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EFFECTS OF CALCIUM CHANNEL BLOCKING AGENTS ON CALCIUM AND CENTRILOBULAR NECROSIS IN THE LIVER OF RATS TREATED WITH HEPATOTOXIC AGENTS

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Abstract—Carbon tetrachloride, chloroform, dimethylnitrosamine, thioacetamide or acetaminophen was each administered to rats in a single hepatotoxic dose. Nifedipine, verapamil or chlorpromazine was administered in association with the hepatotoxic agents to determine if calcium channel blocking agents would prevent an increase in liver cell calcium associated with hepatotoxicity and to determine if these agents would protect against the development of centrilobular necrosis. Following a latent period different for each toxic agent, a 4- to 18-fold increase in liver cell calcium content had occurred by 24 hr. The calcium increase and the centrilobular necrosis (mean histologic score) were correlated. A relatively high calcium to necrosis ratio was obtained with dimethylnitrosamine, thioacetamide and acetaminophen. A lesser calcium to necrosis ratio was obtained with chloroform and carbon tetrachloride, the two toxic agents that destroyed the intracellular calcium sequestration activity of the liver endoplasmic reticulum. Nifedipine or chlorpromazine, administered prior to and 7 hr after the toxic agent, completely prevented the centrilobular necrosis caused by thioacetamide, carbon tetrachloride and acetaminophen; almost completely prevented necrosis with dimethylnitrosamine; and provided partial protection against chloroform toxicity. Two doses of verapamil provided partial protection against necrosis when carbon tetrachloride was the toxic agent and provided almost complete protection with dimethylnitrosamine. A reduction in liver cell calcium was associated with the protective action of the three calcium channel blocking agents. These findings are compared with earlier studies of the protective effects of calcium channel blocking agents in cardiac ischemia.

The administration of an acute toxic dose of carbon tetrachloride to the rat is associated with large increases in the calcium content of the rat liver 24 hr later [1–4]. This increase is completely reversible when a nonlethal dose of carbon tetrachloride is employed [2] and has been demonstrated by both chemical and histochemical means [4]. A measurable increase in calcium content of the liver has also been found following the administration of galactosamine [5] and thioacetamide [6, 7]. The rise in liver cell calcium content conceivably may contribute to the cell injury process associated with toxic agents or it may be a relatively late consequence of cell injury, one that is secondary to the failure of cell membrane function and the already lethal impairment of the cell.

A number of pharmacologic agents have the ability to impede calcium channels in cardiac and smooth muscles [8–11]. Binding studies with nitrendipine further suggest that similar channels may be present in a large variety of tissues [12–15]. In a recent study [1], three calcium channel blocking agents, nifedipine, chlorpromazine or verapamil, were administered to rats in conjunction with carbon tetrachloride. With all three compounds there was a considerable decrease in the liver calcium accumulation and in the centrilobular necrosis associated with the administration of carbon tetrachloride.

Carbon tetrachloride and many other hepatotoxic agents are metabolized by the liver endoplasmic reticulum to reactive intermediates. These intermediates interact with components of the liver cell and, in turn, initiate a train of events leading to cell injury manifested, in part, by varying degrees of centrilobular necrosis. In the present study, several of these hepatotoxic agents (dimethylnitrosamine, thioacetamide, chloroform and acetaminophen) were investigated and were compared with carbon tetrachloride. The objective of this investigation was to determine if an increase in liver calcium content associated with the onset of centrilobular necrosis is a common phenomenon with hepatotoxic agents and if nifedipine, chlorpromazine and verapamil would prevent an increase in the liver cell calcium content and also serve to protect against the onset of centrilobular necrosis.

In the present study, all five of the hepatotoxic agents were associated with a large increase in liver cell calcium content 24 hr after administration of the toxic agent. All five agents produced a centrilobular hepatic necrosis in the rat. The calcium increase and the centrilobular necrosis were considerably reduced in the rats receiving the calcium channel blocking agents. This protective effect of calcium channel blocking agents resembled the protective effects of these agents in experimental ischemia in cardiac tissue.

MATERIALS AND METHODS

The rats used in these experiments were male Sprague–Dawley (200–400 g) from Sasco, Inc. (St. Louis, MO). Carbon tetrachloride was obtained

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from Fisher Scientific (Fair Lawn, NJ). Chlorpromazine HCl, thioacetamide, acetaminophen and D-sorbitol were purchased from the Sigma Chemical Co. (St. Louis, MO). Dimethylnitrosamine was purchased from the Aldrich Chemical Co. (Milwaukee, WI). Nifedipine and verapamil HCl were gifts from Pfizer, Inc. (Brooklyn, NY) and nitrendipine was a gift from Miles Laboratory, Inc. (New Haven, CT). $^{45}CaCl_2$ (8 mCi/mg calcium) and aquasol employed in radioactive assay were obtained from the New England Nuclear Corp. (Boston, MA). Sucrose used in fractionation procedures was ultrapure (ribonuclease free) grade from Schwarz/Mann (Spring Valley, NY).

