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Ca-Efflux, from direct membrane injury by CCl_4, is elicited by amphiphilic vehicles in vitro

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Abstract. Direct membrane injury by CCl_4, in situations excluding metabolic activation, was evaluated in saponin-permeabilized hepatocytes and in microsomes by measuring immediate Ca^{2+} efflux. A good correlation appears between the Ca^{2+} efflux and the level of CCl_4 in the membrane and also the variations in fluidity. Mixtures of CCl_4 with water-soluble vehicles were used to improve the dispersion of CCl_4 in the medium. The mixtures varied in their ability to elicit the membrane effects of CCl_4. The performance of ethanol and, to a lesser degree, other alcohols, suggests the existence of a water stable structural organization between CCl_4 and these amphiphilic vehicles, facilitating the transfer of CCl_4 to the membrane.
Key words. Carbon tetrachloride; alcohols; solvent effect; hepatocytes; microsomes; Ca^{2+} efflux.

It has been postulated that CCl_4 and other hepatotoxic compounds are responsible for cell death through a perturbation of calcium homeostasis[1-3]. One of the earliest consequences is an increase of cytosolic Ca^{2+} and a serious inhibition of the capacity to sequester calcium of the endoplasmic reticulum. These effects were demonstrated by in vivo administration of CCl_4[4-8] and were reproduced in vitro by addition of CCl_4 to suspensions of hepatocytes or liver microsomes[2,5,9]. Metabolic activation of CCl_4 is considered to be the initial event leading to loss of calcium homeostasis, but various findings[10,11] suggest that the rapid Ca^{2+} release[9,12,13] induced by high concentrations of CCl_4 (>1 mM) is mediated by direct solvent effects. It cannot be excluded that these injuries participate in the in vivo toxicity when CCl_4 is administered at doses higher than 1.5 ml/kg. However, the changes observed in hepatic calcium homeostasis have usually been attributed to metabolic radical activation[4-8] in spite of the high hepatic levels of CCl_4 reached (2–6 mM)[14-16]. In fact, it is not known what the intramembrane CCl_4 concentrations are and whether they are able to have direct effects on calcium homeostasis. In vitro systems are useful to study the direct effects, but although a fine dispersion of CCl_4 in the aqueous medium may be appropriate for the investigation of its activity on the membrane, we have poor knowledge of the mechanisms involved and the conditions allowing maximum interaction[9,10,12,13,17,18]. The aim of this work is to demonstrate that the mode of CCl_4 presentation to the membrane systems contributes significantly to the CCl_4 solvent effect described. We paid particular attention to the relationships between the physical state of CCl_4 in the medium, the transfer to the membrane, and Ca^{2+} mobilization in hepatocytes and microsomes.

Materials and methods

Materials. Radioactive $^{45}CaCl_2$ (25 mCi/mg Ca) and $^{14}CCl_4$ (3.4 mCi/mmole) were obtained from Amersham (Les Ulis, France) and spin labels from Syva International (Palo Alto, CA). Collagenase was obtained from Boehringer Mannheim (Meylan, France). HEPES, creatine phosphate, creatine kinase, Mg-ATP, saponin and albumin were obtained from Sigma Chemical (La Verpilliere, France). Alcohols were purchased from Alltech Associates (Deerfield, IL.) and halocarbons from Merck (Darmstat, Germany). All other reagents were commercial products of the highest available grade of purity.

Preparation of hepatocytes and microsomes. Hepatocytes were isolated, by a collagenase perfusion technique[19], from male Sprague Dawley rats weighing 200–250 g. Microsomal membranes were prepared[5] from a 10% liver homogenate in ice-cold 3 mM EDTA, 154 mM KCl at pH 7.4. The pellets were washed three times in calcium-free incubation buffer. Proteins were determined using Lowry's method[20].

