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EFFECTS OF CARBON TETRACHLORIDE ON CALCIUM HOMEOSTASIS

A CRITICAL RECONSIDERATION

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Abstract—The incubation of isolated rat hepatocytes with 0.172 mM carbon tetrachloride caused a rapid decrease in the calcium content of both mitochondrial and extramitochondrial compartments. However, the release of Ca^{2+} from the intracellular stores was not associated with an increase in the cytosolic Ca^{2+} levels as measured by activation of phosphorylase *a* or by Quin-2 fluorescence. A rapid rise in hepatocyte free calcium was only observed with concentrations of CCl_4 higher than 0.172 mM.

The lack of activation of phosphorylase *a* was not due to the inhibition of the enzyme by CCl_4, since in CCl_4-treated hepatocytes the phosphorylase activity could be stimulated by glucagon, butyryl–cAMP or by the increase of cell calcium induced by the addition of A23187.

Ca^{2+}-dependent ATPase of plasma membranes was only slightly affected in the early phases of poisoning with CCl_4 when both mitochondrial and extramitochondrial calcium pools were already lowered. This led to the conclusion that calcium released from intracellular organelles could be extruded from the cells in sufficient amounts to prevent the increase of the cytosolic levels.

A rise in hepatocyte free calcium was observed during the second hour of incubation with CCl_4, concomitantly with the appearance of both LDH leakage and plasma membrane blebbing. The addition of EGTA to the medium prevented both the increase in cytosolic Ca^{2+} and the blebbing suggesting that they were a consequence of an influx of calcium into the cells. However, neither EGTA nor the addition of inhibitors of calcium-dependent phospholipase A_2 or non-lysosomal proteases were able to protect against cell death. These latter results suggested that the alterations of calcium distribution induced by CCl_4 in isolated hepatocytes were not a primary cause of the toxic effects, although they did not exclude that a sustained rise in cytosolic Ca^{2+} could contribute in the progression of cell injury.

One of the earliest consequences of CCl_4 intoxication is a dramatic inhibition of the calcium sequestering capacity by the endoplasmic reticulum vesicles. This effect is already evident after 5–10 min from the *in vivo* administration of CCl_4 or from its addition *in vitro* to suspensions of liver microsomes or isolated hepatocytes [1, 2].

In the latter, the incubation with CCl_4 also affected the Ca^{2+} uptake by mitochondria [3] probably as a consequence of a direct attack of free radical intermediates on the organelles.

Speculations about the consequences of such early damages of calcium homeostasis suggested that the inhibition of microsomal calcium uptake would result in an elevation of the cytosolic Ca^{2+} levels which would then lead to the onset of the cell injuries caused by carbon tetrachloride [4].

Although partially contradicted by subsequent studies [5, 6], this hypothesis was reinvestigated by Long and Moore who reported that cultured hepatocytes exposed to CCl_4 presented an elevation in the cytosolic free calcium, which paralleled the inhibition of the calcium pump in the endoplasmic reticulum [7, 8]. They also showed that the leakage of intracellular enzymes followed the rise of free Ca^{2+} in the hepatocyte [7]. However, the concentrations of CCl_4 (1–3 mM) used in these latter studies were much higher than those sufficient to impair the calcium sequestering capacity of isolated hepatocytes [9] as well as to cause cell damage [9, 10]. On the other hand, CCl_4 at concentrations above 0.5 mM is known to nonspecifically damage the hepatocytes probably as consequence of its solvent properties [11].

Considering the importance of discriminating between the toxic mechanisms related to the metabolic activation of CCl_4 and nonspecific effects we have investigated the alterations of Ca^{2+} homeostasis induced in isolated hepatocytes by the exposure to low concentrations of CCl_4. Particular attention was paid to the possible relationships between these events and the appearance of cell injury.

MATERIALS AND METHODS

Collagenase Type I, Ca^{2+} ionophore A23187, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), arsenazo III, Quin-2 tetracetoxymethyl ester, chlorpromazine, mepacrine, leupeptine, antipain dihydrochloride, *N*-6-aminohexyl-5-chloro-1-naphthalene sulphonamide (W7), vasopressin, glucagon, dibutyryladenosine-3′5′cyclic monophosphate, glycogen, glucose-1-phosphate and caffeine were

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obtained from Sigma Chemical Co. (St Louis, MO). Percoll and Affi-Gel polyacrylamide beads were supplied, respectively, by Pharmacia (Uppsala, Sweden) and by Bio-Rad Laboratories (Richmond, CA).

All other reagents were of the highest purity grade and were purchased from Merck (Darmstadt, F.R.G.).

