Physiol. Res. 49: 261-268, 2000

Evaluation of Calcium Channel Blockers as Potential Hepatoprotective Agents in Oxidative Stress Injury of Perfused Hepatocytes

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Received June 15, 1999
Accepted August 5, 1999

Summary

The aim of this study was to investigate the effects of calcium channel blockers on *tert*butyl hydroperoxide (TBH) induced liver injury using isolated perfused rat hepatocytes. Rat hepatocytes were immobilized in agarose threads and perfused with Williams E medium. Hepatocyte injury was induced by the addition of *tert*butyl hydroperoxide (1 mM) to the perfusion medium 30 min after the addition of either verapamil or diltiazim. Hepatocyte injury was observed by monitoring the functional and metabolic competence of hepatocytes or by ultrastructural morphological examination of hepatocytes. Verapamil (0.5 mM) reduced lactate dehydrogenase leakage in TBH-injured hepatocytes as compared to the controls (154±11 % vs. 247±30 %). Lipid peroxides production was reduced after verapamil pretreatment as compared to the controls and oxygen consumption was increased by pretreatment of hepatocytes with verapamil. Verapamil pretreatment increased the protein synthesis activity at both levels of granular endoplasmic reticulum and free polysomes in cytoplasm and decreased ATPase activity. Diltiazem was qualitatively effective as verapamil. It is concluded that in hepatocyte oxidative injury, calcium channel blockers exhibited hepatoprotective properties. The hepatoprotective effect of calcium channel blockers was accompanied by a decrease in ATPase activity, which may implicate a normalization of Ca^{2+}_i after TBH intoxication.

Key words

Verapamil • Diltiazem • *Tert*butyl hydroperoxide • Hepatoprotection • ATPase

Introduction

It is established that exaggerated calcium influx into cells is an important signal that may lead to cell death. The essential role of calcium for the maintenance of the metabolic competence of cells has been extensively studied. A number of hepatotoxic effects of drugs is associated with an increase in intracellular calcium (Thomas and Reed 1989, Nicotera *et al.* 1992). Even in ischemic cell death, the involvement of triggering factors, which include calcium, has recently been reviewed (Kristián and Siesjö 1998). Some studies suggested that calcium channel blockers (CCB) do possess a protective role against certain types of liver injuries (Lefer and Stahl

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1987, Thurman *at al.*1988, Takei *et al.* 1990, Tokunaga *et al.* 1992).

When the effect of the hepatotoxic substance diamidinothionaphthene (DAMTP) on various hepatocyte calcium compartmentations was examined, the modulatory action of some calcium channel blockers on impaired hepatocyte calcium homeostasis was demonstrated (Sippel *et al.* 1993). It was suggested that CCB prevented cell death as a result of reduction of the cytosolic and mitochondrial calcium content. However, it was concluded in another study that calcium antagonists do not appear to possess a direct protective effect on hepatocyte function or on an overall liver survival after prolonged cold preservation-reperfusion where exaggerated calcium influx into the cells is believed to be one of the main causes of cell death (De Broin *et al.* 1997).

Among several factors, why various investigators have obtained inconsistent results in this area, is the use of *in vitro* or *in vivo* models under conditions which give variable results. Even in clinical trials of some potential hepatoprotective agents in various types of hepatitis, the lack of standardized studies resulted in controversial data. Several *in vitro* models for studying liver physiology, biochemistry, pharmacology and toxicology have been developed. Studies on isolated liver cells offer the advantage of precise control over the sample with accurate quantitative measurements since it is carried out on a known type and number of cells. The recent development of the technique of hepatocyte immobilization, as a nondestructive method for studying the metabolism of hepatocytes, has made it possible to observe a wide array of events under physiological conditions and their course in real time. This efficient perfusion bioreactor model has been used during the present studies, which allows the provision of nutrients to the cells with and without xenobiotics, the removal of cellular waste products, adequate oxygenation and the prevention of artificial cell injury. The system consisting of isolated rat hepatocytes immobilized in a matrix continuously perfused with an appropriate medium has been found to maintain cell viability with excellent metabolic activity for many hours and even days. Perfused immobilized hepatocytes could be used as a liver model to assess the effects of a wide range of chemicals and other liver insults with consistent results.

Our study was undertaken because of the potential usefulness of the above mentioned compounds and the availability of a reliable technique of the hepatocyte bioreactor model. Its general aim was to further investigate the effects of calcium channel blockers on liver injury using isolated rat hepatocytes and to delineate a relationship between the application of these compounds and the modulation of experimentally induced hepatocyte injury.

