentration of about 0.23 μM (data not shown), which is quite close to that predicted by quin2 (0.25 μM, Table 2). Others have reported that, in GH_3 cells, levels of intracellular Ca^{2+} are lower when they are maintained as attached cultures (0.04 μM [37]) than when the same cells are kept as suspensions (0.12 μM, [38]). We speculate that the plasma membrane of cells maintained in monolayer culture may be more impermeable to passive Ca^{2+} influx or, alternatively, may more efficiently extrude intracellular Ca^{2+}; thus, the cultured cells can maintain a larger gradient of extra- to intracellular Ca^{2+}.

Hepatotoxins may increase cytosolic Ca^{2+} levels in rat liver cells in a manner that is different temporally from physiological changes in cytoplasmic Ca^{2+} produced by hormonal agents. In these studies, the alpha$_1$-adrenergic agonist PE was used as a model for hormones that increase cytoplasmic Ca^{2+}. Because results obtained with quin2 (Table 2) indicated that CCl_4 and PE rapidly mobilized Ca^{2+} to approximately the same extent, experiments were conducted to determine whether these agents differed in their durations of action. Quin2 is not suitable for use in experiments where hepatotoxicant effects are monitored for prolonged periods of time; thus, Ca^{2+}-stimulated enzyme activity (phosphorylase *a*) was again determined. CCl_4 exposure produced increased phosphorylase *a* activity that was sustained for $\geq$60 min, whereas PE elevated phosphorylase *a* levels transiently (Fig. 3). CCl_4 may even have mobilized Ca^{2+} to a greater extent than PE, because phosphorylase *a* activity obtained after CCl_4 exposure exceeded that observed following PE at all times examined. This would not have been detected in the experiments with quin2, because quin2 is only useful as an indicator of cytosolic Ca^{2+} up to concentrations of about 1 μM (Fig. 1). In Fig. 3, the curve for phosphorylase *a* activity after CCl_4 treatment may represent Ca^{2+} concentrations directly, or may reflect the activation state of Ca^{2+}-sensitive enzymes. In the early phase (0 to 2.5 min), cytoplasmic Ca^{2+} likely is rising rapidly. During the next phase (2.5 to 5 min), cytosolic Ca^{2+} appears to be declining, perhaps as a result of extrusion by the plasma membrane or accumulation by mitochondria. By the final phase ($\geq$10 min), Ca^{2+} concentrations apparently decreased, or Ca^{2+} could have reached such high levels that Ca^{2+}-sensitive enzyme activity is now inhibited. Thus, increases in cytoplasmic Ca^{2+} following hepatotoxin exposure could be supraphysiologic either in magnitude or duration, or both. Excessive Ca^{2+} could inappropriately stimulate or, if Ca^{2+} reaches extremely high levels, even inhibit Ca^{2+}-sensitive degradative, metabolic, or regulatory processes, and thus initiate or contribute to lethal cell injury.

In summary, these experiments present the first evidence that: (1) DCE, an agent that does not produce lipid peroxidation, as well as CCl_4, can increase cytosolic Ca^{2+} levels in hepatocytes, (2) determinations of cytosolic Ca^{2+} concentrations from phosphorylase *a* activities and quin2 fluorescence yield quantitatively similar results, (3) the resting Ca^{2+} concentration appears to be considerably lower in primary hepatocyte cultures than in freshly isolated hepatocyte suspensions, and (4) the hepatotoxin CCl_4 can produce a sustained, non-physiologic increase in the activity of a Ca^{2+}-sensitive enzyme (phosphorylase *a*). Estimation of cytoplasmic ionized (i.e. biologically active) Ca^{2+} levels will remain an important determination to make when investigating all types of tissue injury (e.g. chemical, ischemic, or traumatic). Disruption of intracellular Ca^{2+} homeostasis may prove to be a mechanism that can initiate or contribute to cellular injury after diverse toxic insults. Prolonged stimulation of Ca^{2+}-responsive biochemical and/or structural cell functions may represent an injurious step common to a wide variety of toxicities and target tissues.

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