Quantification of Ca^{2+} fluxes. The hepatocytes (9 mg protein/ml) were incubated in closed vials under slow stirring at 37 °C in 100 mM KCl, 10 mM NaCl, 1.0 mM KH_2PO_4, 5.0 mM $KHCO_3$, 20 mM HEPES buffer pH 7.2, containing 20 mM creatine phosphate, 10,000 U/l of creatine kinase, 0.3 mM $MgCl_2$ and 3.0 mM Mg-ATP. Plasma membrane was permeabilized at the start of the incubation by addition of saponin (60 mg/l). The microsomes (1 mg protein/ml) were also incubated in closed vials under slow stirring at 37 °C in the same buffer containing 1 mM ATP. For both hepatocyte and microsome preparations, changes in calcium efflux were studied after addition of an aliquot of mixtures of CCl_4 and water-soluble vehicle (methanol,

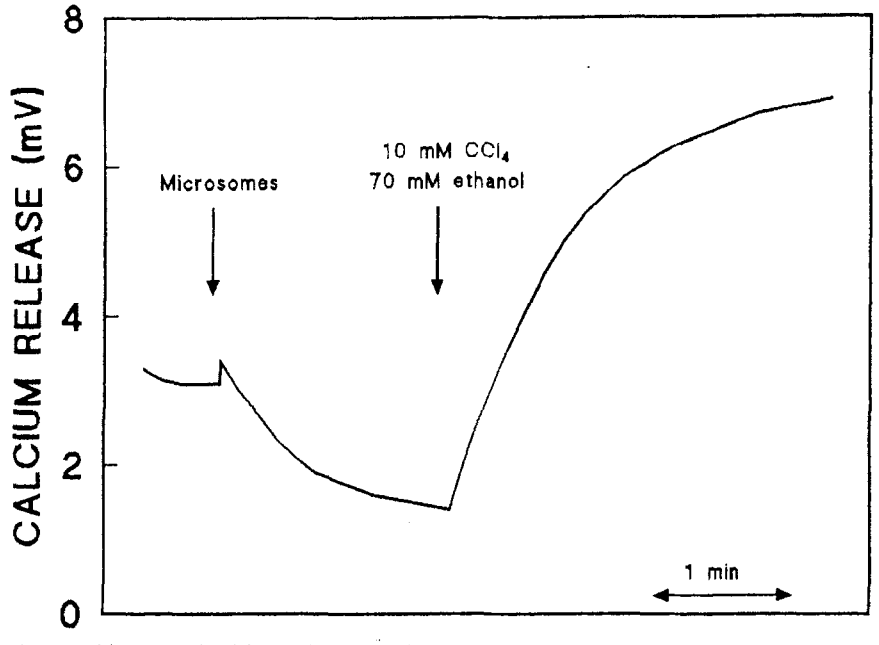

Figure 1. Typical polarographic trace of calcium release by microsomes after exposure to 10 mM CCl_4 dissolved in 70 mM ethanol.

ethanol, propanol, isopropanol, acetonitrile or acetone). Mixtures with other halocarbons ($CBrCl_3$, $CHCl_3$, CH_2Cl_2) were also used. The calcium efflux was measured by polarographic method[21]. The experimental set-up consisted of a thermostatted polypropylene incubation vessel with a magnetic stirrer. A calcium-specific electrode and an Ag-AgCl reference electrode were inserted through the tight lid. The potential of the Ca^{2+} against the reference electrode was measured by means of a millivoltmeter. Figure 1 presents a typical polarographic trace obtained with liver microsomes when exposed to CCl_4 previously dissolved in ethanol. An upward deflection of the trace indicates release of Ca^{2+} into the medium. The rate of Ca^{2+} release (nmoles Ca^{2+}/mg protein/min) was calculated from the initial slope of the curve[9].

Calcium permeability of microsomal membranes was studied in conditions excluding the activity of Ca^{2+}-ATPase. The microsomal membranes (1 mg protein/ml) were resuspended in 30 mM imidazole, 30 mM histidine and 100 mM KCl (I.H. buffer) pH 6.8 at 37 °C, containing 20 μM $^{45}CaCl_2$ (25μCi/μmole), 5 mM $MgCl_2$, 5 mM NaN_3 and 5 mM ATP[22,23]. Passive efflux of $^{45}Ca^{2+}$ was initiated immediately after addition of the CCl_4/ethanol mixture by diluting the loading preparation twenty times in the same buffer. Aliquots were filtered and monitored for radioactivity[23].

