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PROTECTIVE EFFECT OF VARIOUS CALCIUM ANTAGONISTS AGAINST AN EXPERIMENTALLY INDUCED CALCIUM OVERLOAD IN ISOLATED HEPATOCYTES

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Abstract—The effect of the hepatotoxic substance diamidinothionaphthene (98/202) on cytosolic, mitochondrial and extra-mitochondrial calcium distribution was measured in isolated rat hepatocytes. The drastic disturbance of the intracellular calcium homeostasis caused by this substance (increase of the cytosolic and mitochondrial calcium contents and depletion of extra-mitochondrial calcium stores, which at last lead to cell death) gave rise to an investigation of the possible cytoprotective effect of calcium antagonists of various chemical classes: verapamil, diltiazem, and nifedipine on isolated hepatocytes. Our results show that all three calcium antagonists prevented cell death caused by 98/202. The 98/202-induced increase of cytosolic and mitochondrial calcium content was inhibited by all three calcium antagonists. However, only verapamil was able to inhibit the depletion of extra-mitochondrial calcium stores. Since 98/202-induced cell death occurs only in the presence of extracellular calcium, it is concluded that calcium antagonists are also able to inhibit the influx of extracellular calcium in liver cells, which leads to a calcium overload of the cytosol and mitochondria. The various ways of interfering with the calcium homeostasis of liver cells qualifies the hepatotoxic substance 98/202 as a suitable *in vitro* hepatotoxicity model for testing the hepatoprotective effect of different calcium antagonists.

The essential role of calcium for the maintenance of metabolism and function of liver cells is now well established. In recent years, evidence has increased that the hepatotoxic effects of various drugs are associated with or caused by an overload of calcium in the hepatocytes (for review see Refs. 1–3). Recently the hepatotoxic side effects of various trypanocidal diamidino compounds including pentamidine have been evaluated in our laboratory [4–6]. In order to investigate whether the hepatotoxicity of these substances is due to changes in the cellular calcium homeostasis, we examined the cytosolic, mitochondrial and extramitochondrial calcium content of isolated rat hepatocytes after treatment with the hepatotoxic agent diamidinothionaphthene (code No.: 98/202†).

Since the results of these investigations, which are depicted in this paper, showed a great influence of this substance on cellular calcium distribution, the second part of our investigations concerned the influence of calcium channel blockers (calcium antagonists) of various chemical classes on 98/202 disturbed calcium homeostasis of the liver cell. As calcium fluxes in hepatocytes seem to be, at least partially, regulated by calcium channels [7–12], calcium channel blockers could exert a protective effect on liver cells treated with agents producing a calcium overload. Results of several *in vivo* studies showing that calcium antagonists attenuate the hepatocellular damage after various hepatotoxins confirm this assumption [13–15].

There are only a few *in vitro* studies which show a protective effect of calcium channel blockers against hepatotoxins [8, 16–18]. Systematic comparative studies of the influence of different types of calcium channel blockers on the disturbance of intracellular calcium distribution produced by hepatotoxins have not yet been performed. We have therefore investigated the effect of three calcium antagonists, verapamil (a phenylalkylamine), diltiazem (a benzothiapine) and the 1,4-dihydropyridine nifedipine, on the viability, the calcium content of the cytosol, and on mitochondrial and extramitochondrial calcium stores of isolated hepatocytes after treatment with the hepatotoxic substance 98/202.

MATERIALS AND METHODS

Cells and treatments. Hepatocytes were isolated from male Wistar rats (body weight 220–250 g) as described previously [5]. Isolated cells (1×10^6/mL) were incubated at 37° in MEM-Earle-medium (Biochrom, Berlin, Germany) supplemented with 10 mM Hepes, pH 7.4. Some experiments were performed with a similar medium free of calcium. The diamidino-compound 98/202 and the calcium antagonists verapamil, diltiazem and nifedipine were added directly to the cell suspensions. The

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† Abbreviations: 98/202, diamidinothionaphthene; A23187, calcimycin; CCCP, carbonyl-cyanide-*m*-chlorophenylhydrazone.

