Thiol Oxidation and Cytochrome P450-Dependent Metabolism of CCl_4 Triggers Ca^{2+} Release from Liver Microsomes†

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ABSTRACT: Elevation of cytosolic calcium levels has been shown to occur after exposure to hepatotoxins such as CCl_4. This has been associated with inhibition of the Ca^{2+},Mg^{2+}-ATPase which pumps calcium into the endoplasmic reticulum. Elevated cytosolic Ca^{2+} may also result from activation of calcium releasing channels. In the presence of NADPH, CCl_4 produced a concentration-dependent release of calcium from liver microsomes after a lag period. The lag period was shorter with microsomes from pyrazole-treated rats in which CYP2E1 is induced, as compared to saline microsomes. The calcium releasing process appears to be very sensitive to activation by CCl_4 as effective concentrations (e.g., 50 μM) did not affect the Ca^{2+},Mg^{2+}-ATPase or produce lipid peroxidation. Inhibition of the CCl_4-induced release of calcium by 4-methylpyrazole and by anti-CYP2E1 IgG, and the requirement for NADPH, indicates that CCl_4 metabolism is required for the activation of calcium release. The lag period for CCl_4-induced release of calcium was associated with the time required to deplete α-tocopherol from the microsomal membranes; however, lipid peroxidation was not observed at these levels of CCl_4, and the lag period for CCl_4-induced release of calcium was shorter under anaerobic than aerobic conditions, suggesting a possible role for $^{\bullet}CCl_3$ in the mechanism of activation. Production of $^{\bullet}CCl_3$ was observed by ESR spin-trapping experiments with PBN; PBN prevented the CCl_4-induced calcium release, presumably by interacting with $^{\bullet}CCl_3$ and other reactive species. Calcium release was produced by thiol oxidants such as 2,2′-dithiodipyridine. Lipophilic thiols such as mercaptoethanol or cysteamine could partially reverse the CCl_4-induced calcium release, whereas GSH was ineffective. While the IP_3 receptor system is considered as the main regulator of calcium release, liver also contains ryanodine-sensitive calcium releasing stores. The CCl_4-induced calcium release was blocked by ruthenium red, a specific inhibitor of the ryanodine receptor; ruthenium red did not block CCl_4 metabolism to $^{\bullet}CCl_3$. CCl_4 increased the binding of ryanodine, a specific ligand for the ryanodine-sensitive calcium channel. These results suggest that metabolism of CCl_4 to reactive species by cytochrome P450 results in an activation of a ryanodine-sensitive calcium channel, perhaps due to oxidation of lipophilic thiols of the channel. Activation of calcium releasing channels may play a role in the elevated cytosolic calcium levels found in the liver after treatment with hepatotoxins.

The hepatotoxicity of CCl_4 is believed to result largely from the metabolic activation of the halocarbon molecule by cytochrome P450 to $^{\bullet}CCl_3$ radical (McLean & McLean, 1966; McCay et al., 1984; Albano et al., 1982; Connor et al., 1990). The $^{\bullet}CCl_3$ radical has been shown to form adducts with hepatic molecules (Slater, 1966; Gregory, 1966), to oxidize glutathione (Connor et al., 1990) and protein thiols (Suntres & Lui, 1990; Parola et al., 1990), and to induce lipid peroxidation (LPO) (Recknagel & Ghoshal, 1966; Jose & Slater, 1972). It has been documented in numerous studies that CCl_4 rapidly and severely inhibits hepatic calcium sequestration (Srivastava et al., 1990; Yamamoto, 1990 and references therein). Long and Moore (1986) have shown that the cytosolic concentration of Ca^{2+} in hepatocytes exposed to CCl_4 is elevated 100-fold, and this parallels the inhibition of the endoplasmic reticulum Ca^{2+},Mg^{2+}-ATPase. The CCl_4-dependent inhibition of the Ca^{2+},Mg^{2+}-ATPase has been confirmed in several studies, leading to the hypothesis that this is the specific mechanism by which radical intermediates formed from CCl_4 metabolism lead to cell death (Parola et al., 1990; Srivastava et al., 1990; Kodovanti et al., 1991, 1993).

The hypothesis that elevation of free cytosolic Ca^{2+} is a common mechanism in the toxicity of many reactive agents is becoming increasingly persuasive. It has been reported that the oxidation of a small, but critical, pool of protein thiols can cause an irreversible damage to hepatocytes as a result of a rise of cytosolic Ca^{2+} concentration, followed by activation of cytosolic proteolytic systems and phospholipid hydrolysis (Nicotera et al., 1986). Perturbation of hepatic Ca^{2+} homeostasis was also associated with modulation of processes such as gluconeogenesis, glycogenolysis, respiration, and cell division (Kraus-Friedman, 1984; Bygrave & Benedetti, 1993). The concept for a relationship between the oxidation of critical cellular thiols and hepatic Ca^{2+} homeostasis is in agreement with reports showing a CCl_4-induced inactivation of the Ca^{2+},Mg^{2+}-ATPase. Srivastava et al. (1990) have demonstrated that CCl_4 metabolites inhibit the hepatic microsomal Ca^{2+},Mg^{2+}-ATPase by a process that

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can be reversed by GSH,[1] most likely reflecting reduction of disulfide bonds produced on the ATPase as a consequence of thiol oxidation.

