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ACTIVATION OF PHOSPHOLIPASE A_2 BY CARBON TETRACHLORIDE IN ISOLATED RAT HEPATOCYTES*

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Abstract—Freshly isolated rat hepatocytes were exposed to carbon tetrachloride (CCl_4) for periods up to 4 hr. Phospholipase A_2 activity of these preparations was determined by measuring either the release of [^{3}H]arachidonic acid from cellular phospholipids prelabeled with [^{3}H]arachidonic acid or by measuring the formation of [^{14}C]lysophosphatidylethanolamine from cellular lipids prelabeled with [^{14}C]ethanolamine. Through the use of hexane-partition extraction and thin-layer chromatographic analysis of hepatocyte lipid extracts it was found that CCl_4 stimulated phospholipase A_2 activity in a dose- and time-dependent manner. Carbon tetrachloride at concentrations of 0.23 to 1.3 mM produced a 1.4- to 5.3-fold increase in phospholipase activity which was initiated within 30–60 min of incubation at 37°. The role of phospholipase activation as a secondary mechanism of CCl_4-induced hepatocyte injury is discussed.

Carbon tetrachloride (CCl_4) has been widely studied as a hepatotoxic agent. It is recognized that injury to the liver is a consequence of the metabolism of CCl_4 by the liver to highly reactive, free radical intermediates [1, 2]. Subsequent reaction of these radicals within the endoplasmic reticulum results in the initiation of peroxidative degradation of phospholipids and the binding of the electrophilic radicals to membrane lipids and proteins. A loss of several functions of the hepatic endoplasmic reticulum follows shortly thereafter. Protein synthesis, glucose-6-phosphatase, cytochrome P-450 and associated mixed-function oxidases are depressed significantly after exposure to CCl_4 *in vivo* or *in vitro* [1, 2].

Of particular interest has been the finding that calcium sequestration by the endoplasmic reticulum is especially sensitive to CCl_4-induced lipid peroxidation as well as to binding of metabolic products of CCl_4 to lipids and proteins of the endoplasmic reticulum [3, 4]. This has led to the postulation that CCl_4 causes a disruption in cellular calcium homeostasis, since the endoplasmic reticulum is considered to be involved in the regulation of intracellular calcium concentration [5]. Total liver calcium increases after administration of CCl_4 to rats [6–9]. At sublethal doses of CCl_4 the increase in cellular calcium is reversible. Farber and associates [10–12] have postulated that calcium is a common, final link in hepatic injuries induced by several agents which damage the plasma membrane and result in an increase in cellular calcium levels. High calcium, in turn, could lead to activation of membrane phospholipases and ultimately loss of membrane integrity. Phospholipases are widely distributed in membranes [13] and generally involve calcium as an activator of cofactor [13, 14]. Activation of phospholipase-catalyzed breakdown of membrane lipids has been postulated as a causal factor in cell damage in ischemia [11, 12, 15–19], endotoxic shock [20], eosinophil degranulation [21], brain edema [22], and in fibroblast injury associated with exposure to A23187 [23], mercuric ions [24], and polypeptide toxins [25].

During the course of investigation of calcium levels in hepatocytes poisoned with CCl_4, we initiated studies to measure phospholipase activity in these preparations. Results reported herein indicate that exposure of freshly isolated liver cells to CCl_4 caused a dose- and time-dependent increase in phospholipase A_2 activity.

MATERIALS AND METHODS

Animals and materials. Male, Sprague–Dawley strain rats (250–350 g) were obtained from Zivic–Miller Laboratories, Inc., Allison Park, PA. [5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (84–87 Ci/mmole) was obtained from the New England Nuclear Corp., Boston, MA; [1,2-^{14}C]ethanolamine (95 mCi/mmole) was from ICN Pharmaceuticals, Inc., Irvine, CA. Carbon tetrachloride was from the Fisher Scientific Co., Pittsburgh, PA; Waymouth's MB 752/1 medium and heat-inactivated horse serum were from Gibco Laboratories, Grand Island, NY; *N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid (HEPES) was from Research Organics, Inc., Cleveland, OH; collagenase (type I), phospholipase A_2 (*Crotalus adamanteus* venom), and phospholipid and neutral lipid standards were from the Sigma Chemical Co., St. Louis, MO; silica gel G thin-layer chromatographic plates, type K6, were from

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Whatman Chemical Separation Inc., Clifton, NJ; and Scintisol was from Isolab, Inc., Akron, OH.