Table 1. Effects of 98/202 (0.5 mmol/L) on isolated hepatocytes

	Treatment time (min)	A (Results expressed per 10^6 cells/mL) Extracellular calcium + Control	+ Treated	− Control	− Treated	B Results expressed on remaining living cells/mL Extracellular calcium + Control	+ Treated	− Control	− Treated
Cell viability	2	28 ± 3	27 ± 4	29 ± 5	29 ± 5	–	–	–	–
(% dead cells)	5	25 ± 5	44 ± 9*	22 ± 4	32 ± 5	–	–	–	–
	7	28 ± 5	59 ± 10*	29 ± 3	35 ± 10	–	–	–	–
Phosphorylase	2	15.3 ± 1.7	34.7 ± 3.4*	10.3 ± 1.3	21.9 ± 2.7*	21.2 ± 2.7	47.6 ± 2.9*	21.0 ± 3.0	30.8 ± 2.7*
A activity	5	16.5 ± 2.4	27.1 ± 2.4*	12.1 ± 1.5	18.2 ± 3.5*	16.7 ± 2.4	48.3 ± 3.1*	17.7 ± 1.9	27.5 ± 3.8*
(μmolP$_i$/min/g cells)	7	15.2 ± 1.6	22.1 ± 3.4*	13.1 ± 1.1	15.1 ± 3.2	21.0 ± 1.2	53.9 ± 1.6	17.7 ± 3.2	23.2 ± 2.5
Intracellular calcium stores (nmol Ca/mg protein)									
Mitochondrial	7	3.2 ± 0.2	5.1 ± 0.3*	3.2 ± 0.2	4.5 ± 0.3*	4.4 ± 0.3	12.6 ± 0.3*	4.4 ± 0.2	6.9 ± 0.3*
Extramitochondrial	7	1.5 ± 0.7	ND	1.5 ± 0.2	ND	2.1 ± 0.7	ND	2.1 ± 0.1	ND

Data are mean values ± SD.
* $P < 0.05$ significant versus controls.
ND, not detectable.

Table 2. Effects of 98/202 and calcium anatagonists on cellular respiration of isolated hepatocytes

Substances (mmol/L)	Respiration rate (nmol $O_2/10^6$ cells)	
	Basal	Succinate stimulated
Controls	35.4 ± 7.6	90.5 ± 18.8
98/202		
(0.5)	27.7 ± 11.2*	39.9 ± 12.7*
Verapamil		
(0.05)	40.8 ± 3.5	86.8 ± 3.1
(0.1)	34.5 ± 3.2	83.5 ± 9.1
(0.5)	41.8 ± 5.4	93.4 ± 3.9
Diltiazem		
(0.05)	36.0 ± 3.8	81.2 ± 5.1
(0.1)	33.9 ± 8.9	86.7 ± 9.6
(0.5)	33.0 ± 3.1	85.8 ± 5.1
Nifedipin		
(0.05)	34.8 ± 1.9	85.2 ± 8.9
(0.1)	32.1 ± 4.1	88.5 ± 11.6
(0.5)	34.7 ± 13.8	76.3 ± 10.2
98/202 (0.5) +:		
Verapamil		
(0.05)	19.3 ± 3.5*	37.9 ± 13.1*
(0.1)	18.5 ± 8.3*	51.9 ± 7.3*
(0.5)	26.0 ± 2.4*	45.3 ± 6.3*
Diltiazem		
(0.05)	23.2 ± 3.2*	46.2 ± 4.1*
(0.1)	18.9 ± 4.6*	56.9 ± 12.8*
(0.5)	22.5 ± 6.0*	52.4 ± 8.3*
Nifedipin		
(0.05)	23.8 ± 6.3*	42.8 ± 5.6*
(0.1)	18.1 ± 4.6*	46.8 ± 9.6*
(0.5)	22.4 ± 3.9*	48.7 ± 8.4*

Data are mean values ± SD.
* $P < 0.05$ significant versus controls; incubation time: 3 min.

concentrations used were 0.5 mM for substance 98/202 and 0.05, 0.1 and 0.5 mM for the calcium antagonists. In experiments with the combination of calcium antagonists and 98/202, calcium antagonists were added 10 min prior to 98/202. Aliquots for measuring cell viability, cytosolic and compartmental calcium contents were taken 2, 5 and 7 min after addition of substance 98/202.