Besides inhibition of the Ca^{2+},Mg^{2+}-ATPase by reactive metabolites produced from CCl_4 and other toxic agents, increased cytosolic Ca^{2+} levels may result from activation of the calcium channels which are responsible for mobilization of stored calcium. The IP_3R is considered to be the main regulator of Ca^{2+} release in liver. Inositol 1,4,5-trisphosphate (IP_3), a second messenger generated following hormonal stimulation, binds to the IP_3R which is located on the cytosolic interface of an intracellular Ca^{2+}-containing compartment, triggering a release of Ca^{2+} (Berridge, 1993). Recently, it has been shown that the IP_3 channel is considerably more sensitive to the action of IP_3 when its thiol groups are converted to disulfides (Kaplin et al., 1994). Increasing evidence suggests that an IP_3-independent calcium releasing channel also exists in the liver endoplasmic reticulum, which is sensitive to ryanodine (Feng & Kraus-Friedman, 1992; Lilly & Gollan, 1995; Rooney et al., 1989). Ryanodine, an alkaloid that modulates Ca^{2+} release in skeletal (SSR) and cardiac (CSR) sarcoplasmic reticulum, has been shown to specifically bind to the calcium gated calcium release channel (RyR; Coronado et al., 1994). Several differences have been noticed between the ryanodine-induced Ca^{2+} release in liver microsomes, SSR, and CSR, which may be due to the occurrence of different isoforms of the RyR (Feng & Kraus-Friedman, 1992; Lilly & Gollan, 1995). The existence of a liver isoform of the RyR is interesting since the skeletal and cardiac isoforms have cysteine sulfhydryls whose conversion to disulfide(s) opens the channels for Ca^{2+} (Zaidi et al., 1989; Finkel et al., 1993; Galione et al., 1993). If similar reactions occur for the RyR of liver endoplasmic reticulum, increased release of Ca^{2+} from storage sites may play a role in hepatotoxic mechanisms for agents promoting oxidation of protein thiols.

In the present study, we evaluated: (i) whether 2,2′-dithiodipyridine (2,2′-DTDP), a thiol−thiol oxidizing reagent known to activate the SSR and CSR isoforms of the RyR, induces Ca^{2+} release from liver microsomes, and (ii) whether the cytochrome P4502E1-dependent metabolism of CCl_4 causes Ca^{2+} release and modulates ryanodine binding to liver microsomes. CYP2E1 is known to be especially reactive for the bioactivation and hepatotoxicity of CCl_4 (Johansson & Ingelman-Sundberg, 1985; Sohn et al., 1991; Tierney et al., 1992; Teschke et al., 1984). Whether this bioactivation could result in elevated release of Ca^{2+} from microsomal storage sites sensitive to ryanodine, a specific ligand of the RyR, was the major focus of this investigation.

MATERIALS AND METHODS

Reagents. All reagents used were purchased from Sigma Chemical Co. (St. Louis, MO). The solutions used in the experiments were prepared in deionized and Chelex-100-treated water or potassium phosphate buffer. [^{3}H]Ryanodine (70.8 Ci/mmol) was from DuPont, Co. Anti-CYP2E1 IgG was raised against CYP2E1 purified from liver microsomes of pyrazole-treated rats as previously described (Palakodety et al., 1988).

Preparation of Hepatic Microsomes. Sprague-Dawley rats weighing 120−150 g were injected ip with saline (control) or pyrazole (200 mg/kg body wt/day, 2 days). The rats were starved overnight prior to being killed, and liver microsomes were prepared as described previously (Palakodety et al., 1988), resuspended in 0.125 M KCl/0.01 M potassium phosphate or HEPES (pH = 7.4), and stored at −70 °C. All solutions used during the preparation contained 2 mM DTT, 0.15 mM desferrioxamine, leupeptin (0.001 mg/mL), and PMSF (0.1 mM). In all experiments, microsomes isolated from pyrazole-treated rats where used except were indicated otherwise.

ESR Measurements. ESR measurements were performed on a Bruker ECS080 spectrometer with 50 kHz magnetic field modulation at room temperature (22 °C). ESR spectrometer settings were: modulation amplitude 1.0 G, scan time 4 min, time constant 0.64 s, and microwave power 10 mW. For spin-trapping of CCl_4 radical metabolites, PBN was used as reported in Albano et al. (1982). Experiments were carried out in a shaking water bath with samples transferred to the flat cell after 15 min incubation.

Measurement of Ca^{2+} Transport. Ca^{2+} uptake and release from liver microsomes were monitored using a double beam double wavelength spectrophotometer (Perkin Elmer 557) through the differential absorption changes of antipyrylazo III (AP-III) at 720−790 nm (Zaidi, et al., 1989). In a typical experiment, uptake and release of Ca^{2+} were measured in microsomal suspensions (0.56 mg of protein/mL) in 0.1 M phosphate buffer (pH = 7.0), containing KCl (0.1 M), HEPES (20 mM), Mg^{2+} (0.5 mM), ATP (0.5 mM), and an ATP-regenerating system consisting of creatine phosphokinase (30 U/mL) and creatine phosphate (8 mM). Experiments were initiated by the addition of $CaCl_2$ (final Ca^{2+} concentration of 0.06 mM). Control experiments were performed in order to ensure that the compounds used do not interfere with the interaction of Ca^{2+} with AP-III. CCl_4 was dissolved in ethanol, whose final concentration in the microsomal suspension did not exceed 1.5 mM. In control experiments, ethanol (10−100 mM), in the presence of NADPH, did not cause any Ca^{2+} release. For most experiments, the final concentration of CCl_4 was routinely 0.05 mM.