Drug administration. Carbon tetrachloride, dimethylnitrosamine or acetaminophen was administered by stomach tube at a dose of 26 mmoles/kg. Acetaminophen was dissolved in 5% D-sorbitol. The chloroform was administered to rats pretreated with phenobarbital i.p. 80 mg/kg daily for 3 days. The chloroform was administered i.p. at a dose of 3.7 mmoles/kg. Thioacetamide was administered i.p. at a dose of 8.6 mmoles/kg. Chorpromazine HCl, verapamil HCl or nifedipine was given as an intraperitoneal injection at 25 mg/kg rat weight using a 25 mg/ml solution in deionized water or ethanol (nifedipine only). Calcium antagonists were injected 1 hr prior to the toxic agent, but other schedules were also used as indicated.

Preparation of livers. At different intervals animals were decapitated. Livers were perfused *in situ* with 20 ml of isotonic sucrose before removal to a dish of ice-cold isotonic sucrose. An 8.0 g aliquot of the liver was homogenized with 20 ml of isotonic sucrose in a size C glass homogenizing vessel with a Teflon pestle using 10–12 strokes at 1000 rpm (on ice). The homogenate was filtered through four layers of gauze and was centrifuged at 800 rpm (100 *g*) for 10 min. The homogenate supernatant fraction was then fractionated by differential centrifugation.

Fractionation procedure. All fractions and reagents were kept at 0–4°. Low speed spins were performed in a refrigerated Sorvall RC-2 centrifuge using an SS-34 rotor. The 105,000 *g* spin was performed in a Beckman L2-65 ultracentrifuge using a Ti-50 rotor.

The homogenate supernatant fraction was centrifuged at 2500 rpm (755 *g*) for 10 min to produce a combined nuclear and heavy mitochondrial fraction identified as P_1. The P_1 supernatant fraction was centrifuged at 10,000 rpm (12,100 *g*) for 10 min. The light mitochondrial pellet was designated P_2. The P_2 supernatant fraction was centrifuged at 40,000 rpm (105,000 *g*) to produce the microsomal fraction.

Calcium content. Calcium content of rat liver homogenates and subcellular fractions was determined by the method of Moore *et al.* [2]. Homogenates or fractions were mixed 1:1 with 16% trichloroacetic acid and were centrifuged at 27,000 *g* for 10 min. The supernatant fractions were made basic with 1.25 N KOH. The alkaline supernatant fractions were titrated for calcium with 0.1 mM EDTA using calcein (Fisher Scientific) as an end point indicator.

Microsomal calcium uptake. Calcium uptake was measured in the following medium: 100 mM KCl, 30 mM imidazole-histidine buffer (pH 6.8), 5 mM sodium azide, 5 mM $MgCl_2$, 5 mM ATP (pH adjusted with imidazole to 6.8), 20 μM $CaCl_2$, and 0.1 μCi/ml of $^{45}CaCl_2$ in a total of 3 ml. The assay was initiated at 37° by the addition of the subcellular fraction at a concentration of 0.075 to 0.15 mg protein/ml. Aliquots of 500 μl of the incubation mixture were removed at selected time intervals for filtration through 0.45 μm membrane filters (Millipore Corp.). The filters had been prepared with a wash of 0.25 M KCl (2 ml) followed by water (10 ml). Samples were filtered with the aid of a vacuum apparatus and were washed with 0.25 M sucrose (2 ml). Filters were dried and $^{45}Ca^{2+}$ was determined by liquid scintillation spectrophotometry.

Other assays. Protein was estimated according to the method of Lowry *et al.* [16] using bovine serum albumin as standard. Cytochrome *c* oxidase was determined by following the oxidation of cytochrome *c* at 550 nm in the manner described by Beaufay *et al.* [17]. Choline phosphotransferase was assayed using the method of Wuytack *et al.* [18]. For histologic estimates of cell damage caused by toxic agents, liver lobes were fixed in formalin, sectioned and stained with hematoxylin and eosin. Necrosis was scored 1+ to 4+ according to the outward spread from the central vein. The statistical methods employed in evaluating the data of this study are described in Ref. 19. Values for P were determined from Student's *t*-test and Duncan's multiple range test. Values of P for the linear regression studies were determined from the mathematical relation between *t* and *r*.