Male Wistar rats (200–300 g/body wt) were obtained from Nossan (Corenzana, Italy) and fed *ad lib.* with a semi-synthetic diet (Piccioni, Brescia, Italy).

Isolated rat hepatocytes were prepared by collagenase perfusion and suspended in a medium which contained 1 mM $CaCl_2$ [10]. The hepatocyte suspensions (5×10^6 cells/ml) were incubated up to 4 hr in 50-ml Erlenmeyer flasks fitted with a center well. Carbon tetrachloride was added to the center well and allowed to diffuse into the cell suspension. Carbon tetrachloride concentrations were estimated by radioisotope dilution using [^{14}C]CCl_4 as reported in [10].

Intracellular calcium pools were estimated by the arsenazo III assay following the addition of CCCP or A23187, as described by Bellomo *et al.* [12]. Briefly, aliquots of the cell suspension (2.4×10^6 cells) were quickly centrifuged through a layer of Percoll (1.06 final density) in order to remove damaged or dead cells and then resuspended in 2 ml of Ca^{2+}–Mg^{2+}-free Hanks buffer containing 0.04 mM arsenazo III. The changes in the absorbance of the arsenazo III–Ca^{2+} complex were continuously monitored at the wavelength pair of 645–685 nm in a Perkin–Elmer 557 spectrophotometer. The sequential addition of 0.015 mM CCCP and 0.01 mM A23187 led to the release of two distinct quantities of calcium which have been demonstrated to be, respectively, of mitochondrial and extramitochondrial origin [12].

Phosphorylase *a* activity was estimated according to Hue *et al.* [13]. Briefly, 0.8 ml aliquots of hepatocyte suspension were layered on the top of 5 ml Percoll solution (1.06 final density) in Ca^{2+}-free Hanks medium and centrifuged 1 min at 1000 rpm. The supernatant was removed by aspiration and the cell pellet immediately frozen with liquid nitrogen. Cells were allowed to thaw for 5 min at 37° in the presence of 0.8 ml of a lysing buffer containing 100 mM NaF, 20 mM EDTA, 0.5% (w/v) glycogen, 50 mM glycylglycine (pH 7.4) and 0.5% Triton X-100. A portion (0.1 ml) of lysed hepatocytes was incubated for 30 min at 37° with an equal volume of a solution containing 100 mM glucose-1-phosphate, 0.3 M NaF and 1 mM caffeine (pH 6.1).

The reaction was stopped by adding 4 ml ice-cold 2.5% trichloroacetic acid and inorganic phosphate released from glucose-1-phosphate was measured after protein precipitation.

Loading of hepatocytes with Quin-2 was obtained by incubating 3.5×10^6 cells/ml in a medium containing 120 mM NaCl, 5.4 mM KCl, 4.2 mM $NaHCO_3$, 1.3 mM $CaCl_2$ and supplemented with 2% (w/v) bovine serum albumine, 15 mM glucose, 20 mM HEPES, pH 7.4. Quin-2 tetracetoxy methyl ester, 0.05 mM final concentration, was added as a solution in dimethyl sulfoxide and the hepatocytes were incubated for 20 min at 37°. After incubation the cells were washed twice with the medium previously described, but supplemented with 1 mM $MgSO_4$, resuspended at 2×10^6 cell/ml in the same medium and finally exposed to CCl_4.

For the estimation of Quin-2 fluorescence, 1 ml of the suspension was centrifuged at 1000 rpm for 1 min, the cells resuspended in fresh medium and transferred in a quartz cuvette where they were maintained at 37° under continuous stirring. Calibrations and measurements of cytosolic calcium were performed using a Perkin–Elmer LS-5B spectrophotofluorometer as described by Cooper *et al.* [14] except that addition of digitonin (0.012 mg/ml) was used to determine the maximal fluorescence.

Plasma membranes were prepared from isolated hepatocytes by using polyacrylamide beads according to Nicotera *et al.* [15]. Ca^{2+}–ATPase activity in the membranes was assayed by the procedure reported by Lin and Fain [16], but using an incubation medium containing: 25 mM Tris–HCl buffer pH 7.5, 0.38 mM ATP, 1 mM EGTA, 0.5 mM ouabain, 0.01 mM Ruthenium Red and with or without 0.87 mM $CaCl_2$.

Cell damage was evaluated by the leakage of lactate dehydrogenase (LDH) into the incubation medium as previously reported [10] and by the appearance of membrane blebs on the hepatocyte surface.

The stimulation of lipid peroxidation was assessed by the production of malonildialdehyde (MDA) according to Poli *et al.* [10].