The Ca^{2+},Mg^{2+}-ATPase activity was determined by measuring the accumulation of inorganic phosphate during the hydrolysis of ATP (Srivastava et al., 1990). Liver microsomes (0.56 mg/mL) were incubated with CCl_4 (0.05 mM) and NADPH (1 mM) for 20 min in HEPES (0.05 M, pH = 7.4, 37 °C). Subsequently, an aliquot of 0.05 mL was transferred into 1 mL of HEPES (0.05 mM, pH = 7.4), containing ATP (1 mM), ouabain (0.2 mM), $CaCl_2$ (0.06 mM), and $MgCl_2$ (0.05 mM), and the resulting solution was incubated for 20 min at 37 °C. The reaction was stopped by addition of 0.3 mL of 30% (v/v) TCA, and after centrifugation (3 min × 5000*g*), an aliquot of 0.1 mL was transferred into a solution of malachite green (0.8 mL of 0.034% in 1 M HCl). The color development was fixed by addition of 0.1 mL of 1% ammonium molybdate, and absorbance was determined at 640 nm.

PNP Oxidation. PNP oxidation was carried out in a reaction system containing 0.1 M phosphate buffer (pH 7.4,

[1] Abbreviations: dihydrolipoic acid, (DHLA); glutathione, (GSH); cytochrome P4502E1, (CYP2E1); ryanodine receptor, (RyR); phenyl-*tert*-butylnitrone (PBN); inositol 1,4,5-trisphosphate (IP_3); inositol 1,4,5-trisphosphate receptor (IP_3R); dithiothreitol (DTT); 2,2′-dithiodipyridine (2,2′-DTDP); ruthenium red (RR); *p*-nitrophenol (PNP); antipyrylazo III (AP-III).

37 °C), PNP (0.2 mM), liver microsomes (0.5 mg of protein/mL), and NADPH (1 mM) in a final reaction volume of 0.1 mL. The reaction was stopped by addition of 0.06 mL of 30% TCA, and the resulting suspension was centrifuged (5 min × 5000*g*). An aliquot of the supernatant (0.15 mL) was treated with 0.02 mL of 10 M NaOH, and absorbance was determined at 546 nm (Reinke & Moyer, 1985).

[^{3}H]Ryanodine Binding. Liver microsomes were incubated with NADPH (1 mM) for 25 min (0.1 M phosphate buffer, 0.1 M KCl, pH = 7.0, 37 °C) in the presence or in the absence of CCl_4 (0.05 mM). Subsequently, the cytochrome P450 system was inhibited by a stream of CO (2 min), and [^{3}H]ryanodine binding was determined as described previously (Shoshan-Barmatz, et al., 1991). Briefly, a mixture containing NaCl (0.5 M), EGTA (0.5 mM), and [^{3}H]-ryanodine (20 nM) was added to 0.2 mL of the microsomal suspension. After 10 min of incubation at 20 °C, aliquots of 0.1 mL were filtered through 0.22 μm microcentrifuge filters (4 min × 5000*g*; Ultrafree MC, Millipore, Inc.) and washed twice with 0.3 mL of ice-cold washing buffer (10 mM HEPES, 0.2 M NaCl, pH = 7.4; 4 min × 5000*g*). The remaining radioactivity on the filters was determined in an LKB liquid scintillation counter (LKB 1209 Rackbeta). The specific binding was determined as the difference between total binding ([^{3}H]ryanodine alone) and nonspecific binding (20 nM [^{3}H]ryanodine + 0.2 mM [^{1}H]ryanodine). In our experiments, the [^{3}H]ryanodine binding to control microsomes was 243 ± 38 fmol/mg of protein, a value that is in agreement with the studies of Shoshan-Barmatz et al. (1991).

HPLC Detection of α*-Tocopherol*. α-Tocopherol was determined as described by Lang et al. (1986). A 0.20 mL aliquot of the microsomal suspension (1.5 mg of protein/mL) was vortexed with SDS (0.15 mL of 0.1 M), ethanol (0.15 mL), and *n*-hexane (0.30 mL). After centrifugation (3 min × 3000*g*), a portion of the hexane supernatant (0.15 mL, top layer) was removed and dried under nitrogen, redissolved in methanol, and injected into a 20 μL loop of a Rheodine HPLC injector. α-Tocopherol was quantitatively determined using an HP Series III 1090 HPLC system equipped with a C-18 reverse phase column (Microsorb, 4.6 mm × 25 cm, 5 μm; Rainin Instrument Co., Inc.) and fluorescent detector (HP 1046A; λ_{excit} = 295 nm, λ_{emis} = 324 nm, slit widths ± 5 nm). The eluent was methanol at a flow rate of 1.5 mL/min, and synthetic *d,l*-α-tocopherol was used as an external standard (retention time of 7.5 min).

Determination of Protein and Products of LPO. Protein concentration in the microsomal suspensions was determined using the Bio-Rad Protein Assay Kit with bovine serum albumin as a standard. Lipid peroxidation was assayed by measuring the production of TBA-reactive metabolites that are breakdown products of lipid peroxides (Buege & Aust, 1978).