Methods

Animals

Male Wistar rats (200-250 g b.w.) were used throughout the present study. All rats received humane care and were provided with water and standard laboratory chow *ad libitum*. The study protocol complies with the general guidelines for animal care of the First Faculty of Medicine, Charles University in Prague.

Chemicals

The calcium antagonists verapamil and diltiazem, ethylene glycol-bis (b-aminoethylether) N, N, N?, N?-*tetr*aacetic acid (EGTA), bovine serum albumin (BSA), *tert*butyl hydroperoxide, Williams E cell culture medium (with L-glutamine, without phenol red and sodium bicarbonate), diagnostic kits for lactate dehydrogenase leakage measurement, kits for urea biosynthesis measurements, thiobarbituric acid, 1, 1, 3, 3-tetramethoxypropane (TMP) and trypan blue, all from Sigma (St. Louis, MO) were used. Furthermore sea Plaque agarose (low-temperature gelling agarose, FMC, Rockland, ME) and collagenase (Sevac, Prague) was also employed. All other chemicals of analytical grade and were obtained from standard sources.

Isolation of rat hepatocytes

The isolation of hepatocytes was performed by the standard two-phase perfusion method where collagenase was included in the second phase as described by Berry *et al.* (1991).

The hepatocytes were counted and examined for their viability and those which excluded more than 90 % of trypan blue were used.

Immobilization of hepatocytes

Immobilization of hepatocytes was studied in low temperature gelling agars threads and perfusion of the immobilized cells with Williams E medium. The method has been described in detail elsewhere (Farghali and Hynie 1997). Adding of the appropriate drug concentration as mentioned in the Result section began

by the preincubation of immobilized hepatocytes with verapamil or diltiazem. The selected concentrations were based on previously published reports and our preliminary experiments.

Hepatocyte injury

*Tert*butyl hydroperoxide (1-2 mM) was added to the perfusion medium 30 min before the compund of interest (verapamil or diltiazem) for 4 h and the degree of hepatocyte injury was observed as indicated in the Results. This was done by periodical monitoring the functional and metabolic competence of hepatocytes or by their ultrastructural morphological examination in the gel threads at the end of the experiment.

The functional integrity and metabolic competence of hepatocytes

The time course of lactate dehydrogenase (LD) leakage from the immobilized cells into the perfusate after the stabilization and time course of urea synthesis in the perfusate at individual time intervals (as given in the section of the results) till the end of the experiment was measured for each experiment. The oxygen consumption was also assessed periodically by a gas analyzer (ABL 5 Radiometer, Copenhagen) as reported earlier (Farghali and Hynie 1997).

Lipid peroxidation

Lipid peroxides were measured after termination of the experiments. Thiobarbituric acid-reacting substances (malondialdehyde formation, MDA) were determined in the hepatocytes extracted from agarose gel threads by a modification of the method of Ohkawa *et al.* (1979) using 1,1,3,3-tetramethoxypropane as external standard. The level of lipid peroxides was expressed in nmol of malondialdehyde (MDA)/10^6 cells. The values are presented as the means ± S.E.M. The significance of differences was determined using Student's t-test. $P<0.05$ values were considered to be significant.

Histological and electronmicroscopical evaluation of isolated hepatocytes

Samples of hepatocytes immobilized in agarose threads were fixed in a modified Karnovsky's mixture (2 % formaldehyde, 2.5 % glutardialdehyde in 0.08 M sodium cacodylate buffer, pH 7.4) and examined under both light and electron microscopy. The material destined for light microscopy was processed by routine paraffin embedding and sections (8 μm) were stained by hematoxylin-eosin. Samples for electron microscopical examination were treated with 1.33 % OsO_4 in 0.06 M sodium cacodylate buffer (pH 7.2) for another hour and embedded *via* toluene in Epon 812 after dehydration in ethanol. Double-stained ultrathin sections were examined in a JEM 100B electron microscope. ATPase activity was assessed in "tissue chopper" sections (20-40 μm) of agarose threads (embedded in 7 % agar) after one hour fixation in 5 % glutardialdehyde in 0.08 M sodium cacodylate buffer (pH 7.4) according to a modification of the Wachstein and Meisel method (1957) with control experiments for the evaluation of non-specific activity.