Analyses. Cell viability was determined by measuring Trypan blue exclusion. The cytosolic calcium content was estimated by measuring the activity of the calcium dependent phosphorylase according to Hue *et al.* [19], modified by Hoffer and Lowenstein [20], and Long and Moore [21]. This indirect procedure became necessary because of interference of substance 98/202 with fluorescent calcium indicators.

The mitochondrial and extramitochondrial calcium content was determined according to the method of Bellomo *et al.* [22]. In short: calcium from mitochondrial and extramitochondrial stores was released successively by adding in two steps carbonyl-cyanide-*m*-chlorophenyl-hydrazone (CCCP, final concentration 10 μM) and the calcium ionophore calcimycin (A23187, final concentration 10 μM), to the cell suspension. The calcium released successively by CCCP and A23187 was measured spectrophotometrically by recording the absorbance changes at 680 nm resulting from the formation of complexes of calcium with arsenazo III added to the medium at a final concentration of 50 μM.

The effect of 98/202 on the uptake of calcium into mitochondria was measured in cells preincubated with Ruthenium Red (20 μM), an inhibitor of the calcium uniporter of mitochondria. Ruthenium Red was added 30 min before adding the test substance.

Cellular respiration was measured with a Clark oxygen electrode. Substances were added directly into the incubation chamber containing 1×10^6 cells in a final volume of 2 mL buffer as described previously [23]. Succinate in a final concentration of 12.5 mmol/L was used as substrate to stimulate the cellular respiration. Oxygen consumption was measured for 3 min after addition of cells and various test substances and expressed as nmol ⟨O⟩/10^6 cells/min.

Statistics. Mean values ± SD were calculated from 9 to 12 single determinations of four to six different isolations of hepatocytes. Results were analysed statistically by one way analysis of variance or Student's *t*-test, respectively. Differences were regarded as significant if $P < 0.05$.

RESULTS

Effects of 98/202 *(Table* 1)

98/202 decreased cell viability time dependently

in the presence, but not in the absence of extracellular calcium. Intracellular calcium, as measured by increase of phosporylase A activity, was raised significantly as early as 2 min after treatment. This effect was more pronounced in the presence of extracellular calcium. The apparent decrease of phosphorylase A activity after further incubation is deceptive as the activity of the enzyme was related to the whole mass of cells, which also included dead cells. As the number of dead cells increases as a result of 98/202 treatment over time, referring the phosphorylase activity only to the remaining living cells, which retain their enzyme activity, appears more reasonable. Thus it can be shown, that the enzyme activity of the viable cells remained constantly elevated throughout the whole treatment time.

The calcium content of intracellular compartments was also disturbed by substance 98/202. Addition of CCCP and A23187 to untreated cells released 3.2 nmol calcium/mg protein from the mitochondria, i.e. 68% of the total cellular calcium, and 1.5 nmol calcium/mg protein from extramitochondrial compartments. Seven minutes of treatment of the cells with substance 98/202 leads to a significant increase of the mitochondrial calcium content to 4.5 nmol/mg protein in the absence, and to 5.1 nmol calcium/mg protein in the presence of extracellular calcium. Referring the measured calcium content to the number of remaining living cells shows an actual increase to 12.6 nmol calcium/mg protein in the mitochondrial stores in the presence of extracellular calcium and to 6.9 nmol/mg protein in the absence of extracellular calcium. No calcium could be detected in the extramitochondrial calcium stores after 98/202 treatment. Cellular respiration of the hepatocytes 3 min after addition of the toxin was inhibited by 98/202. The basal respiration rate was decreased to 78%, the succinate-stimulated respiration rate to 44% of the control values (Table 2).

Effects of Ruthenium Red on 98/202 *action (Table 3)*

Incubation of the cells with a final concentration of 20 μmol/L Ruthenium Red 30 min prior to 98/202 treatment completely preserved the viability of the cells in the presence of the substance 98/202 (Table 3a). The increase of the mitochondrial calcium content due to substance 98/202 was 60% reduced by Ruthenium Red (Table 3).

Effects of calcium channel blockers on 98/202 *action (Table 4)*

Cell viability. All three calcium channel blockers completely inhibited the 98/202 induced cell cytotoxicity, measured by the ability to exclude Trypan blue from the cells.