Hepatocyte preparation. Hepatocytes were isolated from rats by the collagen perfusion method of Seglen [26] with modification as described by Pencil *et al.* [27]. Basically, a Krebs–Ringer solution containing 0.5 mM ethylene glycol bis(beta-aminoethylether)-*N,N'*-tetraacetic acid (EGTA), 4.0 mM $CaCl_2$, and 0.05% collagenase buffered at pH 7.4 with bicarbonate, was perfused for 20 min through the portal vein of livers removed from anesthetized, heparinized rats. The liver tissue was minced and the cells were filtered through nylon mesh and centrifuged at 50 *g* for 2 min. The cell pellet was washed with several milliliters of Waymouth's MB 752/1 medium buffered at pH 7.4 with 25 mM HEPES. The cells were repelleted by centrifugation, resuspended to a concentration of 10% cells (w/v) (approximately 1×10^7 cells/ml) in Waymouth's medium containing 20% (v/v) heat-inactivated horse serum, and "stabilized" by incubation under 95% O_2:5% CO_2 atmosphere for 20 min at 37°. Cell viability of these preparations, assessed by trypan blue exclusion, was routinely greater than 85%.

Incorporation of [^{3}H]*arachidonic acid or* [^{4}C]*-ethanolamine into hepatocyte lipids.* Stabilized cells were repelleted and resuspended to 10% (w/v) in Waymouth's medium. Typically, 5 μCi of [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid or 5 μCi of [1,2-^{14}C]ethanolamine was added to 25 ml of 10% cells in a 250-ml polypropylene flask and incubated for periods up to 4 hr at 37°. For purposes of phospholipase assay, cells were incubated for 1 hr with the ^{3}H- or ^{14}C-precursors and subsequently exposed to CCl_4. Aliquots of cell suspensions were removed, pelleted, and washed with Waymouth's medium, and the lipids were extracted with chloroform–methanol (2:1) according to Folch *et al.* [28]. The chloroform phase was recovered, concentrated by evaporation with oxygen-free nitrogen, and subjected to thin-layer chromatography (see below).

Hepatocyte incubation and poisoning. Waymouth's medium (2.5 ml) was added to 50-ml polypropylene flasks containing a center tube (6 × 50 mm). Carbon tetrachloride (1.0 to 30 μl) was added to the center tube, and the flasks were stoppered and incubated for at least 15 min to establish equilibrium of CCl_4 between gas and liquid phases. Cells that had been incubated with [^{3}H]arachidonic acid or [^{14}C]ethanolamine for 1 hr were washed, resuspended in Waymouth's medium, and added to the incubation flasks to a final cell concentration of 10% (w/v). Cell suspendions were incubated for periods of up to 4 hr at 37°. Aliquots of cell suspensions were removed for phospholipase assay.

Phospholipase A_2 assay. Phospolipase A_2 activity was determined by measuring either the release of [^{3}H]arachidonic acid from cellular phospholipids prelabeled with [^{3}H]arachidonate, or by measuring the formation of [^{14}C]lysophospholipids from cellular phospholipids prelabeled with [^{14}C]ethanolamine.

When hepatocytes prelabeled with [^{3}H]arachidonate are used, it is crucial to add nonradioactive arachidonate at the start of incubations designed to demonstrate phospholipase A_2 activation (see Fig. 3 and relevant comments in Results). After incubation, aliquots of cell pellets were extracted for their total lipids with chloroform–methanol, 2:1 [28]. The chloroform phase was evaporated to dryness, the lipid residue was dissolved in hexane, and the lipids were fractionated according to Borgström [29]. This hexane-partition lipid separation technique is outlined in Fig. 1. Aliquots of hexane phase I (phospholipids, neutral glycerides, cholesterol, cholesterol esters) or hexane phase II (fatty acids) were assayed for radioactivity by liquid scintillation counting in Scintisol and subjected to thin-layer chromatography (see below). Appearance of ^{3}H-radioactivity in the hexane phase II fraction results from endogenous phospholipase A_2 action which releases [^{3}H]arachidonic acid from the 2-carbon position of the glycerol moiety of phospholipids. In a separate experiment, assay of products after treatment of ^{3}H-labeled lipids with exogenous phospholipase A_2 showed nearly complete loss of radioactivity from the individual phospholipids and the appearance of radioactivity in an area on thin-layer plates corresponding to the fatty acid fraction (see Table 1). Also, chromatographic separation of the hexane phase II fraction in a solvent system designed to separate neutral lipids revealed that, after exposure of CCl_4, over 90% of the radioactivity co-migrated with free fatty acid standards (see Fig. 6).

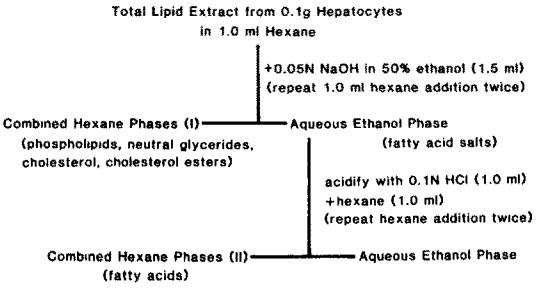

Fig. 1. Isolation of free fatty acids from total lipid extracts of hepatocytes by hexane-partition separation. Adaptation of method of Borgström [29].