RESULTS

The estimation of Ca^{2+} releasable from the mitochondrial or the extramitochondrial compartments by the addition of, respectively, CCCP or A23187 revealed that as early as 15 min from the intoxication of isolated hepatocytes with 0.172 mM CCl_4 there was an appreciable decrease in the calcium content of both these subcellular compartments (Fig. 1). Following 30 min exposure to CCl_4 the loss of calcium from mitochondrial and microsomal pools accounted for about 75% and 50%, respectively (Fig. 1).

The calcium-mediated transition of glycogen phosphorylase from the inactive *b* form to the active *a* form and Quin-2 fluorescence have been used to monitor the changes in cytosolic free calcium concentration during CCl_4 metabolism.

As shown in Fig. 2, phosphorylase *a* activity in isolated hepatocytes was not increased following 15–60 min incubation with CCl_4 when calcium was released from the intracellular compartments. On the contrary, the enzyme activity of CCl_4-treated cells was about 30% lower than that of the control hepatocytes (Fig. 2).

This lack of activation of phosphorylase *a* was not due to an inhibition of the enzyme since in hepatocytes pretreated for 15 min with CCl_4 the activity of phosphorylase *a* was stimulated by the addition of butyryl–cAMP and glucagon or by increasing intracellular calcium with 0.05 mM A23187 (Fig. 3).

Consistent with the effect on phosphorylase *a*, the incubation of Quin-2 loaded hepatocytes in the

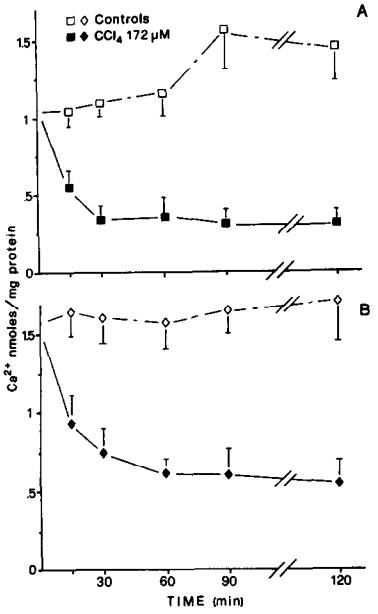

Fig. 1. Content of calcium releasable from the mitochondrial (panel A) and extramitochondrial (panel B) compartments of isolated hepatocytes exposed to CCl_4. Liver cell suspensions (5×10^6 cells/ml) were incubated up to 120 min with or without CCl_4 and the Ca^{2+} content of both intracellular compartments was estimated by the arsenazo III method as described by Bellomo *et al.* [12]. The results are means of 3–4 different experiments ± SD.

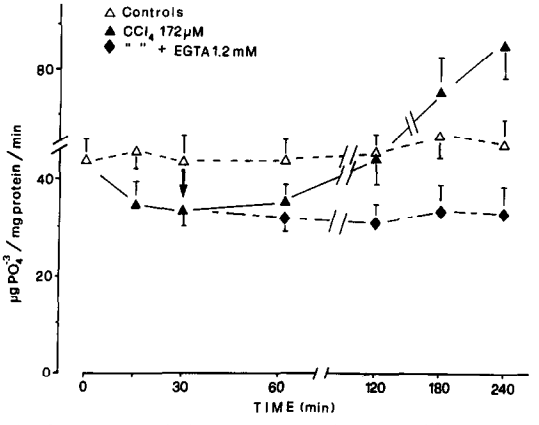

Fig. 2. Activity of phosphorylase *a* in isolated hepatocytes incubated with CCl_4. The activity of phosphorylase *a* was estimated according to Hue *et al.* [13] in the intact cells separated by centrifugation over a Percoll layer ($d = 1.06$). The arrow indicates the addition of EGTA to the cell suspension. The results are means of 3–5 different experiments ± SD.

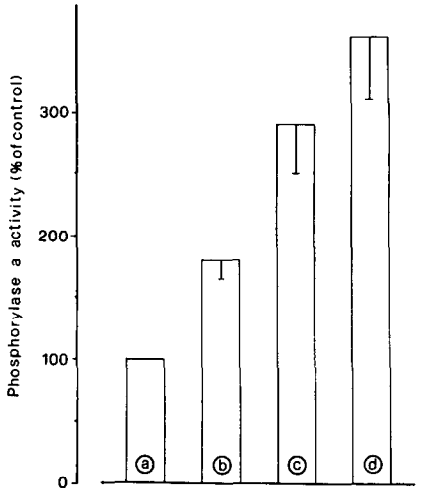

Fig. 3. Effects of various treatments on phosphorylase *a* activity of isolated hepatocytes preincubated with CCl_4. Hepatocytes were pretreated 15 min with 0.172 mM CCl_4 and subsequently incubated 10 min in the presence of: (a) no addition; (b) 0.05 mM A23187; (c) 50 nM glucagon; (d) 0.1 mM dibutyryl-adenosine-3′5′cyclic monophosphate.