RESULTS

2,2′-DTDP-Induced Ca^{2+} Release from SSR and Liver Microsomes. Recent studies suggest the existence of an isoform of the RyR in liver microsomes (Feng & Kraus-Friedman, 1992; Lilly & Gollan, 1995; Rooney et al., 1989). Since thiol reagents trigger Ca^{2+} release from SSR and CSR via oxidation of the RyR thiols (Finkel et al., 1993), experiments were carried out to evaluate whether a similar

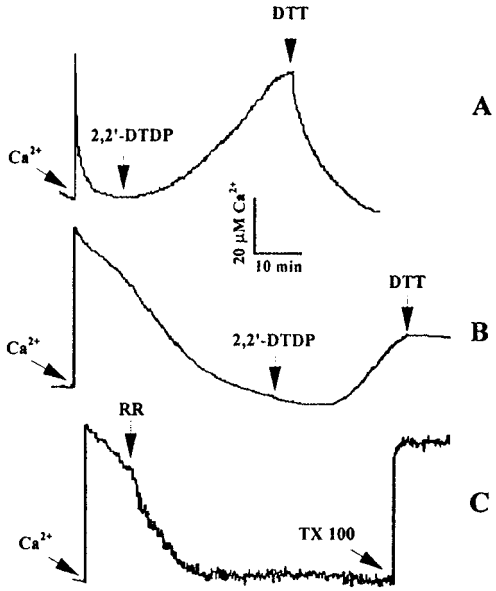

FIGURE 1: Kinetics of calcium uptake and release from SSR and liver microsomes: effects of 2,2′-DTDP, DTT, and ruthenium red. Incubation conditions were 0.1 M phosphate buffer (pH 7.0) at 37 °C, KCl (0.1 M), HEPES (20 mM), $CaCl_2$ (0.06 mM), $MgCl_2$ (0.5 mM), ATP (0.5 mM), phosphocreatine kinase (30 U/mL), and phosphocreatine (8 mM). The recordings shown are typical of four separate experiments (n = 4). (A) SSR (0.5 mg of protein/mL); 2,2′-DTDP (0.05 mM); DTT (10 mM) at 20 °C. (B) Liver microsomes (0.56 mg of protein/mL); 2,2′-DTDP (0.1 mM); DTT (10 mM). (C) Liver microsomes (0.56 mg of protein/mL); ruthenium red (0.020 mM); Triton X-100 (0.15%).

mechanism operates in liver microsomes. The addition of $CaCl_2$ (0.06 mM) to a microsomal suspension containing ATP initially resulted in a rapid increase of the absorbance due to formation of a Ca^{2+}−AP-III complex (Figure 1). Subsequently, the absorbance slowly decreased to its initial value as a result of the Ca^{2+},Mg^{2+}-ATPase-driven transport of Ca^{2+} into the microsomal compartment. No decrease in the absorbance was observed in the absence of ATP (data not shown). Uptake of Ca^{2+} was much more rapid by SSR compared to liver microsomes (Figure 1A compared to Figure 1B). Addition of the thiol oxidant, 2,2′-DTDP, caused release of the accumulated Ca^{2+} after a lag period with both microsomal preparations. The 2,2′-DTDP-induced release of Ca^{2+} was reversed by addition of DTT with SSR (Figure 1A); DTT prevented further Ca^{2+} release with liver microsomes (Figure 1B). Addition of RR, a specific inhibitor of the RyR (Favero et al., 1995), enhanced the rate of Ca^{2+} uptake by liver microsomes (Figure 1C), presumably due to inhibition of Ca^{2+} release (see below). All the accumulated Ca^{2+} was released after disruption of the microsomal membrane with Triton X-100 (Figure 1C).

Effect of CCl_4 on Ca^{2+} Release from Liver Microsomes. Once accumulated, the Ca^{2+} was not released from the liver microsomes even when NADPH was added to initiate electron transfer via the mixed-function oxidation system (data not shown). The addition of CCl_4 in the presence of NADPH to microsomes already loaded with Ca^{2+} resulted in an almost complete release of the accumulated Ca^{2+} after a lag period which was dependent upon the concentration of CCl_4 used over the range of 0.001−0.12 mM (Figure 2). These concentrations of CCl_4 did not produce any Ca^{2+} release in the absence of NADPH (data not shown). The release of Ca^{2+} by such low concentrations of CCl_4 suggests

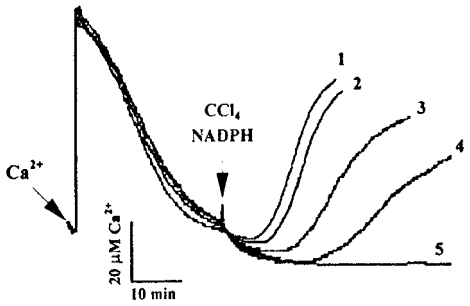

FIGURE 2: CCl_4-induced release of liver microsomal calcium. Experiments were carried out as described in the legend to Figure 1, with the addition of 1 mM NADPH plus the following concentrations of CCl_4 (mM): **1**, 0.12; **2**, 0.05; **3**, 0.025; **4**, 0.01; **5**, 0.001. The content of liver microsomes was 0.56 mg/mL.

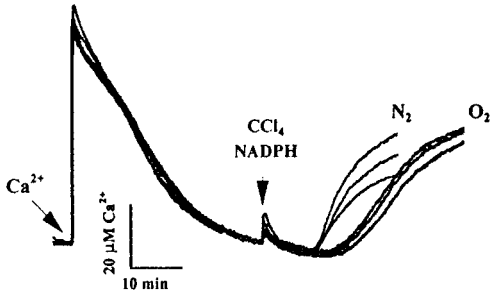

FIGURE 3: CCl_4-dependent Ca^{2+} release from liver microsomes in the presence or in the absence of oxygen. Incubation conditions are the same as indicated in the legend of Figure 1, with the addition of 1 mM NADPH and 0.05 mM CCl_4. The experiments under anaerobic conditions were performed in a cuvette with a Teflon cap after deoxygenation of the incubation buffer with a stream of nitrogen (10 min, 20 °C). Results are from 3 experiments carried out aerobically or anaerobically, using 0.56 mg of microsomal protein/mL.

that the liver microsomal Ca^{2+} channels are very sensitive to activation by metabolites produced from CCl_4 metabolism.