In other experiments where hepatocyte lipids were prelabeled with [^{14}C]ethanolamine, cell plates obtained from aliquots of incubated cell suspensions were extracted with chloroform–methanol (2:1) [28]. The chloroform phase was concentrated and lipids were fractionated by thin-layer chromatography (see below). Radioactivity appearing in lysophospholipid fractions, largely lysophosphatidylethanolamine, is considered to originate from phospholipase A_2 action. The addition of exogenous phospholipase A_2 to [^{14}C]ethanolamine-labeled lipids resulted in a complete conversion of phosphatidylethanolamine and phosphatidylcholine to corresponding lysophospholipid derivatives (Table 1).

Thin-layer chromatography. Lipid separation was carried out on thin-layer plates of silica gel (type K6, 250 μm thickness, Whatman Inc.). Silica gel plates were developed in chloroform/methanol/water (25:20:1) for phospholipid fractionation and in petroleum ether (30–60°)/diethyl ether/acetic acid (80:20:1) for neutral lipid separation [30]. Phospholipid or neutral lipid standards were chromato-

Table 1. Exogenous phospholipase A_2 hydrolysis of [^{3}H]arachidonic acid- or [^{14}C]ethanolamine-labeled hepatocyte lipids

Thin-layer chromatography fraction	Distribution of radioactivity (% of total)			
	[^{3}H]Arachidonic acid-labeled lipid		[^{14}C]Ethanolamine-labeled lipid	
	Control	Plus phospholipase A_2	Control	Plus phospholipase A_2
Lysophosphatidylcholine	1.6	0.8	1.8	13.5
Phosphatidylcholine	46.4	0.6	10.8	0.5
Lysophosphatidylethanolamine	2.7	0.4	3.5	76.8
Phosphatidylinositol	14.5	0.3	1.9	5.4
Phosphatidylethanolamine	16.1	5.0	79.2	2.3
Free fatty acid, neutral lipid	17.5	92.2	2.4	1.2

^{3}H-Labeled or ^{14}C-labeled lipids from hepatocytes incubated with [^{3}H]arachidonic acid or [^{14}C]-ethanolamine (see Materials and Methods) were treated with phospholipase A_2 as follows. Approximately 2 mg [^{3}H]lipids (6.68×10^4 cpm) or 10 mg [^{14}C]lipids (6.42×10^4 cpm) was dissolved in 2.0 ml diethyl ether. Phospholipase A_2 (*Crotalus adamanteus* venom, 10 units to [^{3}H]lipids or 20 units to [^{14}C]-lipids in 0.1 M Tris buffer, pH 8.4) and 4.0 mM $CaCl_2$ were added for a final aqueous volume of 0.25 ml ([^{3}H]lipids) or 0.5 ml ([^{14}C]lipids). A control lipid sample was treated similarly, but buffer was added without phospholipase. The mixtures were incubated (30° for 45 min, [^{3}H]lipids; 25° for 3 hr, [^{14}C]lipids) after which the ether was evaporated and the residue extracted with 5.0 ml of chloroform–methanol (2:1). After the addition of 1.0 ml water the chloroform phase was recovered, evaporated, and reconstituted to 0.05 ml with chloroform. Ten-microliter aliquots were chromatographed on thin-layer plates as described in Materials and Methods. Areas of silicic acid containing lipid corresponding to standard phospholipid migration were removed and assayed directly for radioactivity by liquid scintillation counting.

graphed in lanes adjacent to lipids from cell extracts and visualized by ultraviolet fluorescence with rhodamine 6G dye. Areas of silicic acid containing lipid corresponding to migration of known standards were scraped into vials and assayed for radioactivity in Scintisol by liquid scintillation counting. The R_f values of lipid standards in the phospholipid fractionation system are: lysophosphatidylcholine, 0.04; phosphatidylcholine, 0.12; lysophosphatidylethanolamine, 0.26; and phosphatidylethanolamine, 0.61.