The results are expressed as percent of the controls receiving CCl_4 alone and are means of 3–4 different experiments ± SD.

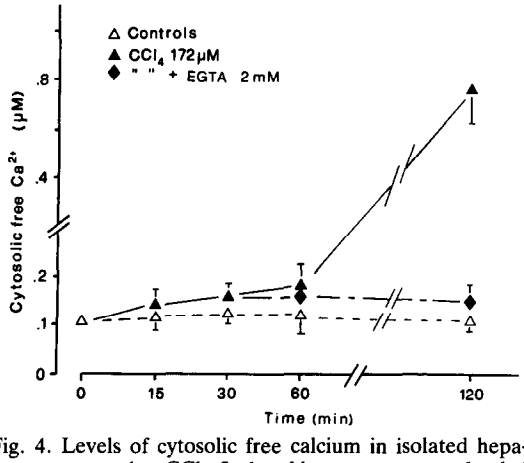

Fig. 4. Levels of cytosolic free calcium in isolated hepatocytes exposed to CCl_4. Isolated hepatocytes were loaded with Quin-2 as described in the Materials and Methods section, and then incubated in the presence of CCl_4 with or without 2 mM EGTA. The results are means of three different experiments ± SD.

presence of CCl_4 for up to 60 min did not show significant variations in the cytosolic levels of calcium (Fig. 4). Such an effect was not the result of an underestimation of the Ca^{2+} concentration due to a nonspecific interaction of CCl_4 with Quin-2, since the sensitivity of Quin-2 to calcium remained unchanged despite the presence of the haloalkane (not shown).

The possibility that calcium released from the mitochondrial and extramitochondrial compartments might be transported out of the cells, preventing the increase in the cytosolic levels, was investigated by measuring Ca^{2+}-translocase activity in the plasma membranes of isolated hepatocytes.

Table 1 shows that after 15 min of incubation with 0.172 mM CCl_4 the activity of high affinity Ca^{2+}-ATPase was decreased by about 30%. Since the concentration of cytosolic free calcium remained unchanged despite the loss of mitochondrial and

Table 1. Activity of high affinity Ca^{2+}–ATPase in plasma membranes prepared from isolated rat hepatocytes incubated with or without CCl_4

	Incubation time (min)		
	5	15	60
Control	0.36 ± 0.05	0.34 ± 0.04	0.38 ± 0.02
CCl_4 0.172 mM	0.39 ± 0.08	0.24 ± 0.06*	0.17 ± 0.07†

The results are expressed as micromoles of phosphate/hr/mg protein and represent the mean of 3–4 different experiments ± SD. Significance by the Student's *t*-test: * $P < 0.05$; † $P < 0.01$.

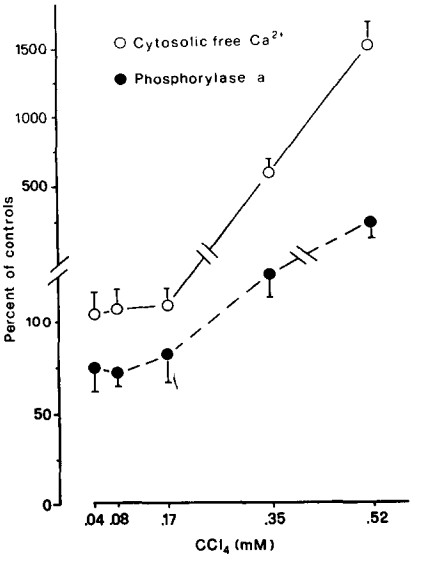

Fig. 5. Effect of different concentrations of CCl_4 on the cytosolic free calcium and on the phosphorylase *a* activity of isolated hepatocytes. Liver cells were incubated 30 min with increasing amounts of CCl_4 added to the center well of the incubation flasks. The concentrations of CCl_4 in the cell suspension were calculated by isotope dilution as reported in [10]. Cytosolic levels of calcium were measured by Quin-2 fluorescence as described in the Materials and Methods section.

The results are expressed as percent of the untreated controls and are the means of three experiments ± SD.