Electron spin resonance (ESR) measurements. Microsomal membranes (18–20 mg protein/ml) were labelled using 5-doxylstearic acid, 12-doxylstearic acid or 16-doxylstearic acid. An aliquot of the spin label in chloroform was transferred into a tube and coated as a thin film by complete evaporation of the solvent under nitrogen. Microsomal suspension in I.H. buffer was added and vortexed at room temperature. The molar concentration lipid/spin label was approximately 200. CCl_4/ethanol mixtures were added to aliquots of labelled membranes and the spectra recorded[24]. The order parameter S (for 5-doxylstearic acid) and the rotational correlation time τ (for 12- and 16-doxylstearic acid) were calculated[25]. A decrease of S or of τ indicates an increase of membrane fluidity.

Microsomal membrane associated CCl_4. Microsomes (1 mg protein/ml) were incubated in I.H. buffer at 37 °C for 1 min in the presence of different mixtures of $^{14}CCl_4$ (1 μCi/ml) and ethanol. After incubation, an aliquot was filtered by centrifugation through 0.45 μm nylon membrane (spin × 8170) for 30 sec, washed with 1 ml of ice-cold incubation buffer and radioactivity counted. This procedure measures the radioactivity associated with microsomal membranes.

Results

Table 1 shows the Ca^{2+} release rates obtained with hepatocytes and microsomes under the effects of CCl_4

Table 1. Effect of various CCl_4/vehicle mixtures (10 mM/70 mM) on Ca^{2+} release from hepatocytes permeabilized with saponin, or from liver microsomes

Vehicle	log P[26]	nmoles Ca^{2+} released/mg protein/min	
		Hepatocytes	Microsomes
Methanol	−0.64	0.24 ± 0.11[a]	0.20 ± 0.05
Ethanol	−0.03	2.04 ± 0.27	0.67 ± 0.09
1-Propanol	0.30	0.22 ± 0.17	0.24 ± 0.07
Isopropanol	0.05	ND	0.18 ± 0.01
Acetonitrile	−0.34	0.15 ± 0.14	0.00 ± 0.00
Acetone	−0.24	ND	0.14 ± 0.03

The vehicles added individually were without effect on Ca^{2+} release. ND: Not determined. [a]Values are means ± SEM of 3 different preparations.