Phosphorylase A activity. Likewise, the increase of phosphorylase A activity caused by 98/202 was reduced to control levels by verapamil, nifedipine, and diltiazem. For all substances the lowest concentration (0.05 mmol/L) was the most effective, among the three types of calcium antagonists verapamil was the best protective substance. The effect of diltiazem was slowest in onset.

Calcium content in intracellular calcium stores (Fig. 1). The calcium antagonists diltiazem and nifedipine completely inhibited the increase of the mitochondrial calcium content due to 98/202 in the absence as well as in the presence of extracellular calcium. The depletion of the extramitochondrial calcium store, however, was not prevented by these two antagonists. In contrast, additional treatment of the cells with verapamil, especially at the lowest concentration used (0.05 mmol/L) completely protected the hepatocytes from the increase of mitochondrial calcium content as well as the depletion of extracellular calcium store by 98/202. 98/202 induced inhibition of cellular respiration was not prevented by any of the calcium antagonists (Table 2).

Effects of calcium channel blockers themselves on cell integrity (Table 5)

Cell viability was not influenced by the three calcium antagonists at the concentrations used (0.05, 0.1 and 0.5 mmol/L) within 17 min treatment time (data not shown). The activity of phosphorylase A was raised by all three antagonists, especially after 12 and 15 min, nifedipine showing the most prominent effect by inducing a 3-fold increase above control levels at a concentration of 0.5 mmol/L within 15 min treatment. All three calcium channel blockers led to a significant decrease of the mitochondrial calcium content of hepatocytes. Verapamil had the most consistent effect in the presence, as well as in the absence of extracellular calcium. The effects of nifedipine and diltiazem were more variable. The cellular respiration rate was not changed by all three calcium channel blockers at the concentrations used (Table 2).

DISCUSSION

Calcium is present in liver cells in various compartments, e.g. cytosol, mitochondria and endoplasmic reticulum, and under normal conditions its homeostasis is precisely maintained. It has been well documented during the last few years, that raised levels of the cytosolic and/or mitochondrial calcium contents caused by various drugs are the reason for hepatocellular injury and cell death [2, 24–27].

Our present results show, that the substance 98/202, which proved to be hepatotoxic *in vivo* and *in vitro* in earlier studies from our laboratory [5, 6], exerts its hepatotoxic action through a disturbance of intracellular calcium homeostasis. The drastic increase of mitochondrial calcium content seems to be the main hepatotoxic event. 98/202 treatment also leads to an activation of phosphorylase A indicating an increase of the cytosolic calcium content. Evidently the increased cytosolic calcium content originates from the extracellular space as well as from intracellular non-mitochondrial calcium stores. In the absence of extracellular calcium, however, activation of phosphorylase A is only transient and is not followed by an increased number of dead cells. The origin of this calcium flux seems to be the extramitochondrial calcium stores, which are fully depleted by 98/202. Therefore, the main

Table 3. Influence of Ruthenium Red on cell viability and mitochondrial calcium content of 98/202 (0.5 mmol/L) treated hepatocytes

	Cell viability (% dead cells)		Mitochondrial calcium content (nmol/mg protein)	
	Controls	Treated	Controls	Treated
A				
Ruthenium Red −	27 ± 3	59 ± 10*	3.2 ± 0.2	4.5 ± 0.3*
Ruthenium Red +	25 ± 4	31 ± 3†	2.7 ± 0.2	3.3 ± 0.4†
B				
Ruthenium Red −	−	−	4.4 ± 0.2	6.9 ± 0.3
Ruthenium Red +	−	−	3.8 ± 0.2	4.8 ± 0.3*†

Data are mean values ± SD.
Mitochondrial calcium content was measured in the absence of extracellular calcium.
A: Results expressed per 10^6 cells/mL.
B: Results expressed on remaining living cells/mL.
* $P < 0.05$ significant versus controls.
† $P < 0.05$ significant versus 98/202 treatment.
Incubation time: 98/202: 7 min. Ruthenium Red was added 30 min prior to 98/202.