Possible interference by peroxidized lipids. We considered the possibility that CCl_4-dependent peroxidation of cell phospholipids might yield products that could possibly co-migrate with lysophosphatidyl derivatives appearing as a result of the action of phospholipase A_2. To test this possibility, we subjected a [^{14}C]ethanolamine-labeled lipid extract from hepatocytes to forced peroxidation by exposure to ultraviolet light. Spectrophotometric analysis of these treated lipids revealed an intense, maximal absorbance difference at 230–240 nm compared to untreated lipid, indicating a high degree of conjugated double-bond structure characteristic of peroxidized lipid. The lipid extract contained 88% of the ^{14}C-label in phosphatidylethanolamine and 8% in phosphatidylcholine before peroxidation. Peroxidation did not alter the chromatographic distribution of ^{14}C-radioactivity; no radioactivity appeared in that region of the thin-layer plate corresponding to lysophosphatides. Hence, peroxidation does not interfere in the chromatographic assay for lysophospholipids.

We were also concerned that potential ^{3}H-labeled breakdown products of peroxidized ^{3}H-labeled lipids would be extracted into the free fatty acid fraction (hexane phase II) of the hexane partition method. To test this possibility, hepatocytes were incubated with [^{3}H]arachidonic acid to label the phospholipids as described above. The labeled hepatocytes were peroxidized by incubation in the presence of added ferrous ions. Confirmation of peroxidation was determined by measurement of malondialdehyde. Lipids were extracted from peroxidized hepatocytes and from non-peroxidized control cells and subjected to hexane-partition separation as outlined in Fig. 1. ^{3}H-Radioactivity in the free fatty acid fraction (hexane phase II) from highly peroxidized hepatocytes was not increased over the corresponding fraction from control cells. Any ^{3}H-labeled breakdown products of lipid peroxidation that were extracted into the total lipid fraction remained in the phospholipid/neutral lipid fraction (hexane phase I) during the hexane partitioning. Therefore, we consider the free fatty acid fraction to be free of contaminating radioactivity arising from peroxidation which would interfere in this assay for phospholipase activity.

Determination of CCl_4 concentration in incubation media. The concentration of CCl_4 in the incubation mixtures is given as values previously obtained under identical experimental conditions in this laboratory [31].

RESULTS

In this study phospholipase A_2 activity was assayed by measuring the appearance in incubated hepatocytes of free [^{3}H]arachidonic acid or [^{14}C]lyso-

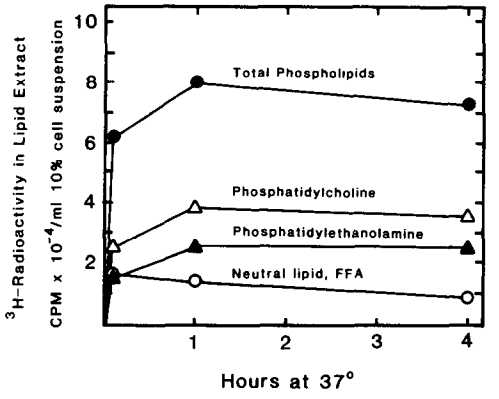

Fig. 2. Incorporation of [^{3}H]arachidonic acid into lipids of isolated hepatocytes. [^{3}H]Arachidonic acid (0.2 μCi/ml 10% cells) was added to hepatocytes and incubated at 37° in Waymouth's 752/1 medium for periods up to 4 hr. Lipids were extracted from aliquots of cell suspensions and fractionated by thin-layer chromatography. Additional details are given in Materials and Methods.

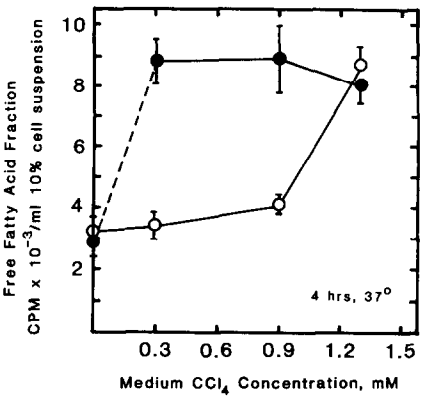

Fig. 3. Stimulation of phospholipase A_2 activity by CCl_4 (0.3 to 1.3 mM) in isolated hepatocyte preparations prelabeled with [^{3}H]arachidonic acid. Free fatty acid radioactivity (hexane phase II fraction, see Fig. 1) was determined after 4 hr of incubation of 10% cell suspensions at 37°. Key: (○) cells without added nonradioactive arachidonic acid, and (●) cells with nonradioactive arachidonic acid added to a 2.2 mM final concentration at start of incubation. Total lipid radioactivity in control cells (no CCl_4) without added nonradioactive arachidonic acid was $4.97 \pm 0.38 \times 10^4$ cpm/ml 10% cells. Total lipid radioactivity in control cells (no CCl_4) with nonradioactive arachidonic acid added was $5.84 \pm 0.49 \times 10^4$ cpm/ml 10% cells. Values are given as mean ± S.E.M. for five separate experiments. Additional details are given in Materials and Methods.