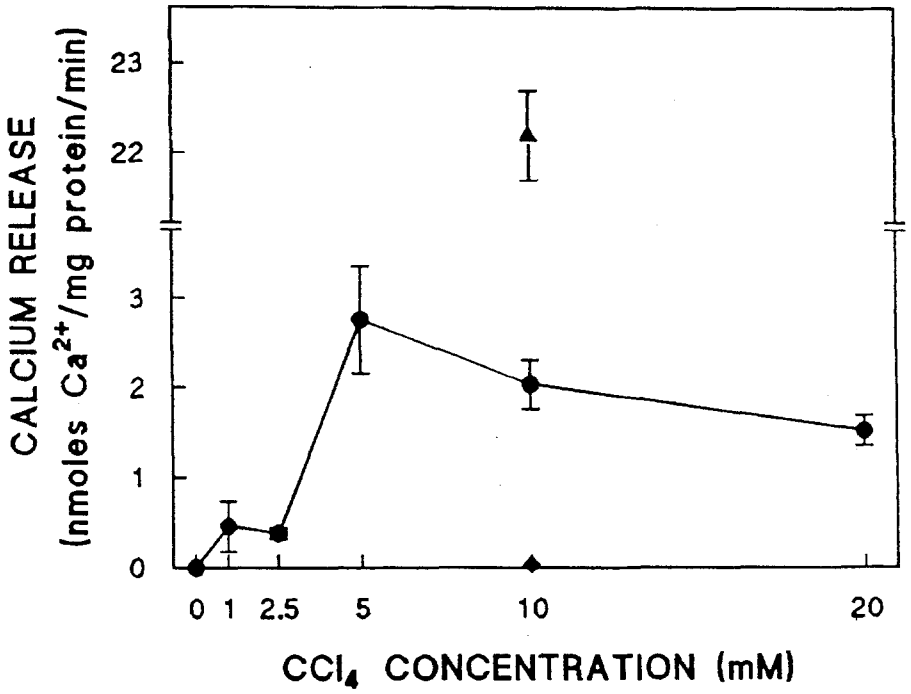

Figure 2. Rate of Ca^{2+} release from saponin-permeabilized hepatocytes after exposure to various concentrations of CCl_4 dissolved in 35 (—◆—), 70 (—●—) or 140 (—▲—) mM ethanol. Each point represents the mean ± S.E.M. of 4 separate experiments.

(10 mM) previously dissolved in different vehicles (70 mM). In the two cases, mixtures with ethanol gave the highest values followed by those with other alcohols while acetone and acetonitrile were less efficient in eliciting CCl_4-induced Ca^{2+} release. The rate of release was not related to the octanol-water partition coefficient (log P) of the vehicles.

We verified that CCl_4 or vehicles added alone were without effect on Ca^{2+} release, likewise when both, CCl_4 and vehicle, were added simultaneously but separately (i.e. two solutions instead a combined mixture).

Figure 2 shows the dose-dependent effect of CCl_4/ethanol mixtures on the release rate of Ca^{2+} from hepatocytes. With 70 mM ethanol added, the release rate of Ca^{2+} was not proportional to the concentration of CCl_4 and the highest values were obtained with 5 mM CCl_4. When the concentration of CCl_4 was 10 mM a maximal release was observed with 140 mM ethanol. So the release seems to be dependent on the concentration of both CCl_4 and ethanol. The effects of CCl_4/ethanol mixtures upon microsomal membranes (table 2) were similar to those observed with hepatocytes: the release rate of Ca^{2+} was maximal when the final concentration of CCl_4 was 10 mM and ethanol was 140 mM. Mixtures of both compounds increased membrane fluidity, measured by the decrease of S parameter for 5-doxylstearic acid, in a dose-dependent manner. Similar increases in membrane fluidity were observed with the two other membrane probes (data not shown). The amount of $^{14}CCl_4$ associated with microsomal membranes in differ-

Table 2. Effects of CCl_4/ethanol mixtures added to liver microsomes

Final concentrations (mM) CCl_4	(mM) Ethanol	Ca^{2+} release (nmoles/mg prot/min)	CCl_4 associated (nmoles/mg prot/min)	Order parameter (S)
–	–	0.00 ± 0.00		0.668 ± 0.0015
–	70	0.00 ± 0.00		0.667 ± 0.0015
–	140	0.04 ± 0.03		0.667 ± 0.0018
1	70	0.00 ± 0.00	14.2 ± 2.17	0.666 ± 0.0020
2.5	70	0.06 ± 0.004	49.7 ± 4.87	0.666 ± 0.0015
5	70	0.34 ± 0.08***	79.5 ± 18.91	0.661 ± 0.0023
10	35	0.07 ± 0.04	56.5 ± 12.97	0.666 ± 0.0020
10	70	0.62 ± 0.09***	73.5 ± 15.65	0.658 ± 0.0015*
10	140	1.06 ± 0.08***	129.7 ± 15.49	0.655 ± 0.0014***
20	70	0.50 ± 0.11***	139.2 ± 15.34	0.653 ± 0.0015***

Values are means ± SEM of 4 different preparations and comparisons were made using Student's t-test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus control (without CCl_4 or ethanol).