RESULTS

Administration of the five hepatotoxic agents led to an increase in the calcium content of the rat liver and this increase paralleled the development of centrilobular necrosis. The administration of nifedipine, verapamil or chlorpromazine served to prevent or to reduce both the calcium change and the development of centrilobular necrosis.

Carbon tetrachloride, the initial hepatotoxic agent employed in this study, had been chosen because of its previously established effects on liver calcium content [1–4]. The four additional hepatotoxic agents in the present study were selected because they cause a centrilobular necrosis and, like carbon tetrachloride, they are metabolized to reactive toxic intermediates by the liver endoplasmic reticulum. The 26 mmoles/kg dose employed with carbon tetrachloride was an appropriate nonlethal toxic dose employed in earlier studies [1–4]. The same dose level was employed in the present study with dimethylnitrosamine and acetaminophen. This dose level was decreased for chloroform and thioacetamide since it represented a lethal dose for these compounds.

The changes in liver calcium content and the development of centrilobular necrosis following a single acute dose of hepatotoxic agent are presented in Fig. 1. The histological score for necrosis describes the zones that are affected. One plus represents the first three cell layers from the central vein, two plus the next three cell layers, and at four plus extends

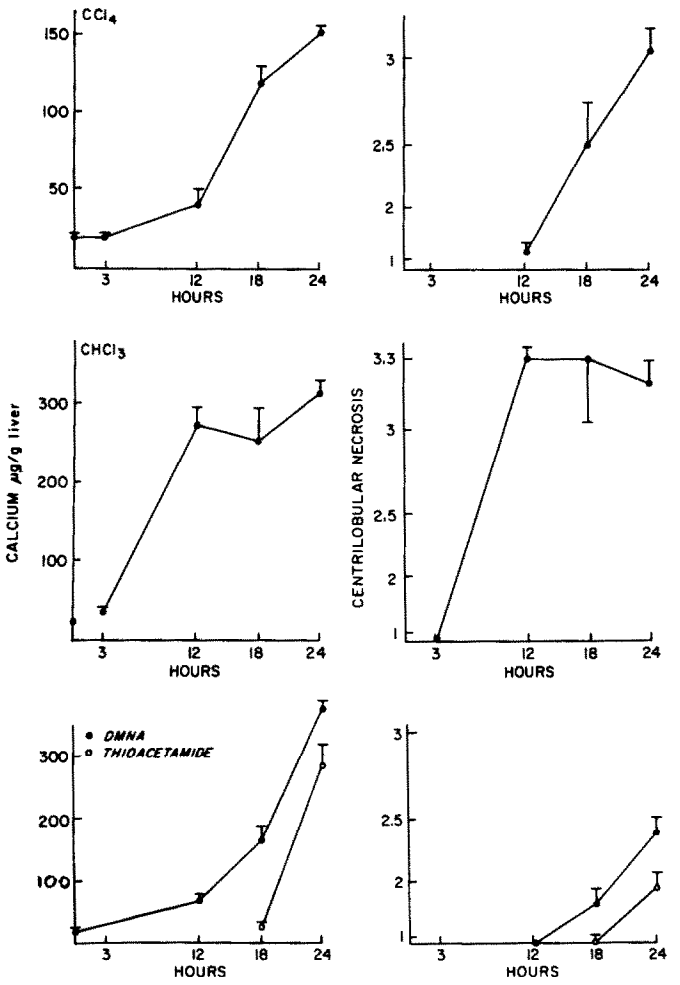

Fig. 1. Graphic time course of the changes in liver cell calcium content and the development of liver cell necrosis following administration of hepatotoxic agents. Calcium content is expressed as μg/g liver. Centrilobular necrosis is expressed as the mean histological score from 0 to 4+. Necrosis is plotted on an expanded scale representing the cubic function of the histological score. Dosage: CCl_4, 26 mmoles/kg; $CHCl_3$, 3.7 mmoles/kg; dimethylnitrosamine, 26 mmoles/kg; and thioacetamide, 8.6 mmoles/kg.

to the outermost cell layers of the lobule. Cells extend out in three dimensions and cell involvement would roughly approximate a cubic function of the linear histological score.

The changes in liver calcium content and liver cell necrosis were temporally associated (Fig. 1). With chloroform, a large increase in both calcium content and necrosis occurred between 3 and 12 hr after administration of the toxic agent. With carbon tetrachloride and dimethylnitrosamine this increase began between 12 and 18 hr after administration of the toxic agent. With thioacetamide and acetaminophen this increase occurred between 18 and 24 hr after administration of the toxic agent.

Normally, a part of the intracellular liver calcium is sequestered in the endoplasmic reticulum [20, 21]. Two of the hepatotoxic agents examined, carbon tetrachloride and chloroform, destroyed the calcium sequestering activity of the liver endoplasmic reticulum (Table 1). These two toxic agents were evaluated separately from the other three.