phospholipid from lipids prelabeled with [^{3}H]-arachidonic acid or [^{14}C]ethanolamine. The incorporation of [^{3}H]arachidonic acid into lipids is given in Fig. 2. Significant uptake of arachidonic acid occurred within minutes and remained associated with phospholipids for the 4-hr incubation at 37°. Radioactivity was incorporated mainly into phosphatidylcholine (40–50%) and phosphatidylethanolamine (25–35%). Treatment of [^{3}H]arachidonic acid-labeled lipids with exogenous phospholipase A_2 indicated that ^{3}H-incorporation was principally into the 2-position of the glycerol moiety since chromatographic analysis before and after hydrolysis showed a nearly complete loss of ^{3}H-radioactivity from phospholipids and an appearance of label in the free fatty acid fraction (see Table 1). Subsequent thin-layer chromatographic analysis of the control neutral lipid/free fatty acid portion prior to phospholipase A_2 treatment has shown that radioactivity in this fraction is associated mostly with triglyceride and is less than 5% as free fatty acid.

Exposure of isolated hepatocytes, prelabeled with [^{3}H]arachidonic acid, to CCl_4 resulted in a 3-fold stimulation of phospholipase A_2 activity (Fig. 3) at concentrations of CCl_4 in the medium as low as 0.3 mM. This amount of radioactivity released from phospholipids and recovered as free fatty acids from control and experimental cells represents about 5 and 15%, respectively, of the total counts present in hepatocyte lipids. However, in the absence of added nonradioactive arachidonic acid, stimulation of phospholipase activity was evident only at the highest concentration of CCl_4 tested. Arachidonic acid is rapidly incorporated into hepatocyte lipids (see Fig. 2). The addition of nonradioactive arachidonic acid services to increase the intracellular pool of this fatty acid such that any radioactivity, present as [^{3}H]arachidonic acid in the intracellular pool of free fatty acids, would be diluted out. Without this maneuver, [^{3}H]arachidonic acid, liberated from phospholipids by phospholipase A_2, would immediately be reincorporated into newly synthesized lipids.

When the intracellular pool of arachidonate is expanded by addition of nonlabeled arachidonate, the [^{3}H]arachidonate appearing as a result of phospholipase A_2 activity appears in the free fatty acid fraction after lipid extraction and hexane partitioning.

In another set of experiments, phospholipase A_2 activation was studied using a lower range of CCl_4 levels, 0.07 to 0.33 mM (Fig. 4). Carbon tetrachloride at 0.23 and 0.33 mM produced a 1.4- to

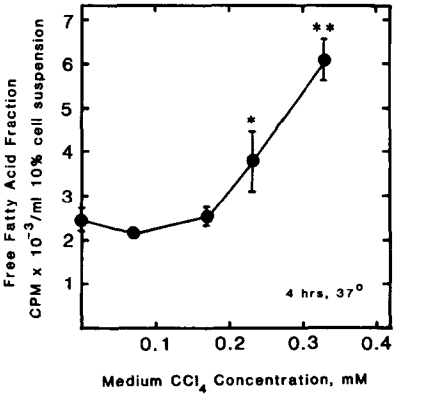

Fig. 4. Stimulation of phospholipase A_2 activity by CCl_4 (0 to 0.3 mM) in isolated hepatocyte preparations prelabeled with [^{3}H]arachidonic acid. Conditions were similar to those of Fig. 3. Total lipid radioactivity of control cells (no CCl_4) was $4.43 \pm 0.31 \times 10^4$ cpm/ml 10% cells. Values are given as mean ± S.E.M. for four separate experiments. Statistical significance between control and experimental values was: *$P < 0.10$, **$P < 0.001$.

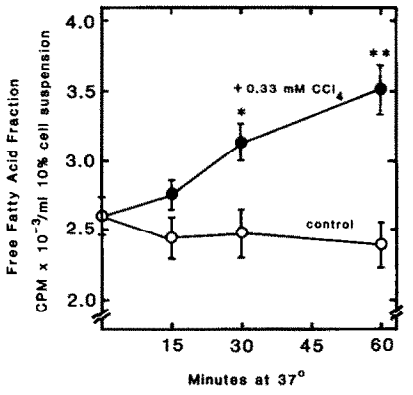

Fig. 5. Time course of phospholipase A_2 stimulation by CCl_4 in isolated hepatocytes prelabeled with [^{3}H]arachidonic acid. Conditions were similar to those of Fig. 3. Key: (○) control; and (●) plus 0.33 mM CCl_4. Total lipid radioactivity at the start of the incubation was $4.49 \pm 0.26 \times 10^4$ cpm/ml 10% cells. Values are given as mean ± S.E.M. for four (control) or five (experimental) separate experiments. Statistical significance between control and experimental values was: $^*P < 0.025$, and $^{**}P < 0.005$.