CCl_4 is metabolized to $^{\bullet}CCl_3$ radical which can react with oxygen to form the peroxyl radical ($^{\bullet}OOCCl_3$). Both radicals have been shown to induce LPO in liver microsomes (Recknagel & Ghoshal, 1966; Jose & Slater, 1966). To determine whether the observed Ca^{2+} release induced by CCl_4 is due to LPO-dependent permeabilization of the microsomal membranes, experiments were carried out under anaerobic conditions. Compared to the controls carried out under air, the lag time for CCl_4-dependent release of Ca^{2+} was shorter under anaerobic conditions (Figure 3). Under aerobic reaction conditions and with these concentrations of CCl_4, there was no detectable accumulation of LPO products (formation of thiobarbituric acid-reactive products, data not shown). However, CCl_4 metabolism did produce a marked loss of α-tocopherol from the microsomal membranes (Figure 4), which probably accounts for the absence of accumulation of LPO products. It is of interest that the lag time prior to stimulation of Ca^{2+} release by CCl_4 is similar to the time required for significant decreases in the concentration of α-tocopherol produced by CCl_4, e.g., 10−20 min.

To relate the CCl_4-induced Ca^{2+} release to activation of the RyR in liver microsomes, the effect of RR was determined. RR strongly prevented the Ca^{2+} release stimulated by CCl_4 (Figure 5A). The spin-trapping agent, PBN, which reacts with a variety of radical species, including $^{\bullet}CCl_3$, was also very effective in preventing CCl_4-induced release of Ca^{2+} (Figure 5A). With respect to thiols, DHLA had some protective effect as it increased the lag period for

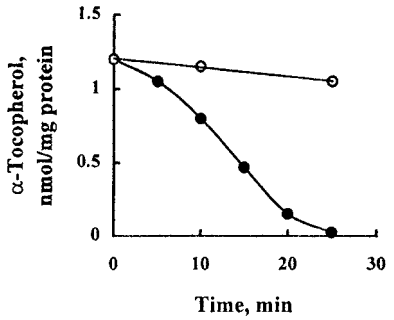

FIGURE 4: Decrease of liver microsomal α-tocopherol content by CCl_4. The CCl_4 (0.05 mM)-induced loss of α-tocopherol was monitored by HPLC as described in Materials and Methods in the absence (○) or presence (●) of 1 mM NADPH. The content of liver microsomes was 1.5 mg of protein/mL.

the increase in Ca^{2+} release produced by CCl_4, whereas GSH was not effective (Figure 5A). Lipophilic thiols such as mercaptoethanol or cysteamine, however, partially reversed the Ca^{2+} release induced by CCl_4, provided ATP was added to pump the Ca^{2+} back in; ATP alone did not reverse the Ca^{2+} release (Figure 5B).

Since the prevention of the CCl_4-induced Ca^{2+} release by RR is important in linking the actions of CCl_4 to activation of the RyR, it was necessary to validate that the actions of RR were on the RyR rather than inhibition of the CYP450 reductase/CYP450 system or/and to a direct interaction with radical metabolites of CCl_4. The first possibility was ruled out because RR did not inhibit the CYP2E1-dependent hydroxylation of PNP at concentrations (0.02−0.03 mM) which prevented the CCl_4-induced Ca^{2+} release (data not shown). In order to evaluate whether ruthenium red reacts with radical metabolites of CCl_4, ESR experiments using PBN as a spin-trap were conducted. PBN has been used successfully to detect $^{\bullet}CCl_3$ radicals in liver microsomes, in isolated hepatocytes, and by injecting it intraperitoneally into rats after they had been treated with CCl_4 (Albano et al., 1982). After incubation of microsomes (10 mg of protein/mL) with CCl_4 (about 10 mM) and NADPH (1 mM), an ESR spectra with a triplet of doublets was observed (Figure 6). Its hyperfine structure is consistent with the results obtained by Albano et al. (1982), and allows the assignment of the adduct as that formed by addition of $^{\bullet}CCl_3$ to PBN. No adducts were detected in the absence of CCl_4 (Figure 6). RR had no effect on the formation of PBN−CCl_4 adducts, suggesting that its inhibitory effect on the CCl_4-induced Ca^{2+} release is not due to alteration of the halocarbon metabolism and/or interception of $^{\bullet}CCl_3$ radicals. An additional experiment, suggesting that the inhibitory effect of RR is due to its interaction with a Ca^{2+} channel, is that it blocked further calcium efflux when added to a reaction system already in the midst of the CCl_4-stimulated Ca^{2+} release process (data not shown).

Effect of CCl_4 on Ca^{2+},Mg^{2+}-ATPase and Ryanodine Binding. CCl_4 was added to microsomes already loaded with Ca^{2+}; therefore, effects on the Ca^{2+}, Mg^{2+}-ATPase would not be responsible for the elevated levels of Ca^{2+} in the medium of the microsomal suspensions after addition of CCl_4. However, inhibition of the ATPase by CCl_4 would prevent reuptake of the Ca^{2+} released by CCl_4 and could magnify the Ca^{2+} release effect produced by CCl_4. The effect of CCl_4 on activity of the Ca^{2+},Mg^{2+}-ATPase was therefore evaluated. At a concentration of 0.05 mM, CCl_4