When the centrilobular necrosis, measured 3, 12 and 18 hr after administration of CCl_4 and $CHCl_3$, is presented as a cubic function of the histological score, liver cell necrosis correlates linearly with calcium content (Fig. 2). Liver cell necrosis at 12, 18 and 24 hr after administration of the other three hepatotoxic agents also was correlated linearly with calcium content. This second linear regression line is steeper (Fig. 2). With both regression lines some increase in the calcium occurred before there was any necrosis.

Calcium and necrosis were followed for 72 hr with carbon tetrachloride. At 72 hr the calcium level had returned to normal but there remained a residual necrosis with a mean score of 1.4 ± 0.18.

When the calcium channel blocking agents nifedipine, verapamil or chlorpromazine were administered prior to the toxic agent in a single dose or in two doses prior to and 7 hr after the toxic agent, there was a considerable reduction in both the calcium content and necrosis (Fig. 3).

When administered 1 hr before and 7 hr after the hepatotoxic agent (Tables 2 and 3), essentially com-

Table 1. Hepatotoxic agents and Mg^{2+}-ATP-dependent calcium sequestering activity of rat liver microsomes

	Calcium uptake (nmoles/mg protein/30 min)
Control	148 ± 10.3 (13)
Carbon tetrachloride, 24 hr	20.3 ± 4 (5)
Chloroform, 12 hr	31 ± 6 (6)
Dimethylnitrosamine, 24 hr	124 ± 5 (6)
Acetaminophen, 24 hr	132 ± 6 (4)
Thioacetamide, 24 hr	130 ± 12 (4)

The toxic agents were administered in a single dose to the rats at the dosage levels described in the text. At 12 or 24 hr the animals were killed and liver microsomes were prepared. Mg^{2+}-ATP-dependent calcium uptake was measured as described in the text. Values are means ± S.E.; the numbers in parentheses indicate the number of experiments.

plete protection was obtained with nifedipine or chlorpromazine against necrosis measured at 24 hr for carbon tetrachloride, thioacetamide and acetaminophen. More than 90% reduction was obtained in the centrilobular necrosis caused by dimethylnitrosamine (Table 3) when nifedipine, verapamil or

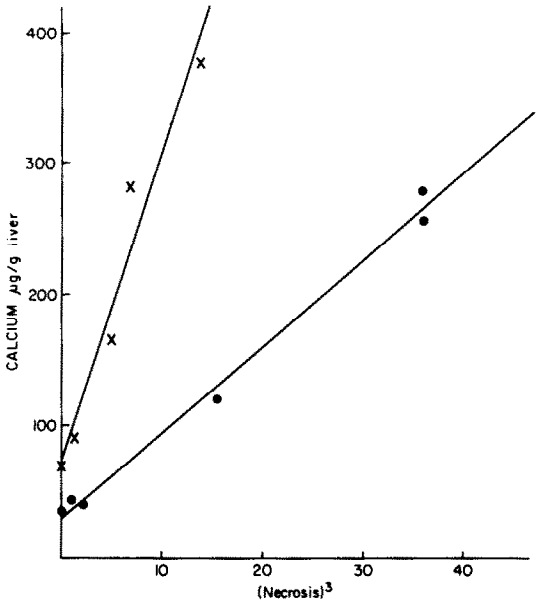

Fig. 2. Linear regression of liver cell calcium (y axis) plotted against the cubic function of the mean histologic score for centrilobular necrosis (x axis) following the administration of hepatotoxic agents. Key: (●) $CHCl_3$ at 3, 12 and 18 hr, CCl_4 at 12 and 18 hr and the low dose of CCl_4 at 24 hr; and (×) dimethylnitrosamine at 12, 18 and 24 hr, thioacetamide at 24 hr and acetaminophen at 24 hr. The r value for $CHCl_3$ and CCl_4 is 0.996 ($P < 0.01$) and the r value for dimethylnitrosamine, thioacetamide and acetaminophen is 0.973 ($P < 0.01$).