Table 4. Effects of calcium antagonists on 98/202 action

	Cell viability (% dead cells)			Phosphorylase A activity (μmol P_i/min/g cells)		
Substances (mmol/L)	Treatment time (min)			Treatment time (min)		
	2	5	7	2	5	7
Controls	26 ± 2.3	25 ± 2.2	26 ± 2.2	16.4 ± 1.2	17.0 ± 0.7	17.2 ± 1.0
98/202						
(0.5)	27 ± 4.0	44 ± 9.0*	59 ± 10.0	47.6 ± 3.4*	48.3 ± 1.3*	53.9 ± 1.1*
98/202 (0.5) +:						
Verapamil						
(0.05)	24 ± 1.6	26 ± 3.1†	29 ± 3.2†	17.8 ± 1.4†	16.8 ± 1.4†	17.7 ± 0.8†
(0.1)	26 ± 3.5	29 ± 4.8†	27 ± 3.2†	22.3 ± 1.3†	19.2 ± 0.1†	17.9 ± 1.0†
(0.5)	26 ± 5.0	26 ± 2.0†	30 ± 4.2†	24.6 ± 0.9*†	19.2 ± 2.1†	16.7 ± 1.3†
Diltiazem						
(0.05)	27 ± 2.0	29 ± 2.0†	28 ± 2.5†	24.3 ± 2.1*†	20.0 ± 1.9†	20.6 ± 1.4†
(0.1)	25 ± 2.0	31 ± 1.6†	30 ± 2.4†	27.9 ± 1.4*†	20.7 ± 1.4†	19.8 ± 1.5†
(0.5)	28 ± 1.8	30 ± 1.7†	32 ± 1.8†	36.3 ± 1.3*†	26.0 ± 1.3*†	19.3 ± 2.1†
Nifedipin						
(0.05)	25 ± 4.8	26 ± 2.5†	28 ± 3.3†	21.5 ± 1.8†	18.6 ± 0.8†	15.1 ± 2.0†
(0.1)	25 ± 4.2	30 ± 3.3†	32 ± 3.3†	23.9 ± 3.3†	22.2 ± 1.7†	21.1 ± 1.9†
(0.5)	25 ± 6.6	34 ± 3.3†	37 ± 3.3†	23.8 ± 2.0†	24.5 ± 1.3†	24.1 ± 1.3†

Data are mean values ± SD.
* $P < 0.05$ significant versus controls.
† $P < 0.05$ significant versus 98/202 treatment.

source of the accumulated calcium is obviously the extracellular space, as only in the presence of extracellular calcium 98/202 leads to an increase of phosphorylase A activity, which is markedly above the maximum activity that can be achieved "physiologically" by the α_1-adrenoreceptor agonist phenylephrine (31.8 ± 4.5 μmol P_i/g cells/min). The increased cytosolic calcium is transported into the mitochondria, which leads to a 3-fold increase of mitochondrial calcium content, and is probably also partially extruded from the cell via Ca^{2+}-ATPases.

The mitochondrial calcium accumulation may lead to a decrease of the mitochondrial membrane potential, which in turn is followed by a decrease of the transmembrane proton gradient [1]. Thus, the electrochemical forces needed for ADP-phosphorylation are diminished, which results in a decrease of oxygen consumption. Indeed, an inhibition of cellular respiration rate can be seen after 98/202 treatment. The resulting energy deficiency may then diminish the active extrusion of the excess calcium from mitochondria and cytosol. These processes may lead to mitochondrial damage and ultimatively to cell death [1, 3, 28, 29].

Thus, it appears that 98/202 treatment enhances the influx of extracellular calcium into the cell, either by opening calcium channels or leading to leaks in the cytoplasmic membrane, and depletes the