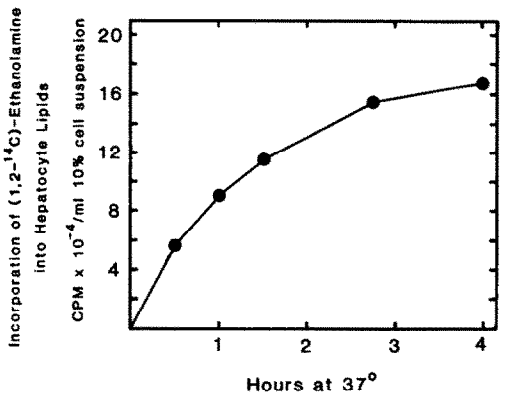

Fig. 7. Incorporation of [1,2-^{14}C]ethanolamine into lipids of isolated hepatocytes. [^{14}C]Ethanolamine, 0.2 μCi/ml 10% cells, was added to hepatocytes and incubated at 37° in Waymouth's MB 752/1 medium. At several intervals, lipids were extracted from aliquots of cell suspensions. Thin-layer chromatography of these lipid extracts (see Materials and Methods) revealed that phosphatidylethanolamine and phosphatidylcholine contained approximately 80 and 15% of the radioactivity respectively.

2.3-fold increase in hepatocyte phospholipase A_2 activity, respectively. Lesser concentrations of CCl_4 were without effect over the 4-hr, 37° incubation.

The time course of phospholipase A_2 stimulation by 0.33 mM CCl_4 is given in Fig. 5. A small rise in activity was seen as early as 15 min with an increase at 60 min of 1.4-fold times that of the zero time control.

Additional thin-layer chromatography analysis of the hexane phase II fraction (free fatty acids) extracted from hepatocytes exposed to CCl_4 is given in Fig. 6. Over 90% of the ^{3}H-radioactivity in this fraction co-migrated with fatty acid standards. This confirms the validity of using the hexane partition method [29] for isolating free fatty acids in the determination of phospholipase A_2 activity of hepatocytes prelabeled with [^{3}H]arachidonic acid.

An alternative method for measuring phospholipase A_2 activity was to isolate the lysophospholipid products of this hydrolysis after exposure to CCl_4. For this purpose hepatocellular lipids were labeled with [^{4}C]ethanolamine. The incorporation of [^{14}C]ethanolamine into lipids is given in Fig. 7. Although abundant uptake was evident, the rate of incorporation was not as rapid as when [^{3}H]arachidonic acid was used (see Fig. 2). Subsequent thin-layer chromatographic separation of these lipids revealed that phosphatidylethanolamine and phos-

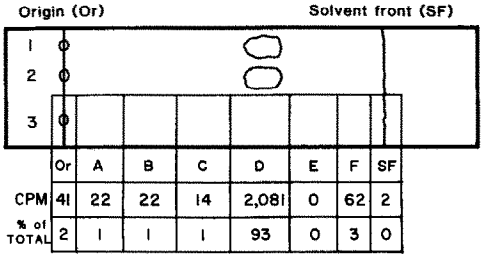

Fig. 6. Thin-layer chromatographic analysis of free fatty acid fraction (hexane phase II fraction, see Fig. 1) of [^{3}H]arachidonic acid-labeled lipids extracted from isolated hepatocytes exposed to 0.33 mM CCl_4 for 4 hr at 37°. Incubation conditions were as given in Figs 3 and 4. Thin-layer silica gel G plates (Whatman K6) were developed in a solvent system designed to separate neutral lipids (see Materials and Methods). Approximately 10 μg of palmitic acid or arachidonic acid was placed on the origin of lanes 1 and 2 respectively. An aliquot of the free fatty acid extract of cells exposed to CCl_4 was placed on lane 3. After development, lanes 1 and 2 were sprayed with rhodamine 6G dye, and lipid fluorescence was visualized under ultraviolet light. Areas of silica gel in lane 3, as indicated, were scraped into vials, and radioactivity was determined by liquid scintillation counting.