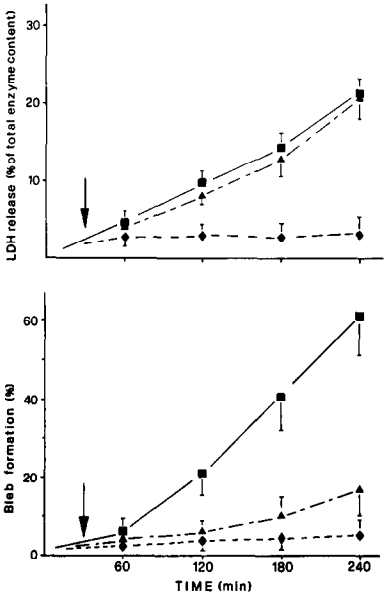

Fig. 6. Leakage of LDH and plasma membrane blebbing in isolated hepatocytes incubated with CCl_4 in the presence or in the absence of 1.2 mM EGTA. The calcium chelator was added (arrow) 30 min after the exposure to 0.172 mM CCl_4 in order to not interfere with the metabolism of the haloalkane. The symbols represent: ◆ control hepatocytes incubated without CCl_4; ■ cells receiving 0.172 mM CCl_4; ▲ cells exposed to CCl_4 and subsequently treated with 1.2 mM EGTA.

The results are the means of three different experiments ± SD.

extramitochondrial calcium pools, it is conceivable to argue that the observed inhibition of the plasma membrane Ca^{2+}–ATPase was not high enough to significantly impair the active extrusion of the calcium released intracellularly.

These results were apparently in contrast with the rise in cytosolic Ca^{2+} observed by Long and Moore in hepatocytes exposed to high concentrations of CCl_4 [7, 8]. However, when the action of CCl_4 on phosphorylase *a* activity and Quin-2 fluorescence were assayed in liver cells exposed to increasing amounts of the haloalkane we found that concentrations of CCl_4 above 0.172 mM were necessary to enhance cytosolic free calcium (Fig. 5).

Furthermore, even with 0.172 mM CCl_4 the cytosolic free Ca^{2+} levels began to rise after the second hour of treatment (Figs 2 and 4) reaching micromolar concentrations. This increase was preceded by a more marked inhibition of plasma membrane Ca^{2+}–ATPase (Table 1) and appeared to be entirely due to an influx of calcium from the extracellular compartment since the chelation of calcium with EGTA completely prevented both the activation of phosphorylase *a* and the increase in Quin-2 fluorescence (Figs 2 and 4).

The changes in the intracellular distributions of calcium occurring immediately after the addition of CCl_4, were not accompanied by evident alterations of hepatocyte integrity, as evaluated by the leakage of lactate dehydrogenase (LDH) and by plasma membrane blebbing (Fig. 6). Signs of cell damage became evident only during the second hour of treatment (Fig. 6) and were associated with the elevation

Table 2. Effect of inhibitors of calcium-activated enzymes on the stimulation of lipid peroxidation and the hepatocyte damage induced by CCl_4

Treatments	MDA production (nmol/10^6 cells)	LDH (% leakage)	Cell blebbing (%)
None	0.34 ± 0.13	7.2 ± 2.2	4
CCl_4 0.172 mM	2.17 ± 0.25	18.0 ± 2.6	48
CCl_4 0.172 mM + chlorpromazine 0.05 mM	1.03 ± 0.45	18.3 ± 3.1	40
CCl_4 0.172 + mepacrine 0.05 mM	1.35 ± 0.71	17.3 ± 4.0	44
CCl_4 0.172 mM + leupeptin 0.1 mM	2.04 ± 0.24	18.7 ± 2.6	46
CCl_4 0.172 mM + antipain 0.05 mM	1.50 ± 0.31	18.5 ± 2.1	55
CCl_4 0.172 mM + W7 0.05 mM	2.68 ± 0.36	23.0 ± 3.6	60

Isolated hepatocytes were incubated 4 hr with CCl_4 and the inhibitors as described in the Materials and Methods section. Stimulation of lipid peroxidation was evaluated by the production of malonildialdehyde (MDA). LDH leakage is expressed as percent of the total enzyme content of hepatocytes assayed after cell lysis with 0.05% Triton X-100. Cell blebbing represents the percent of the hepatocytes showing plasma membrane blebs over the total number of hepatocytes excluding Trypan Blue (approximately 60–70% of all the hepatocytes).

The hepatocytes incubated 4 h with the inhibitors alone did not show stimulation of lipid peroxidation and LDH leakage was about 6–9%. The values represent the mean of three different experiments ± SD.

of cytosolic free calcium.

Blocking the rise of cytosolic calcium with EGTA protected the hepatocytes against the blebbing, but did not prevent the leakage of LDH induced by CCl_4 (Fig. 6). On the other hand, the addition of 1.2 mM EGTA alone was not found to affect hepatocyte integrity.