Table 5. Influence of calcium antagonists on isolated rat hepatocytes

	Phosphorylase A activity (μmol P_i/min/g cells)			Intracellular calcium stores (nmol Ca/mg protein) Extracellular calcium			
	Treatment time (min)			+		−	
				Mitochondrial	Extramito	Mitochondrial	Extramito
				Treatment		time (min)	
	12	15	17	17	17	17	17
Controls	12.6 ± 1.4	14.0 ± 1.7	13.3 ± 1.3	3.2 ± 0.14	1.5 ± 0.18	3.7 ± 0.19	1.7 ± 0.1
Vera (mmol/L)							
0.05	11.2 ± 1.3	14.5 ± 1.6	12.0 ± 2.5	3.1 ± 0.12	1.4 ± 0.21	3.3 ± 0.25	2.0 ± 0.12
0.1	11.9 ± 1.4	11.5 ± 1.3*	12.5 ± 3.1	2.6 ± 0.18*	1.5 ± 0.12	2.6 ± 0.16*	2.2 ± 0.11
0.5	16.6 ± 2.5*	17.6 ± 2.0*	11.2 ± 1.6	2.7 ± 0.10*	1.4 ± 0.19	2.3 ± 0.16*	1.9 ± 0.12
Dil (mmol/L)							
0.05	13.7 ± 1.1	13.5 ± 1.8	10.3 ± 1.6	3.1 ± 0.14	1.4 ± 0.16	3.9 ± 0.15	1.1 ± 0.19
0.1	17.2 ± 1.1*	20.1 ± 1.8*	14.3 ± 0.6	2.7 ± 0.21	1.7 ± 0.19	2.9 ± 0.2	2.0 ± 0.14
0.5	19.7 ± 1.2*	15.6 ± 1.2	14.3 ± 1.2	2.5 ± 0.22	1.5 ± 0.18	3.8 ± 0.14	1.7 ± 0.19
Nif (mmol/L)							
0.05	15.0 ± 1.8	13.0 ± 1.5	13.5 ± 0.9	3.1 ± 0.18	1.5 ± 0.18	3.7 ± 0.18	1.7 ± 0.10
0.1	14.9 ± 1.2	14.9 ± 1.2	14.2 ± 2.3	3.2 ± 0.12	1.6 ± 0.11	3.7 ± 0.09	1.5 ± 0.09
0.5	31.4 ± 3.5*	35.3 ± 2.9*	26.9 ± 1.7*	2.4 ± 0.15	1.4 ± 0.12	2.8 ± 0.08	1.6 ± 0.12

Data are mean values ± SD.
* $P < 0.05$ significant versus controls.
Vera: verapamil; dil: diltiazem; nif: nifedipine.

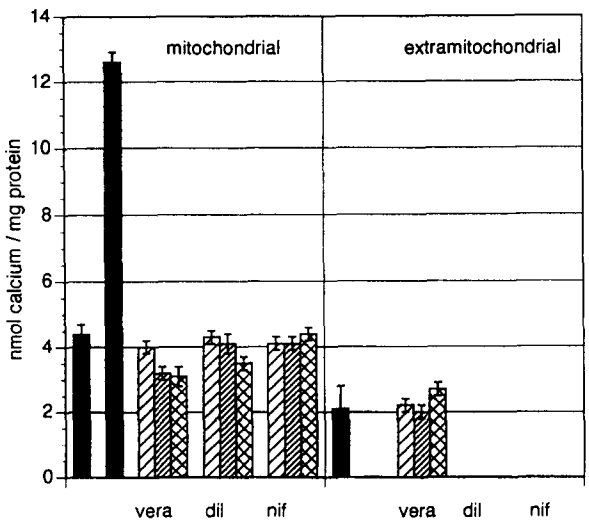

Fig. 1. Effects of calcium antagonists on 98/202 action on intracellular calcium stores. (■) Controls; (▩) 98/202; 98/202 (0.5 mmol/L)+: verapamil (vera), diltiazem (dil and nifedipine (nif) at concentrations: (▨) 0.05, (▨) 0.1 and (■) 0.5 mmol/L. Data are mean values ± SD. Results are expressed on remaining living cells. Incubation time: 98/202, 7 min; calcium antagonists 10 min prior to 98/202.

extramitochondrial calcium stores leading to increased calcium levels in the cytosol and in the mitochondria. Evidently these calcium levels are deleterious to liver cells and are no longer compatible with cell viability. This assumption is confirmed by the results with Ruthenium Red, an inhibitor of this uptake system, which significantly inhibited the 98/202 induced uptake of increased cytosolic calcium into mitochondria. The number of dead cells after 98/202 is significantly decreased after Ruthenium Red treatment, which confirms our hypothesis that the calcium overload of the mitochondria leads to cell death and is an essential factor in 98/202 induced hepatotoxicity.