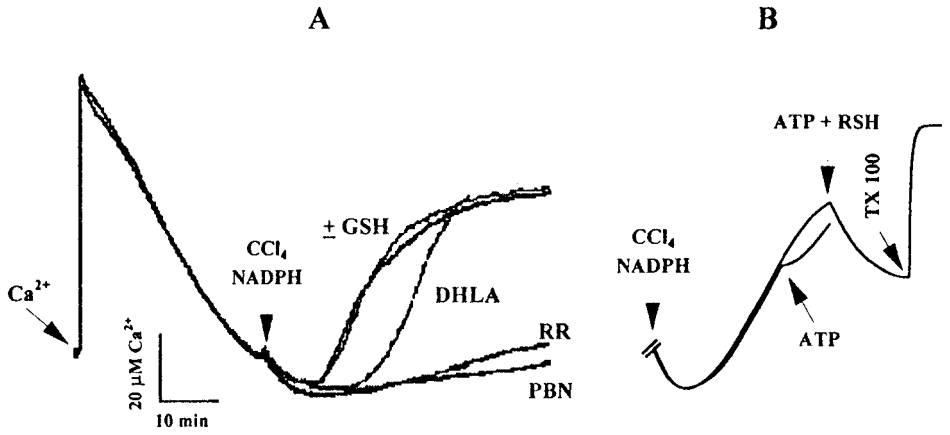

FIGURE 5: Effect of thiols, ruthenium red, and PBN on the NADPH plus CCl_4-induced release of Ca^{2+} from liver microsomes. Incubation conditions are the same as indicated in the legend to Figure 3. (A) Final concentration of additions was: ruthenium red, 0.02 mM; PBN, 25 mM; GSH, 4 mM; and DHLA, 4 mM. (B) Final concentration of additions was: ATP, 0.5 mM; and cysteamine, 5 mM.

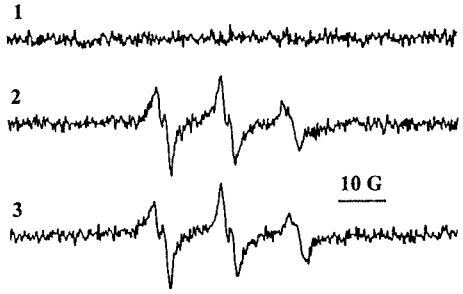

FIGURE 6: EPR spectra of PBN spin adducts formed by the metabolism of CCl_4. Liver microsomes (10 mg of protein/mL) were incubated for 15 min at 37 °C with 1 mM NADPH (spectrum 1), or NADPH plus 10 mM CCl_4 in the absence (spectrum 2) or presence (spectrum 3) of 0.03 mM ruthenium red.

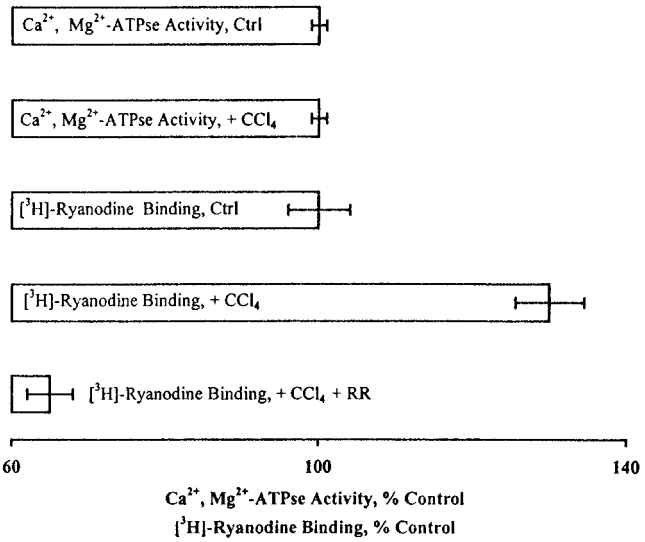

FIGURE 7: Effect of CCl_4 on Ca^{2+},Mg^{2+}-ATPase activity and [^{3}H]-ryanodine binding to liver microsomes. The assays for determination of [^{3}H]ryanodine binding and the activity of the Ca^{2+},Mg^{2+}-ATPase are described in the Materials and Methods section. The data shown are the average from three experiments ± standard error. The activity of the Ca^{2+},Mg^{2+}-ATPase in control microsomes was 9.7 ± 0.2 nmol of P/(min·mg of protein); the [^{3}H]ryanodine binding to control microsomes was 243 ± 38 fmol/mg of protein.

had no effect on enzyme activity (Figure 7), although this concentration of CCl_4 induced Ca^{2+} release (Figure 2).

Another method besides activation of RR-sensitive Ca^{2+} release to assess interaction of CCl_4 with the RyR is to determine the effect of CCl_4 on binding of [^{3}H]ryanodine to liver microsomes; indeed, CCl_4 plus NADPH incubation resulted in a 30% increase in [^{3}H]ryanodine binding (Figure 7). Neither CCl_4 alone or NADPH alone affected [^{3}H]-ryanodine binding (data not shown). To relate the [^{3}H]-ryanodine binding to the RyR, the effect of RR was determined. RR, at a concentration of 0.03 mM, inhibited [^{3}H]ryanodine binding in the presence of CCl_4 plus NADPH by 50% (Figure 7).