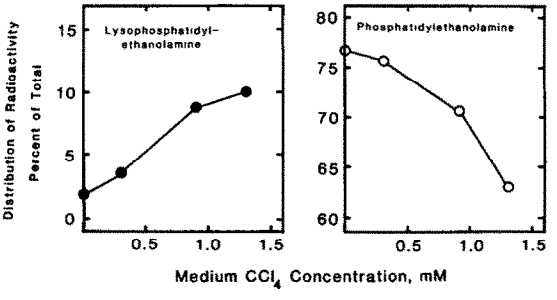

Fig. 8. Stimulation of phospholipase A_2 activity by CCl_4 in isolated hepatocyte preparations prelabeled with [^{14}C]-ethanolamine. Radioactivity in lysophosphatidylethanolamine (●) and phosphatidylethanolamine (○) was determined after 4 hr of hepatocyte incubation at 37° in the presence of 0 to 1.3 mM CCl_4. Lipid extracts of hepatocytes were fractionated by thin-layer chromatography, and radioactivity was measured by liquid scintillation counting. Total radioactivity in lipids of control (no CCl_4) hepatocytes before fractionation was 1.39×10^5 cpm/ml 10% cells. Additional details are given in Materials and Methods.

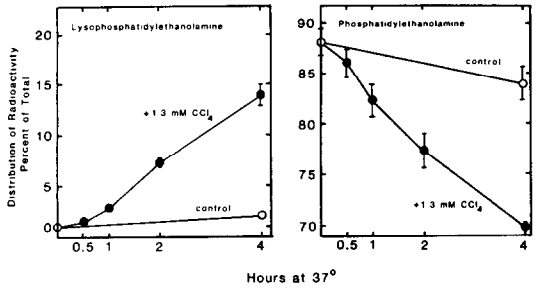

Fig. 9. Time course of phospholipase A_2 stimulation by CCl_4 in isolated hepatocytes prelabeled with [^{14}C]ethanolamine. Conditions were similar to those of Fig. 8. Key: (○) control; and (●) plus 1.3 mM CCl_4. Lipid radioactivity at the start of incubation averaged 1.22×10^5 cpm/ml 10% cells. Values given are the mean ± range for two separate experiments.

phatidylcholine contained 75–80% and 8–15% of the radioactivity respectively. The addition of exogenous phospholipase A_2 to lipids labeled with [^{14}C]ethanolamine generated products that were identified as lysophosphatidylethanolamine and lysophosphatidylcholine (see Table 1). Isolation of these lyso-derivatives after exposure of hepatocytes to CCl_4 was considered to be evidence of phospholipase A_2 action.

Exposure of liver cells, prelabeled with [^{14}C]-ethanolamine, to CCl_4 also resulted in a stimulation of phospholipase A_2 activity (Fig. 8). Carbon tetrachloride at concentrations of 0.3, 0.9 and 1.3 mM caused 1.9-, 4.6-, and 5.3-fold increases in lysophosphatidylethanolamine, respectively, after 4 hr of incubation at 37°, compared to control hepatocytes incubated in the absence of CCl_4. Also, levels of phosphatidylethanolamine decreased in a corresponding manner.

The time course of CCl_4-induced phospholipase A_2 activity is illustrated in Fig. 9. A modest increase in [^{14}C]lysophosphatidylethanolamine was observed at 60 min of incubation with a 6.6-fold rise over control at 240 min. A corresponding loss of radioactivity in phosphatidylethanolamine was seen over the same period in hepatocytes exposed to CCl_4.

DISCUSSION

Carbon tetrachloride produces a wide array of dysfunction and injury in the liver both *in vivo* [1, 2] and in isolated hepatocytes [32, 33]. The initial event in the toxigenic sequence is metabolism of CCl_4 by the hepatic cytochrome P-450, mixed-function oxidase system to reactive radicals ($\cdot CCl_3$ and $\cdot OOCCl_3$). These radicals initiate peroxidation of lipids and bind to lipids and proteins of the endoplasmic reticulum. Losses in glucose-6-phosphatase activity, cytochrome P-450 concentration and associated activities, protein synthesis, and calcium sequestration have been observed after exposure to CCl_4. Carbon tetrachloride metabolism and the immediate consequences appear to be confined within an envelope surrounding the cytochrome P-450 complex of the endoplasmic reticulum. However, CCl_4-induced injury eventually encompasses the entire hepatocyte. It appears necessary to postulate that the initial, localized lipid peroxidation and binding of CCl_4 metabolic products evoke secondary mechanisms which ultimately account for the wide-spread pathological consequences of the action of CCl_4. The significance of this paper is that it points to activation of phospholipase A_2 as a probably crucial secondary mechanism.

Results reported in Fig. 3–5, 8, and 9 show clearly that in isolated hepatocytes CCl_4 induced phospholipase A_2 activity in a dose- and time-dependent manner. It is of interest that CCl_4 at concentrations as low as 0.23 to 0.33 mM is able to stimulate phospholipase activity. These concentrations correspond to levels of CCl_4 obtained in portal blood of rats given moderate intragastric doses of CCl_4 (0.6 to 2.5 ml CCl_4/kg) (E. Glende, unpublished). As discussed in a previous report [27], we believe that the range of CCl_4 concentrations used in these experiments is toxicologically meaningful. Carbon tetrachloride at less than 1.3 mM is most likely initiating pathological responses as a result of its metabolism rather than by means of direct solvent action.