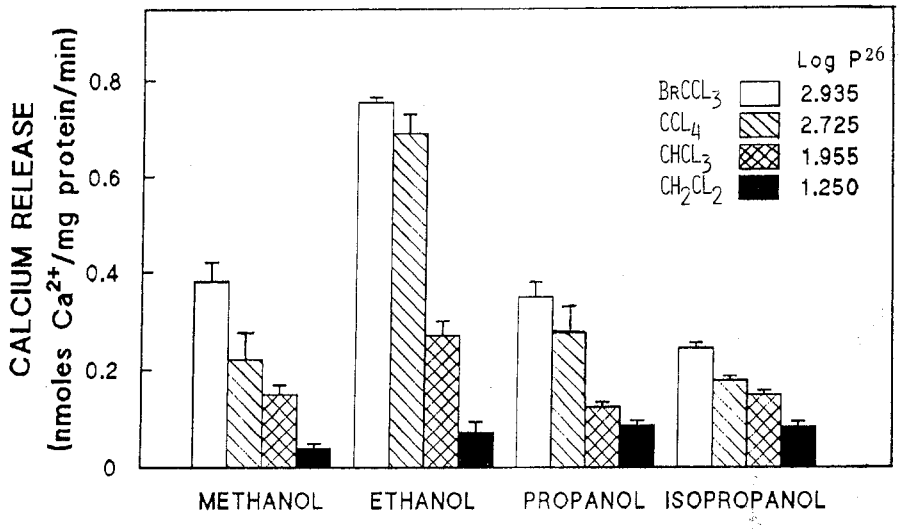

Figure 3. Effect of several hydrophobic halocarbons (10 mM) dissolved in amphiphilic alcohols (70 mM) on Ca^{2+} release from hepatic microsomes. Results are the mean ± SEM of 3 separate experiments.

ent situations gives a maximal value of 139 nmoles. From these results we calculated the correlation coefficient between the release rate of Ca^{2+} after CCl_4/ethanol treatement and CCl_4 associated with microsomal membranes ($r = 0.08$) or the order parameter S ($r = 0.85$).

In the effect observed, the log P of CCl_4 seems to be more important than the log P of vehicles as shown in figure 3. For a series of halocarbons, the greater their log P the higher the calcium efflux. On the other hand maximum eliciting effect was observed with ethanol, confirming the independence from log P of the vehicle. Results obtained by polarography were confirmed by the measurements of passive $^{45}Ca^{2+}$ efflux (table 3) made in conditions increasing permeability changes. The initial calcium content after loading was 7.6 ± 0.6 nmoles of Ca^{2+}/mg proteins (mean ± SEM of six different preparations). Efflux of Ca^{2+} from membranes treated with CCl_4/ethanol mixtures was more rapid and more complete than was the case with control membranes, especially from microsomes exposed to 10 mM CCl_4/140 mM ethanol mixtures, which showed total Ca^{2+} release after 0.25 min of exposure. In contrast, 1 mM CCl_4/70 mM ethanol or 10 mM CCl_4/35 mM ethanol mixtures or 140 mM ethanol alone gave values similar to those obtained for control membranes.

Table 3. Passive calcium efflux from $^{45}Ca^{2+}$ loaded liver microsomes initiated after addition of CCl_4/ethanol mixtures. The results are expressed as percentage of the initial value

Final concentrations (mM)		Time (min)					
CCl_4	Ethanol	0.25	0.5	1.0	2.0	5.0	20.0
–	–	69.3	61.4	58.3	44.6	17.4	8.5
1	70	74.8	56.6	50.4	46.1	24.8	4.2
5	70	44.2	33.2	21.6	12.3	4.2	1.1
10	35	69.7	65.5	51.2	44.0	27.1	6.2
10	70	47.1	36.0	31.7	11.0	5.6	1.6
10	140	4.8	2.9	2.2	1.6	0.7	0.6
20	70	50.1	33.4	25.8	14.0	4.6	0.5
–	140	70.3	60.5	56.4	45.5	20.2	7.2

Values are means of 2 different preparations.

Discussion

Previous studies had demonstrated that the exposure of isolated hepatocytes to high concentrations of CCl_4 caused a rapid Ca^{2+} efflux[9,12,13] related to a decrease of Ca^{2+} in both mitochondria[27,28] and endoplasmic reticulum[4,5,28]. In our experiments, the effluxes of calcium were independent of metabolic activation (absence of NADPH from the microsomal incubation medium) and occurred as rapidly as one minute after addition of halocarbon, as observed by others[9,13]. However, the concentrations are one order of magnitude higher than those reached in experimental conditions where injury is induced under metabolic activation of CCl_4[6,17,18,28].