Relationship between CYP2E1-Dependent Metabolism of CCl_4 and Ca^{2+} Release from Liver Microsomes. CYP2E1 is known to be especially reactive in catalyzing the metabolism of CCl_4 (Johansson & Ingelman-Sundberg, 1985; Sohn et al., 1991; Tierney et al., 1992; Teschke et al., 1984). It would be anticipated that microsomes enriched in CYP2E1 would be more reactive than control microsomes in promoting the ability of CCl_4 to induce Ca^{2+} release. A comparison was made between the ability of microsomes from pyrazole-treated rats and saline controls to release Ca^{2+} after incubation with CCl_4 plus NADPH. Oxidation of PNP was elevated about 4- to 5-fold after pyrazole treatment, validating induction of CYP2E1 (Figure 8A). The lag period for CCl_4-induced release of Ca^{2+} was considerably shorter with microsomes from pyrazole-treated rats than saline controls (about 16 min versus 28 min) (Figure 8B). 4-Methylpyrazole is an effective inhibitor of CYP2E1-catalyzed oxidation of substrates, including CCl_4 (Dai & Cederbaum, 1995); the lag period for CCl_4-induced release of Ca^{2+} was increased in the presence of 2 mM 4-methylpyrazole, while 5 mM 4-methylpyrazole completely prevented Ca^{2+} release stimulated by CCl_4 (Figure 8C). In a similar manner, anti-CYP2E1 IgG increased the lag period required for CCl_4 to induce release of Ca^{2+}, whereas preimmune IgG had no effect (Figure 8D). Higher concentrations of antibody could not be tested because the preimmune IgG began to show significant inhibition.

DISCUSSION

The intracellular stores from which Ca^{2+} is released consist of three major components: a Ca^{2+},Mg^{2+}-ATPase that sequesters calcium; Ca^{2+}-binding proteins that store Ca^{2+} (calsequestrin and calreticulin); and the specific IP_3R and/or RyR that release Ca^{2+} back to the cytosol. Recently, it has been reported that liver contains IP_3-independent ryanodine-sensitive calcium stores. Feng and Kraus-Friedman (1992) have demonstrated the existence of high affinity

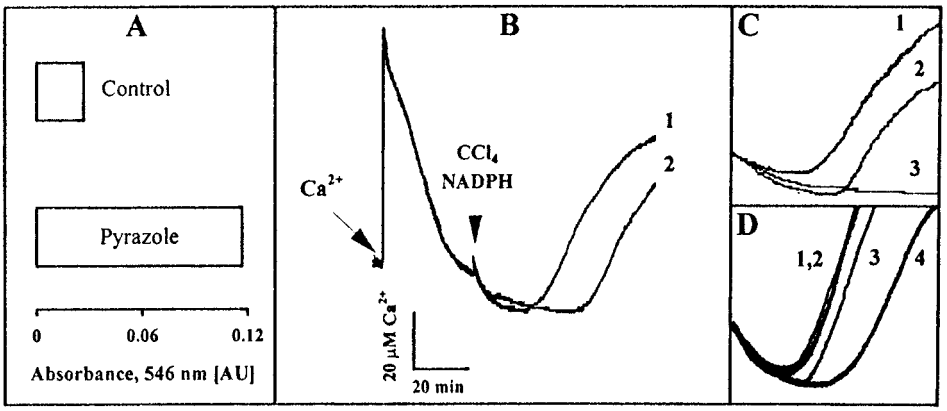

FIGURE 8: Effect of pyrazole induction of CYP2E1 on CCl_4-induced Ca^{2+} release from liver microsomes. Rats were treated with pyrazole to induce CYP2E1 as described in Materials and Methods. Basic incubation conditions are described in the legend to Figure 3 (A) NADPH-dependent hydroxylation of PNP by control microsomes or by microsomes isolated from pyrazole-treated rats. (B) CCl_4 (10 μM)-dependent release of Ca^{2+} by control microsomes (curve 2) and by microsomes isolated from pyrazole-treated rats (curve 1). (C) Effect of 4-methylpyrazole on CCl_4-dependent release of Ca^{2+} by microsomes from pyrazole-treated rats. Experiments were carried out in the presence of 0 (curve 1), 2 mM (curve 2), and 5 mM (curve 3) 4-methylpyrazole. (D) Effect of anti-CYP2E1 IgG on CCl_4-dependent release of Ca^{2+} by microsomes from pyrazole-treated rats. Experiments were carried out in the absence of IgG (controls, curve 1), or with 0.36 mg/mL preimmune IgG (curve 2), 0.18 mg/mL anti-CYP2E1 IgG (curve 3), and 0.36 mg/mL anti-CYP2E1 IgG (curve 4). IgG concentrations of 0.36 mg/mL would be equivalent to about 1 mg of IgG/nmol of total cytochrome P450 under experimental conditions.

ryanodine binding sites in liver microsomes and the presence in the microsomal membrane of a protein with molecular weight that is comparable to that of the skeletal muscle isoform of the RyR. Subsequently, Lilly and Gollan (1995) have shown that ryanodine induces Ca^{2+} release from liver microsomes that can be explained by the existence of an isoform of the RyR. The activity of both IP_3R and RyR can be influenced by their redox status (Galione et al., 1993). The carboxyl-terminal cytoplasmic tail of the IP_3R contains two cysteines which are present in a TXCFICG sequence motif that is highly conserved in all subtypes of IP_3Rs and RyRs (Berridge, 1993). Those cysteines are strong candidates to be the target for the redox regulation of both receptors. It has been documented that Ca^{2+} effluxes across the IP_3R and the RyR can be modulated as a result of thiol/disulfide conversion. Thimerosal, a sulfhydryl oxidizing reagent, has been shown to activate both the IP_3R and the RyR (Galione et al., 1993). The RyR of SSR and CSR can be also activated by 2,2′-DTDP, H_2O_2, Cu^{2+}, Ag^+, and Fe^{2+}/ascorbate (Zaidi et al., 1989; Favero et al., 1995; Salama & Abramson, 1984; Stoyanovsky et al., 1994).