Table 2. Effects of carbon tetrachloride, chloroform and calcium channel blockers (25 mg/kg) on calcium content and centrilobular necrosis

	Calcium (µg/g liver)	Centrilobular necrosis (mean score)
24 hr Carbon tetrachloride		
Control	156 ± 5 (39)	3.05 ± 0.1 (20)
Nifedipine		
−1 hr	112 ± 13 (5)	2.3 ± 0.3 (6)
−1 and 7 hr	39 ± 1.7 (12)	0(3)
Nitrendipine		
−1 hr		1.3 ± 0.25 (7)
−1 and 7 hr		0.5(2)
Verapamil		
−1 hr	102 ± 17 (10)	2.3 ± 0.7 (7)
−1 and 7 hr	71 ± 3.6 (7)	1.9 ± 0.1 (10)
Chlorpromazine		
−1 hr	109 ± 8 (24)	0.75 ± 0.4 (4)
−1 and 7 hr	81 ± 18 (10)	0(3)
24 hr Chloroform		
Control	316 ± 14 (13)	3.2 ± 0.1 (10)
Nifedipine		
−1 and 7 hr	95 ± 31 (5)	2 ± 0.05 (9)
Chlorpromazine		
−1 and 7 hr	91 ± 18 (7)	2.1 ± 0.3 (8)

The experimental values, with the exception of verapamil (−1 hr:) necrosis, are significantly different ($P < 0.01$) from their respective controls. Values are means ± S.E.; the numbers in parentheses indicate the number of experiments.

Table 3. Effects of dimethylnitrosamine, thioacetamide, acetaminophen and calcium channel blockers (25 mg/kg) on calcium content and centrilobular necrosis

	Calcium (μg/g liver)	Centrilobular necrosis (mean score)
24 hr Dimethylnitrosamine		
Control	376 ± 15 (29)	2.4 ± 0.13 (24)
Nifedipine −1 and 7 hr	166 ± 7 (5)	1.1 ± 0.4 (8)
Verapamil −1 and 7 hr	190 ± 9 (9)	1.0 (9)
Chlorpromazine −1 and 7 hr	97 ± 16 (8)	0.6 ± 0.2 (8)
24 hr Thioacetamide		
Control	285 ± 36 (11)	1.9 ± 0.2 (13)
Nifedipine −1 and 7 hr	32 ± 4 (7)	0.28 ± 0.18 (7)
Chlorpromazine −1 and 7 hr	33 ± 2 (9)	0(9)
24 hr Acetaminophen		
Control	90 ± 15 (11)	1.1 ± 0.25 (12)
Nifedipine −1 and 7 hr	23 ± 1.8 (4)	0(4)
Chlorpromazine −1 and 7 hr	21 ± 0.6 (2)	0(2)

The experimental values are significantly different ($P < 0.01$) from their respective controls. Values are means ± S.E.; the numbers in parentheses indicate the number of experiments.

Table 4. Effects of hepatotoxic agents on distribution of calcium, cytochrome *c* oxidase and choline phosphotransferase activity in liver cell particulate fractions

	Calcium		Cytochrome *c* oxidase (%)	Choline phospho-transferase (%)
	(μg/g liver)	(%)		
Control				
P_1	14 ± 0.65 (11)	65 ± 3	70 ± 3 (14)	38 ± 1.6 (11)
P_2	3.9 ± 0.2 (11)	18 ± 1	25 ± 2 (14)	20 ± 1.2 (11)
P_3	3.7 ± 0.4 (11)	17 ± 1.7	5 ± 0.2 (14)	42 ± 1.6 (11)
Dimethylnitrosamine				
P_1	271 ± 7 (8)	75 ± 2		
P_2	83 ± 7 (8)	23 ± 2		
P_3	7 ± 1.7 (8)	2 ± 0.5		
Thioacetamide				
P_1	196 ± 5 (8)	71 ± 2		
P_2	72 ± 5 (8)	26 ± 2		
P_3	8 ± 0.3 (8)	3 ± 0.2		
Carbon tetrachloride				
P_1	124 ± 11 (8)	83 ± 7	72 ± 4 (15)	
P_2	21 ± 2 (8)	14 ± 1	23 ± 2 (15)	
P_3	5 ± 2 (8)	3 ± 1.2	5 ± 0.5 (15)	

Values are means ± S.E.; the numbers in parentheses indicate the number of experiments. Measurements were made 24 hr after the administration of the hepatotoxic agents.