It has been suggested that high levels of cytosolic Ca^{2+} lead to cell damage by inducing the activation of phospholipase A_2 [18] and of the cytosolic proteases [19]. In isolated hepatocytes exposed to CCl_4, however, neither plasma membrane blebbing nor LDH leakage were prevented by the addition of inhibitors of phospholipase A_2 such as chlorpromazine and mepacrine, as well as by the addition of leupeptin and antipain, two well-known inhibitors of non-lysosomal proteases (Table 1). A similar lack of protection was also observed using the calmodulin inhibitor *N*-6-aminohexyl-5-chloro-1-naphthalene sulphonamide (W7).

DISCUSSION

Previous studies have demonstrated that the exposure of isolated rat hepatocytes to concentrations of CCl_4 ranging between 0.1 and 0.2 mM reproduced *in vitro* many of the cellular alterations observed in the liver during the *in vivo* intoxication [9, 10, 20]. Among these effects, the incubation of hepatocytes with 0.172 mM CCl_4 causes a severe alteration of calcium homeostasis characterized by the depletion of both the mitochondrial and extramitochondrial Ca^{2+} pools. Several mechanisms are likely to be responsible for such damage. For instance, the inactivation of the Ca^{2+}–ATPase in the endoplasmic reticulum is the main cause of the lowering of microsomal calcium content [1, 2, 9], but, according to Benedetti and co-workers [21], the inhibition of glucose-6-phosphatase which also occurs during the early phases of CCl_4 intoxication [20] might contribute by affecting the retention of calcium by the endoplasmic reticulum.

In a previous study we have reported that the loss of calcium from mitochondria is the result of the inhibition of the uniport uptake system [3]. It is possible that such an effect might be the consequence of a direct attack of free radical intermediates since we have observed that CCl_4 is reductively activated to $CCl_3^{\cdot}$ by the mitochondrial respiratory chain [22].

The damage of the enzymatic systems transporting Ca^{2+} into the intracellular organelles is associated with a decrease of calcium-dependent ATPase functions in the plasma membrane. In the early phases of the intoxication, however, the inhibition of this latter enzyme does not appear to be as severe as that of calcium transport in both the microsomes and mitochondria. This chronological difference and the presence of ATP levels sufficient to support the function of plasma membrane Ca^{2+}-translocase [3] might explain the steady levels of cytosolic free calcium present during the release of Ca^{2+} from the intracellular organelles.

We suggest that the increase in cytosolic calcium reported by Long and Moore [7, 8] is a peculiar effect of the high concentrations of CCl_4 which can not be exclusively ascribed to the impairment of the microsomal stores. The stimulation of both Quin-2 fluorescence and phosphorylase *a* activity were, in fact, evident within 1 min of haloalkane addition, well before the damages related to the metabolic activation of CCl_4 could reasonably take place. Moreover, the concentrations of CCl_4 sufficient to impair Ca^{2+} uptake by the endoplasmic reticulum [7, 9] are much lower than those which were reported to rise cytosolic calcium.

Hoek *et al.* [23] have recently demonstrated that ethanol and other organic solvents cause activation of phosphorylase *a* which peaks 20–30 sec after addition of the solvent to isolated hepatocytes. Inositol 1,4,5 triphosphate and cytosolic free Ca^{2+} increased concomitantly with the phosphorylase activity, indicating that ethanol was able to stimulate phosphoinositide-specific phospholipase C. Consistently, Snyder and Lamb [24] reported that 5 mM CCl_4 activates phospholipase C by simple interaction

with liver plasma membranes suggesting that, similarly to ethanol, high concentrations of CCl_4 might trigger the formation of diacylglycerol and phosphoinositols leading to a rapid rise in cytosolic free calcium and in phosphorylase *a* activation.

According to this hypothesis, the source of Ca^{2+} mobilized by high concentrations of CCl_4 appears to be the endoplasmic reticulum. In fact, the pretreatment of isolated hepatocytes with 0.172 mM CCl_4 completely abolished the rapid increase in cytosolic free Ca^{2+} and the phosphorylase activation induced by 0.5 mM CCl_4 (not shown).

Isolated hepatocytes exposed to 0.172 mM CCl_4 presented an activation of phosphorylase *a* only in the late phases of the intoxication as a result of an influx of Ca^{2+} from the extracellular space. Tsokos-Kuhn and co-workers [25] have demonstrated that a marked increase in Ca^{2+} permeability takes place in the plasma membranes isolated from the livers of rats treated with CCl_4. Nonetheless, experiments in our laboratory suggest that the progressive depletion of cellular ATP associated with the impairment of Ca^{2+} transport mechanisms are probably the main causes for the increase in the cytosolic Ca^{2+} [26].