The findings of the various ways which 98/202 interferes with the maintenance of hepatocellular calcium homeostasis prompted us to investigate if this substance may act as a suitable tool to study the effects of calcium channel blocking agents on calcium fluxes accompanying drug-induced hepatotoxicity. The cytoprotective effect of calcium channel blockers in the liver has been documented *in vivo* by several

authors [8, 13–15]. We now tested comparatively three calcium channel blockers of different chemical groups, the 1.4 dihydropyridine nifedipine, the benzothiapine diltiazem and the phenylalkylamine verapamil on their ability to protect isolated hepatocytes from the cytotoxic effects by substance 98/202 described above.

Our results show that verapamil, diltiazem and nifedipine are equally effective in preventing the cell death caused by substance 98/202. As the increase of cytosolic calcium content after 98/202 treatment is mainly due to the influx of calcium from the extracellular space, it is safe to assume, that all three inhibit the inward movement of calcium across the cytoplasmic membrane of the hepatocytes. It remains to be differentiated, however, whether this is due to a blockade of receptor-operated calcium channels as assumed by Hughes *et al.* [10] or a membrane stabilizing effect [30]. The high concentrations needed in our experiments are in favour of an inhibition of receptor-operated channels, because receptor-operated calcium channels are generally less sensitive than voltage-operated channels to calcium channel blockers, and their inhibition requires concentrations lying in the same order of magnitude as in our experiments [31, 32]. In addition, all three calcium channel blockers prevented the accumulation of calcium in the mitochondria produced by 98/202. This effect seems for once to be a secondary effect as a consequence of the inhibition of the influx of extracellular calcium into the cytosol. This means that either the calcium extrusion through the plasma membrane will now cope with the reduced calcium inflow, so that mitochondria need not take up excess calcium, or the uptake of calcium into the mitochondria is inhibited directly by the calcium channel blockers. The finding that the calcium antagonists themselves, especially verapamil, lowered the mitochondrial calcium content even when the cytosolic calcium content (phosphorylase A activity) was enhanced, means it is likely that the calcium channel blockers can interfere with the mitochondrial calcium uptake system.

The depletion of the extramitochondrial calcium stores by 98/202 is affected differently by the three calcium channel blockers. Only verapamil is able to inhibit the complete depletion of these stores, diltiazem and nifedipine have no effect on this action of substance 98/202. This indicates, that the membranes of these stores, which mainly represent the endoplasmic reticulum [33] contain a calcium export system, which is sensitive only to the phenylalkylamine-type calcium antagonist verapamil and not to the representatives of the two other classes, the dihydropyridine nifedipine or the benzothiapine diltiazem.

It is evident that, in spite of the general importance of the endoplasmic reticulum for various cell functions, e.g. protein synthesis [34], the effect of verapamil on these stores is not essential for its overall cytoprotective potency, so that interference with the calcium sequestration of the endoplasmic reticulum seems not to be the main effect of the acute hepatotoxicity of substance 98/202. These findings are in agreement with reports of Nicotera *et al.* [1] with the antioxidant 2,5-di-tert-butyl-1,4-benzohydroquinone. Nevertheless, the integrity of the endoplasmic reticulum and its calcium sequestering capacity may be important for the maintenance of cell functions depending on intracellular signal transduction. In this way verapamil is superior to nifedipine and diltiazem in normalizing the intracellular calcium distribution.

The inhibition of cellular respiration of 98/202 is not affected by all three calcium antagonists. Thus, it seems to be the consequence of a direct interference of 98/202 with the respiration chain of the mitochondria as already seen for other diamidine compounds [5, 35]. This effect is, however, less pronounced than that produced by other diamidines and obviously is not sufficient to cause cell death on its own. It appears that, as described above, the additional calcium overload caused by 98/202 seems to contribute essentially to the breakdown of mitochondrial function.

In conclusion, the various ways of interfering with cellular calcium homeostasis of substance 98/202, e.g. influx of extracellular calcium into the cell, depletion of nonmitochondrial calcium stores, and increase of mitochondrial calcium content, qualifies this substance as a suitable *in vitro* hepatotoxicity model for testing the ability of calcium antagonists of various types to protect isolated hepatocytes from calcium overload and cell death.

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