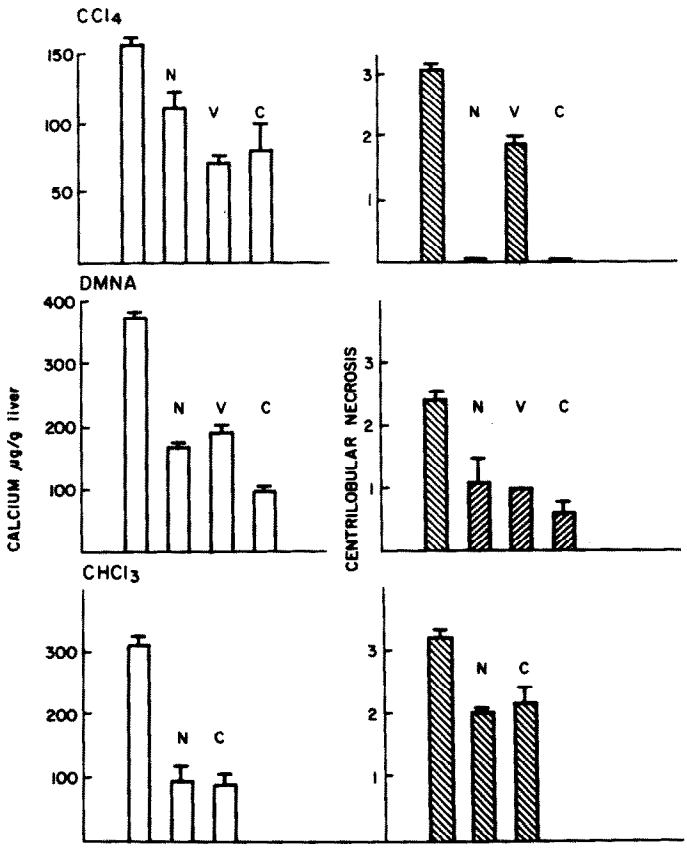

Fig. 3. Protective effect of calcium channel blocking agents on the liver cell calcium content and the mean histologic score of centrilobular necrosis 24 hr after the administration of hepatotoxic agents. Calcium channel blocking agents were administered 1 hr before and 7 hr after the hepatotoxic agent. N is nifedipine, V is verapamil and C is chlorpromazine. The specific values and their standard errors are to be found in Tables 3 and 4. Because the necrosis extends in three dimensions, the percent of protection is determined from the cubic function of the histological score. Partial protection was greater than it appears on the chart.

chlorpromazine was employed. Partial protection was obtained with nifedipine and chlorpromazine against the chloroform-induced centrilobular necrosis (Table 2). Verapamil provided partial protection when it was tested with carbon tetrachloride. Nitrendipine, a dihydropyridine analogue of nifedipine, was tested with carbon tetrachloride, and it greatly reduced centrilobular necrosis. When administered as a single dose, it appeared to be more effective than nifedipine (Table 2). Most of the calcium and necrosis data that were obtained when channel blockers and carbon tetrachloride or chloroform were administered coincided with the linear regression slope obtained in the absence of a channel blocking agent (Fig. 4).

In several instances the protection against centrilobular necrosis was greater than that anticipated from the observed calcium reduction and the linear regression slope. This included the effect of one or two doses of chlorpromazine with carbon tetrachloride where protection against cellular necrosis was complete and the reduction in calcium was incomplete (Table 2). The two doses of nifedipine and verapamil reduced necrosis more than the calcium when dimethylnitrosamine was the toxic agent (Table 3). No increase in calcium occurred and no necrosis occurred when nifedipine or chlorpromazine was administered along with either thioacetamide or acetaminophen (Table 3).

In the control livers of the present study, the microsomal fraction contained 5% of the mitochondrial marker activity, 42% of the endoplasmic reticulum marker activity, and 17% of the particulate calcium (Table 4). Algebraic estimate from these data indicated that 33% of liver cell particulate calcium was apparently localized in the endoplasmic reticulum and 67% was localized in the mitochondria. This was in agreement with an earlier report [20].

The high levels of liver calcium 24 hr after administration of the hepatotoxic agents appeared to be associated with the liver mitochondria. Table 4 shows the distribution of the calcium in three particulate fractions: P_1, nuclear + heavy mitochondrial; P_2, light mitochondrial; and P_3, microsomal. The distribution of calcium 24 hr after administering the hepatotoxic agents paralleled the distribution of the cytochrome *c* oxidase mitochondrial enzyme marker. Although subcellular redistribution of calcium in the course of the cell fractionation is a potential source of artifact in the data of Table 4, the differences between the toxic agents and the control are very large, and the results are fairly consistent over a diverse range of calcium levels in the liver.