The alterations in calcium compartmentation and the subsequent rise in cellular free Ca^{2+} are considered to be critical events in the onset of irreversible cell injury [19, 27–29] by triggering the activation of calcium-dependent phospholipase A_2, of non-lysosomal proteases as well as of calcium-dependent endonucleases [18, 19, 30]. This applies to several conditions including the oxidative stress induced by quinones, the cytotoxicity of cistamine and methylprednisolone-mediated tymocyte death [30–32].

In isolated hepatocytes poisoned with CCl_4, however, the alterations of calcium homeostasis occurring shortly after the addition of the toxic compound did not seem to affect cell integrity, and did not contribute to cause fat accumulation [6], which is the other toxic response of the liver to carbon tetrachloride [10, 20, 33]. Moreover, plasma membrane blebbing and LDH leakage occurring in the late phase of the intoxication, although chronologically associated with calcium accumulation, were not prevented or delayed by inhibitors of phospholipase A_2 and of non-lysosomal proteases.

Therefore, we postulated that the changes in calcium distribution induced by low concentrations of CCl_4 cannot be ascribed as the exclusive and the primary cause of hepatocyte death. It is possible, however, that substained levels of cytosolic Ca^{2+} might act as an additional mechanism which could contribute to the progression of cell injuries.

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REFERENCES

1. Moore L, Chen T, Knapp HR and Landon EJ, Energy dependent calcium sequestering activity in rat liver microsomes. *J Biol* **250**: 4562–4568, 1975.
2. Lowrey K, Glende EA and Recknagel RO, Destruction of liver microsomal calcium pump activity by carbon tetrachloride and bromotrichloromethane. *Biochem Pharmacol* **30**: 135–140, 1981.
3. Albano E, Bellomo G, Carini R, Biasi F, Poli G and Dianzani MU, Mechanisms responsible for carbon tetrachloride-induced perturbation of mitochondrial calcium homeostasis. *FEBS Lett* **192**: 184–188, 1985.
4. Recknagel RO, A new direction in the study of carbon tetrachloride hepatotoxicity. *Life Sci* **33**: 401–408, 1983.
5. Brattin JW, Glende EA and Recknagel RO, Pathological mechanisms in carbon tetrachloride hepatotoxicity. *J Free Radicals Biol Med* **1**: 27–38, 1985.
6. Pencil SD, Brattin WJ, Glende EA and Recknagel RO, Carbontetrachloride dependent-inhibition of lipid secretion by isolated hepatocytes: characterization and requirement of bioactivation. *Biochem Pharmacol* **33**: 2425–2429, 1984.
7. Long RL and Moore L, Elevated cytosolic calcium in rat hepatocytes exposed to carbon tetrachloride. *J Pharmacol Exp Ther* **238**, 186–191, 1986.
8. Long RL and Moore L, Cytosolic calcium after carbon tetrachloride, 1,1-dichloroethylene and phenylephrine exposure. *Biochem Pharmacol* **36**: 1215–1221, 1987.
9. Pencil SD, Glende EA and Recknagel RO, Loss of calcium sequestration capacity in endoplasmic reticulum of isolated hepatocytes treated with carbon tetrachloride. *Res Commun Chem Pathol Pharmacol* **36**: 413–428, 1982.
10. Poli G, Gravela E, Albano E and Dianzani MU, Studies on fatty liver with isolated hepatocytes. *Exp Mol Pathol* **30**: 116–127, 1979.
11. Berger LM, Bhatt H, Combes B and Estabrook RW, CCl_4-induced toxicity in isolated hepatocytes: the importance of direct solvent injury. *Hepatology* **6**: 36–45, 1986.
12. Bellomo G, Jewell SA, Thor H and Orrenius S, Regulation of intracellular calcium compartmentation: studies with isolated hepatocytes and *t*-butyl-hydroperoxide. *Proc Natl Acd Sci U.S.A.* **79**: 6842–6846, 1982.
13. Hue L, Bontemps F and Hers HG, The effect of glucose and of potassium ions on the interconversion of the two forms of glycogen phosphorylase and of glycogen synthetase in isolated rat liver preparations. *Biochem J* **152**: 105–114, 1975.
14. Cooper RH, Coll KE and Williamson JR, Differential effects of phorbol ester on phenylephrine and vasopressin-induced Ca^{2+} mobilization in isolated hepatocytes. **260**: 3281–3287, 1985.
15. Nicotera P, Moore M, Bellomo G, Mirabelli F and Orrenius S, Demonstration and partial characterization of glutathione disulfide-stimulated ATPase activity in the plasma membrane fraction from rat hepatocytes. *J Biol Chem* **260**: 1999–2002, 1985.
16. Lin SH and Fain JN, Purification of (Ca^{2+}–Mg^{2+}–ATPase from rat liver plasma membranes. *J Biol Chem* **259**: 3016–3020, 1984.
17. Carafoli E and Tiozzo RA, A study of energy-linked calcium transport in liver mitochondria during CCl_4 intoxication. *Exp Mol Pathol* **9**: 131–140, 1969.
18. Glende EA and Pushpendran CK, Activation of phospholipase A_2 by carbon tetrachloride in isolated rat hepatocytes. *Biochem Pharmacol* **35**: 3301–3307, 1986.
19. Nicotera P, Hartzell P, Davis G and Orrenius S, The formation of plasma membrane blebs in hepatocytes exposed to agents that increase cytosolic Ca^{2+} is mediated by the activation of a non-lysosomal proteolytic system. *FEBS Lett* **209**: 139–144, 1986.
20. Poli G and Gravela E, Lipid peroxidation in isolated hepatocytes. In: *Free Radicals, Lipid Peroxidation and*