Carbon tetrachloride-induced phospholipase activity was significant within 30 min of exposure when [^{3}H]arachidonic acid-labeled hepatocytes were used (Fig. 5). With [^{14}C]ethanolamine-labeled liver cells, the appearance of [^{14}C]lysophosphatidylethanolamine was evident 60 min after exposure (Fig. 9). If phospholipase-induced degradation of membrane lipid is to be considered as a secondary mechanism of toxicity, evidence of phospholipase activity must precede or be concurrent with the appearance of cell injury. Based on CCl_4-induced hepatocyte injury, measured as glutamic-oxaloacetic transaminase released during 0.5 to 5 hr of incubation under similar conditions in this laboratory [31], the appearance of CCl_4-stimulated phospholipase activity is not inconsistent with this requisite. Routine determination of hepatocyte viability was not carried out for the experiments reported in this study. Previous work [31] and other unpublished studies from this laboratory indicate that, at concentrations of CCl_4 at 1.3 mM or above, a 70% or greater loss of cell viability is observed (trypan blue exclusion or GOT release) after 3 hr at 37°. At 0.3 to 0.9 mM, CCl_4 induces a 10–20% loss of viability after 3 hr at 37°. However, a much closer examination of the temporal relationship between CCl_4 cell injury and phospholipase activation must be carried out.

Stimulation of phospholipase A_2 by CCl_4 appears to be similar in magnitude to phospholipid deacylation induced in cultured fibroblasts by calcium and ionophore A23187 [23]. In the study by Shier and DuBourdieu [23], 15% hydrolysis of phospholipids, brought about by millimolar extracellular concentrations of calcium in the presence of 5 μM A23187, was associated with death of all cells. In our work, CCl_4 induced a release of [^{3}H]arachidonic acid in the range of 3–10% of the total ^{3}H-radioactivity incorporated into lipids (Figs. 3–5) or the hydrolysis of as much as 8–13% of the [^{14}C]ethanolamine-labeled lipids to [^{14}C]lysophosphatidylethanolamine. Thus, it seems not unlikely that the degeneration of membranes of subcellular organelles and of the cell

membrane resulting from CCl_4 poisoning may be due, in part, to phospholipase A_2 activation. In the case of the endoplasmic reticulum, CCl_4-induced losses of glucose-6-phosphatase and mixed-function oxidase activities are generally attributed to lipoperoxidative membrane damage or to binding of CCl_4 metabolic products to lipids and proteins of the endoplasmic reticulum. However, phospholipase-catalyzed lipid degradation may also contribute to the pathophysiological action of CCl_4. Alteration of hepatic microsomal lipid by the addition of phospholipase A_2 has been shown to significantly depress residual glucose-6-phosphatase activity [16, 34] and calcium sequestration activity [16]. Activation of endogenous phospholipase activity by incubation of liver microsomes with 5 mM $CaCl_2$ leads to a reduction in glucose-6-phosphatase and cytochrome P-450 content [14]. Thus, it appears that activation of phospholipase A_2 may be an important link in the chain of causality leading from the initial events of CCl_4 metabolism to ultimate death of the cell. The biochemical details of phospholipase A_2 activation are unknown. A CCl_4-dependent disturbance of hepatocellular calcium homeostasis [3, 4], coupled with the lipid peroxidation [35–37], may be involved.

In a series of reports, Lamb and co-workers [38–40] have presented evidence that CCl_4 promotes phospholipase C activity *in vitro* and *in vivo*. Homogenates of hepatocytes exposed to CCl_4, and in particular the plasma membrane fraction (1000 *g* cell fraction) derived therefrom, exhibited enhanced phospholipase activity upon subsequent incubation [38, 39]. Further analysis indicated that phospholipase C activation by CCl_4 was independent of CCl_4 metabolism and resulted from a direct, physical action of CCl_4 [39]. In the present study, we were not able to detect CCl_4-induced phospholipase C activation in isolated hepatocyte preparations. Exposure of ([^{14}C]ethanolamine)-phosphatidylethanolamine-labeled cells to CCl_4 did not yield the radioactive products expected from phospholipase C action such as [^{14}C]phosphorylethanolamine which would appear in the water–methanol phase of the Folch-style lipid extraction method employed (unpublished data).

We propose that activation of phospholipase A_2 may be an important secondary mechanism of CCl_4 hepatotoxicity leading to extended membrane damage, dysfunction, and cell death. Further work is needed to establish the subcellular location of the activated phospholipase A_2, and to work out the mechanisms linking CCl_4 metabolism to phospholipase A_2 activation.

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