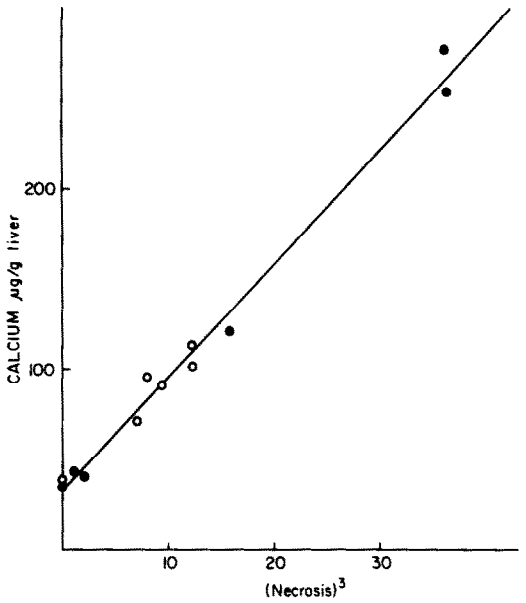

Fig. 4. Linear regression of liver cell calcium (y axis) plotted against the cubic function of the mean histologic score for centrilobular necrosis (x axis) following the administration of hepatotoxic agents and calcium channel blocking agents. Key: (●) $CHCl_3$ at 3, 12 and 18 hr, CCl_4 at 12 and 18 hr and the low dose of CCl_4 at 24 hr; and (○) CCl_4 at 24 hr with nifedipine and verapamil at one or two doses and $CHCl_3$ at 24 hr with two doses of nifedipine or chlorpromazine. The r value is 0.995 ($P < 0.01$). The values of specific data points and their standard errors are to be found in Table 2.

DISCUSSION

Following the administration of hepatotoxic agents, there was a lengthy latent period followed by a progressive increase in liver cell calcium content associated with cell necrosis; at 24 hr after administration, the necrosis was varied, ranging from 1+ with acetaminophen to 3+ with chloroform and carbon tetrachloride. The early administration of several calcium channel blocking agents prevented or reduced both the calcium increase and the cell necrosis. In this study, toxic liver cell injury by a variety of hepatotoxic compounds was prevented by the administration of several chemical agents already in established clinical usage.

Two experimental models are presented to evaluate the findings of the hepatotoxicity study. In the first, it is postulated that the elevation of calcium content is a cause of cell injury. Initially, the entire liver lobule may be attacked by the toxic agent. There is some support for this in the observation that carbon tetrachloride and chloroform almost totally destroyed endoplasmic reticulum calcium sequestration in the liver. All five of the toxic agents in the present study interacted with the liver endoplasmic reticulum. The toxic agents may initially increase calcium in all of the liver cells.

There was a variable lag period before the tissue calcium content rose. This varied from less than 12 hr with chloroform to more than 18 hr with thioacetamide and acetaminophen. The sensitivity to calcium entry would be greatest in the less oxygenated centrilobular zone of the liver. Reduced oxygenation may trigger an ever increasing rate of calcium entry leading to irreversible change. A calcium increase that would occur in the outer zones of the liver lobule would be more readily reversible.

In a second model, the hepatotoxin-induced cell necrosis is primary, and the calcium entry into liver cells is chiefly a consequence of lethal cell injury. This model also fits the close relation observed between necrosis and the increase in liver cell calcium. It is, however, not consistent with the two separate linear regression slopes in Fig. 2. If necrosis alone were to determine the large scale calcium entry, with all of the compounds there would be similar calcium levels for the same degree of necrosis.

Six experimental data points deviate from the linear regression slopes and are assessed in terms of the two experimental models. Twenty-four hours after administration of carbon tetrachloride, the rate of liver cell calcium increase was diminishing and 72 hr after the administration of carbon tetrachloride it was back to normal. The rate of increase of necrosis at 24 hr was not diminishing and there was a residual necrosis present at 72 hr. This does not fit the model where the increase in calcium is secondary to the necrosis. The slow down in the calcium increase with carbon tetrachloride at 24 hr may reflect tissue recovery in the outer zones of the liver lobule. Chlorpromazine completely prevented the carbon tetrachloride induced necrosis but it permitted some increase in cell calcium content. Again this does not fit the model where a calcium increase is secondary to the necrosis. With dimethylnitrosamine, nifedipine and verapamil reduced the necrosis more than it reduced the calcium increase.

It is uncertain from this study whether the tissue calcium rise is uniform throughout the liver lobule or whether there is a gradient distribution. Earlier histologic studies with the electron microscope indicate that, at 24 hr after carbon tetrachloride, thioacetamide and dimethylnitrosamine, the large increase in calcium that can be visualized by histologic staining is associated with the centrilobular zone [22]. The relationship between calcium and zonal necrosis in these studies is a visible one. With carbon tetrachloride, there is also a large but transitory increase in calcium 1 hr after administration which is normal again by 2 hr [23]. Histologic staining shows that this early calcium increase is diffusely distributed throughout the liver lobule [23].

The events in the liver study were strikingly similar to events previously described in cardiac ischemia. The reperfusion of myocardial tissue after prolonged ischemia causes extensive and often irreversible cell damage [24–28]. The ischemia itself is not associated with a large increase in calcium [24, 25]. The reperfusion of the myocardium following ischemia is associated with a rapid massive gain in calcium, which is postulated to be a contributory factor in cell death [24–28]. The administration of verapamil, nifedipine or diltiazem at the beginning of the experimental period minimizes the subsequent increase in calcium and the associated cell damage [25, 29–32]. A similar pattern of events was observed in renal ischemia [33], liver cell ischemia [34, 35] and in heart

tissues perfused with a calcium free medium and then reperfused with a medium containing normal levels of calcium [36]. In the kidney and liver cell ischemia studies, calcium channel blocking agents were employed and cell injury was prevented or reduced [33–35].