Our results showed that addition of CCl_4/ethanol mixtures to the microsomal suspension induced Ca^{2+} release in a similar manner to that observed with isolated hepatocytes. Nevertheless, hepatocytes released a higher level of Ca^{2+} than did microsomes, which is in agreement with several intracellular Ca^{2+} pools being affected.

Various possibilities could be proposed to explain the mechanism of calcium release by solvent membrane effects. The activation of phosphoinositide-specific phospholipase C involved in the mobilization of intracellular Ca^{2+} by CCl_4 and other solvents has been suggested[29,30]. However, this does not seem to be the main mechanism involved, because the results obtained with hepatocytes and microsomes are similar in spite of the fact that phospholipase C activity can only take place in hepatocytes. Inhibition of Ca^{2+}-ATPase cannot be excluded when Ca^{2+} release is studied by the polaro-

graphic method. However, experiments with $^{45}Ca^{2+}$ demonstrated that Ca^{2+} release is mediated essentially by an increased membrane permeability.

Two facators seem to be relevant in explaining the Ca^{2+} release observed: the halocarbon partition coefficient (fig. 3) and the vehicle used (table 1). The partition coefficient of the vehicle does not seem to be an important factor in the expression of the CCl_4 induced effects. The present report demonstrates that the physical state in which CCl_4 is delivered into the medium plays an important role in the effects described. The dispersion of CCl_4 in the aqueous medium is the major factor explaining the particular effect of mixtures with ethanol. Other amphiphilic molecules such as acetone and acetonitrile, which are good solvents for CCl_4 but lead to a worse dispersion in the medium, are practially without effect on CCl_4-induced Ca^{2+} release.

In various studies on in vitro injury, CCl_4 was delivered to the medium solubilized in ethanol 70–170 mM[9,12,13,31]. For others, CCl_4 was delivered as 20% solutions in ethanol[10,11], fixing the ethanol concentration instead. Unlike authors of previous reports[9,12,13] we found a release rate of Ca^{2+} which was not proportional to the final CCl_4 concentration, maximal release occurring when 5–10 mM CCl_4 was present. Further increments of CCl_4 were not effective, unlike ethanol, which hints at the existence of an optimal CCl_4/ethanol ratio. Our results suggest that sequential steps are involved in the direct effect of CCl_4 when dissolved in ethanol. Hydrophobic molecules like CCl_4 dissolved in amphiphilic compounds, such as short chain alcohols, could have some structural organization[32] when delivered in an optimal ratio. The results observed could be explained if this organization is partly conserved when the mixture is added to a polar medium like water. The stability of the structures could depend on the relative affinity of the amphiphilic compound towards water and hydrophobic molecules such as halocarbons. Our results concerning Ca^{2+} release favour the existence of more stable structures when ethanol is used. An optimal CCl_4/ethanol ratio is probably necessary to accomplish maximal transfer from the medium to the membrane. The amount of CCl_4 interacting with the membranes is dependent on the concentration of ethanol, as can be shown by the variation in membrane fluidity and the amount of CCl_4 associated with the membrane. Our results show that a better understanding of the interaction between vehicles, hydrophobic compounds and water could help to optimize the experimental conditions, allowing a maximal transfer to active membrane structures.

The relevance of observations of halocarbon-induced direct toxicity to the pathogenesis of liver injury appears limited, although similar changes in microsomal membrane fluidity are observed[33]. The existence of considerable variations in individual susceptibility to ethanol-induced liver damage can be attributed to genetic and dietary factors, but may also be due to interactions with other hepatotoxic agents. Thus, doses of ethanol and of hepatotoxic agents that by themselves do not produce hepatoxicity could become dangerous for the liver. The concentrations of ethanol and CCl_4 used in this work were comparable to the blood ethanol levels reported in man during drinking sprees[34] and to the peak hepatic CCl_4 concentration achieved in vivo when 2.0–2.5 ml/kg is administered to rats intragastrically[14–16].

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