Cancer (Eds. McBrien DCH and Slater TF) pp. 215–241, Academic Press, London, 1982.
21. Benedetti A, Fulceri R, Ferro M and Comporti M, On a possible role for glucose-6-phosphatase in the regulation of liver cell cytosolic calcium concentration. *Trends Biochem Sci* **10**: 284–285, 1986.
22. Tomasi A, Albano E, Banni S, Botti B, Corongiu F, Dessi' MA, Iannone A, Vannini V and Dianzani MU, Free radical metabolism of carbon tetrachloride in rat liver mitochondria. *Biochem J* **246**: 313–317, 1987.
23. Hoek JB, Thomas AP, Rubin R and Rubin E, Ethanol-induced mobilization of calcium by activation of phosphoinositide-specific phospholipase C in intact hepatocytes. *J Biol Chem* **262**: 682–691, 1987.
24. Snyder JW and Lamb RG, The role of lipid peroxidation in the chemical-dependent activation of hepatic phospholipase C *in vitro*. *Res Commun Chem Pathol Pharmacol* **55**: 3–15, 1987.
25. Tsokos-Kuhn JO, Smith CV, Mitchell JR, Tate CA and Entmann ML, Evidence for increased membrane permeability of plasmalemmal vesicles from livers of phenobarbital-induced CCl_4-intoxicated rats. *Mol Pharmacol* **30**: 444–451, 1986.
26. Albano E, Carini R, Parola M, Bellomo G, Poli G and Dianzani MU, Increase in cytosolic free calcium and its role in the pathogenesis of hepatocyte injury induced by carbon tetrachloride. In: *Free Radicals in Liver Pathology* (Eds. Poli G, Cheeseman KH, Dianzani MU and Slater TF). Advances in the Biosciences, Vol. 76, pp. 45–54. Pergamon Press, Oxford, 1989.
27. Jewell SA, Bellomo G, Thor H, Orrenius S and Smith MT, Bleb formation in hepatocytes during drug metabolism is caused by disturbance in thiol and calcium ion homeostasis. *Science* **217**: 2157–2160, 1982.
28. Farber JL, Membrane injury and calcium homeostasis in the pathogenesis of coagulative necrosis. *Lab Invest* **47**: 114–123, 1984.
29. Bellomo G, Jewell SA, Smith MT, Thor H and Orrenius S, Perturbation of Ca^{2+} homeostasis during hepatocyte injury. In: *Mechanisms of Hepatocyte Injury and Death* (Eds. Kepler D, Pepper H, Bianchi L and Reutter W) Falk Symposium 38, pp. 119–128. MPT Press, Lancaster, 1984.
30. Mirabelli F, Salis A, Vairetti M, Bellomo G, Thur H and Orrenius S, Cytoskeletal alterations in human platelets exposed to oxidative stress are mediated by oxidative and calcium dependent mechanisms. *Arch Biochem Biophys*, in press.
31. Nicotera P, Hartzell P, Baldi C, Svensson S, Bellomo G and Orrenius S, Cystamine induced toxicity in hepatocytes through the elevation of cytosolic Ca^{2+} and the stimulation of non-lysosomal proteolytic system. *J Biol Chem* **261**: 14628–14635, 1986.
32. McConkey DJ, Nicotera P, Hartzel P, Bellomo G, Wyllie AH and Orrenius S, Glucocorticoids activate a suicide process in tymocytes through an elevation of cytosolic Ca^{2+} concentration. *Arch Biochem Biophys* **269**: 365–370, 1989.
33. Poli G, Albano E and Dianzani MU, The role of lipid peroxidation in liver damage. *Chem Phys Lipids* **45**: 117–142, 1987.