The cardiac events are interpreted as a sequence of changes initiated by a dramatic fall in ATP (about 90%) resulting from a period of ischemia [24, 25]. This large fall in ATP reduces ion homeostasis and there is a net gain in both sodium and calcium. The increase in free cytosolic calcium depletes the limited supply of ATP by stimulating ATPase activity associated with ion extrusion pumps. On reperfusion of the ischemic cardiac tissue, mitochondrial substrate oxidation energizes the sequestration of the increased intracellular calcium into the mitochondria. This mitochondrial calcium sequestration occurs in preference to the mitochondrial formation of ATP [24, 25] and further depletes the intracellular ATP.

The appropriate administration of a calcium channel blocking agent early in this sequence reduces a part of the calcium entry, presumably that served by voltage dependent calcium ion channels or by other calcium channels that may prove sensitive to those agents. This calcium reduction at the beginning of ischemia spares the limited supply of ATP in the cell and prevents an initial large decrease of intracellular ATP from escalating into an irreversible series of events.

In the present liver study, it is postulated that metabolic perturbation by the toxic agents also disrupts the cellular mechanisms which serve to maintain a low intracellular cytosol calcium. Increasing uptake of calcium into the liver cell mitochondria progressively diminishes the formation of ATP by the mitochondria. The central zone of the liver lobule which has a lesser oxygen supply is most susceptible to this toxic cellular injury. Calcium channel blocking agents function by reducing the initial early entry of calcium into the liver cells and prevent the spiralling pathologic consequences of an excessive calcium entry on liver cell energy metabolism. Wands *et al.* [37] have shown that an *in vivo* dose of carbon tetrachloride like that employed in this study progressively diminishes rat liver cell ATP content and this is paralleled by a fall in the liver cell membrane potential.

The effective protection from hepatotoxic cell injury by the calcium channel blocking agents is striking. This protective action may stand as strong supporting evidence for the actual participation of calcium in the process of cell injury. The protection is important, in spite of an incomplete understanding of the mechanism. The present findings did not explain why in several instances the reduction of necrosis with calcium channel blocking agents exceeded their effect on liver cell calcium. It may be compatible with a model where there is a modest increase of calcium that is uniform throughout the liver and a much larger increase that is specifically associated with the zonal necrosis area.

Endoplasmic reticulum calcium sequestering activity was virtually absent in the chloroform and carbon tetrachloride treated livers. The early loss of this activity with chloroform and carbon tetrachloride has been described previously [2, 38, 39]. The free cytosol calcium in liver cells is 0.2 μM [20, 40], and the endoplasmic reticulum has the capacity to reduce the cytosol calcium to this level [21]. In the absence of endoplasmic reticulum, the mitochondria have the capacity to maintain the cytosol calcium at about 0.5 μM [21]. With the large calcium increase in toxic liver cell injury, the cytosol calcium must increase. This increase would be greater in the absence of endoplasmic reticulum calcium sequestering activity. This may explain why cell injury associated with the increase of calcium is several times greater following the administration of carbon tetrachloride and chloroform than it is with the other toxic agents. Chloroform induced cell injury was more rapid in onset and somewhat greater than that encountered with the other agents. This may be why only partial rather than complete protection was achieved with nifedipine or chlorpromazine when chloroform was the hepatotoxic agent.

A very important clinical mechanism of action of many calcium channel blocking agents is the dilation of blood vessels which enhances local vascular circulation. Such an action in the liver could enhance the oxygenation of the centrilobular region of the liver lobule that appears to be susceptible to hepatotoxic cell injury. Chlorpromazine protected very well in this experimental liver hepatotoxicity study. At a 20 mg/kg dose level, the chlorpromazine effect on rat blood pressure (and presumably the effect on blood vessels in general) was much less than that of nifedipine and verapamil (unpublished observation).

In studies of isolated cells, the circulatory parameter is absent. In one such study [41], calcium was found to be essential for carbon tetrachloride-induced cell death in cultured hepatocytes. Similarly, calcium was essential for cell death in cultured hepatocytes when other toxic agents were employed [42].

The prevention of cell injury with calcium channel blocking agents may, on further study, be found to have a useful role in such situations as ischemia associated with cardiac surgery, storage of kidneys for transplant, and possibly even cerebral ischemia as well as some types of chemical toxic injury. Conceivably agents that are more specific for liver and kidney may be found within the chemical group designated as calcium channel blocking agents.

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