In order to evaluate whether Ca^{2+} release in liver microsomes can be dependent on thiol oxidation, experiments with 2,2′-DTDP, a compound known to activate the skeletal and cardiac isoforms of the RyR without affecting the activity of the corresponding Ca^{2+},Mg^{2+}-ATPases (Zaidi et al., 1989), were carried out. A 2,2′-DTDP-induced Ca^{2+} release was found with liver microsomes; however, the effect on liver microsomal Ca^{2+} release slightly differed from the Ca^{2+} release induced under similar experimental conditions in SSR: in liver microsomes 0.10−0.15 mM 2,2′-DTDP was required for induction of Ca^{2+} release whereas 0.02−0.05 mM 2,2′-DTDP was sufficient in experiments with SSR. The 2,2′-DTDP-induced Ca^{2+} release from SSR was, in agreement with previous reports (Zaidi et al., 1989; Finkel et al., 1993), fully reversed by DTT, while in liver microsomes the release was only blocked by the disulfide reductant. These different effects may relate to accessibility of sensitive thiols of the RyR of SSR and of liver microsomes to 2,2′-DTDP and to DTT; e.g., the CCl_4-induced release of Ca^{2+} could only be reversed by lipophilic thiols. These results suggest that in liver microsomes there are Ca^{2+} stores that could release Ca^{2+} ions under conditions which result in thiol oxidation.

The metabolism and toxicity of CCl_4 have been extensively investigated. It is well documented that CCl_4 metabolism increases the cytosolic Ca^{2+} concentration and inhibits the hepatic Ca^{2+},Mg^{2+}-ATPase by oxidizing its -SH groups (Long & Moore, 1986; Srivastava et al., 1990; Yamamoto, 1990). Currently, however, there is no information on the action of CCl_4 metabolites on the two principal intracellular calcium channels responsible for mobilization of stored calcium, the IP_3R and the RyR. It is possible that the CCl_4-dependent augmentation of the cytosolic Ca^{2+} concentration may be due, in part, to a CCl_4-dependent oxidative activation of a Ca^{2+} releasing channel. Experiments to evaluate this possibility were the basis of the current study. Metabolism of CCl_4 by liver microsomes resulted in Ca^{2+} release after a lag period. The lag period was lower with microsomes from pyrazole-treated rats compared to saline controls; pyrazole treatment induces CYP2E1, and CCl_4 is an effective substrate for metabolism by CYP2E1. The concentrations of CCl_4 which induce Ca^{2+} release are considerably lower than the concentrations required to inhibit the Ca^{2+},Mg^{2+}-ATPase or to induce lipid peroxidation (Srivastava et al., 1990), suggesting that the Ca^{2+} releasing channels may be very sensitive to activation by CCl_4-derived metabolites. That CCl_4 metabolism is required for inducing Ca^{2+} release is suggested by the requirement for NADPH, by the shorter lag period with pyrazole microsomes, and by the inhibition of the releasing effects of CCl_4 by 4-methylpyrazole and by anti-CYP2E1 IgG.

Lipid peroxidation does not appear to be the mechanism by which CCl_4 metabolism induces microsomal Ca^{2+} release. At concentrations of 50 μM, CCl_4 did not produce an increase in TBARS. Ca^{2+} release induced by CCl_4 was enhanced under anaerobic conditions, suggesting a prominent role for $^{\bullet}CCl_3$, but not $^{\bullet}OOCCl_3$, in the CCl_4 mechanism. PBN, which was effective in demonstrating the production of $^{\bullet}CCl_3$, prevented the CCl_4-induced Ca^{2+} release most likely as a consequence of trapping $^{\bullet}CCl_3$. The lag period required for CCl_4-catalyzed Ca^{2+} release correlated with the time

required for the consumption of the endogenous α-tocopherol content of the liver microsomes. A possible explanation is that $^{\bullet}CCl_3$ preferentially reacts with α-tocopherol; once the α-tocopherol is depleted, $^{\bullet}CCl_3$ may now react with the Ca^{2+} releasing channels, activating Ca^{2+} release. In view of the activation of Ca^{2+} release by 2,2′-DTDP, it is possible that $^{\bullet}CCl_3$ reacts with and oxidizes channel thiol groups to activate Ca^{2+} release. The ability of lipophilic thiols such as cysteamine to reverse the CCl_4-induced release, whereas hydrophilic thiols such as GSH or DTT were ineffective, suggests that $^{\bullet}CCl_3$-sensitive epitopes/thiols in a lipophilic domain may play a role in regulating Ca^{2+} release. GSH has been shown to protect the Ca^{2+},Mg^{2+}-ATPase from inhibition by CCl_4 metabolites (Srivastava et al., 1990) at concentrations which did not prevent CCl_4-induced Ca^{2+} release.

Two experimental observations suggest that the RyR may play a role in the CCl_4-induced Ca^{2+} release. The Ca^{2+} release is blocked by ruthenium red, a specific inhibitor of the cardiac and skeletal isoforms of the RyR. Ruthenium red did not block formation of $^{\bullet}CCl_3$−PBN spin adducts, indicating that it did not inhibit CCl_4 metabolism to this reactive species. Metabolism of CCl_4 increased the binding of ryanodine to the liver microsomes by a reaction suppressed by ruthenium red. Ryanodine is a specific ligand for RyR. Further studies involving purification and characterization of the liver RyR are necessary to evaluate whether $^{\bullet}CCl_3$ specifically interacts with the RyR, especially with critical thiyl residues. Possible activation of IP_3 receptors also remains to be evaluated. It is interesting to speculate that activation of hepatotoxins to reactive species by cytochrome P450 may cause oxidative modification of Ca^{2+} releasing channels such as the RyR, which triggers release of Ca^{2+}, and that such activation may contribute to the increased cytosolic Ca^{2+} concentration found in the liver after hepatotoxin treatment.

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