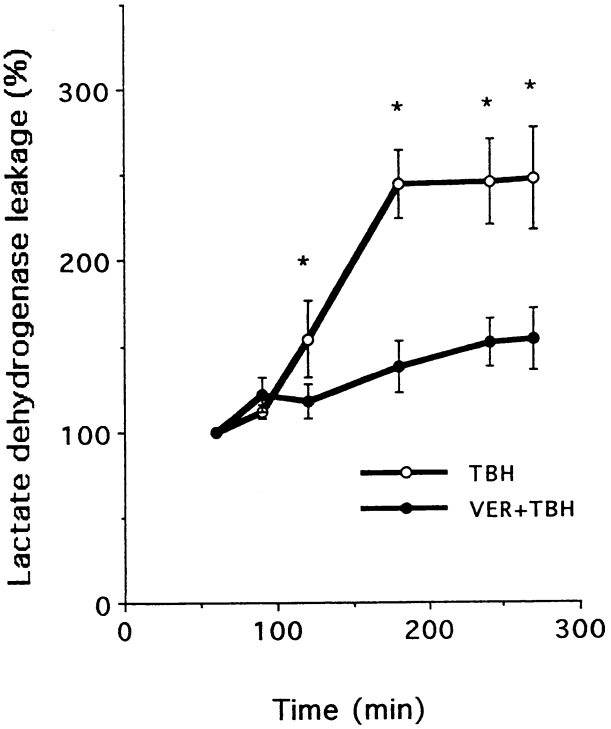

Fig. 1. *The time course of lactate dehydrogenase leakage into the perfusion medium of immobilized hepatocytes treated with TBH alone (control) and those pretreated with verapamil (VER) that was followed by TBH (mean ± S.E.M. n=6-9). The asterisks indicate significant difference (P<0.05).*

Results

At the end of 270 mın lasting perfusion period, verapamil (0.5 mM) reduced lactate dehydrogenase leakage in TBH-injured hepatocytes as compared to the controls (154±11 % vs. 247±30 %) (Fig. 1). The production of lipid peroxides was reduced after verapamil pretreatment from 4.5±0.4 to 2.1±0.15 nmol/10^6 cells. Figure 2 shows that the rate of oxygen consumption was increased at all time intervals when hepatocytes were pretreated with verapamil. Urea synthesis by hepatocytes with both treatments did not differ (data not shown).

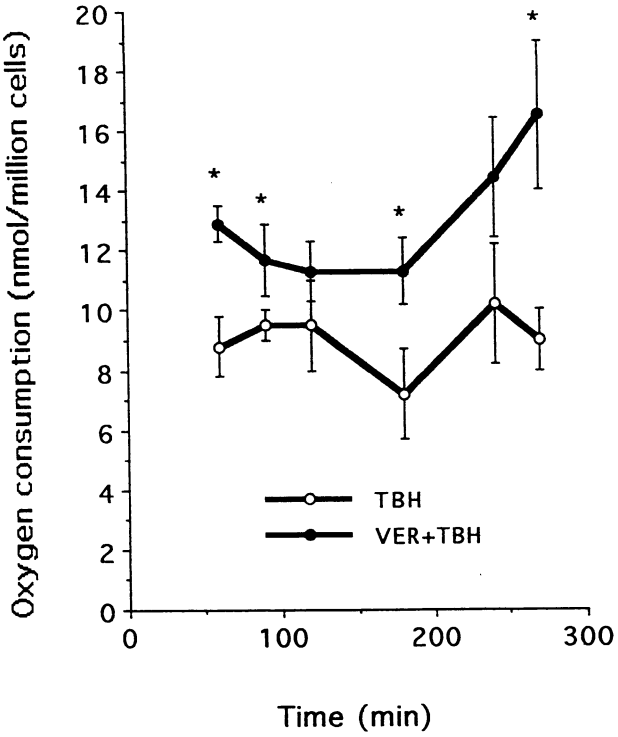

Fig. 2. *The time course of the rate of oxygen consumption by hepatocytes under TBH intoxication (control) and those pretreated with verapamil (VER) that was followed by TBH. (mean ± S.E.M, n=6-9). The asterisks indicate significant difference (P<0.05).*

Verapamil pretreatment increased protein synthesis at both levels of granular endoplasmic reticulum and free polysomes in the cytoplasm (Figs 3 and 4). Electron microscopy revealed a lower ATPase activity due to verapamil pretreatment. Diltiazem was qualitatively as effective as verapamil but differed quantitatively. It reduced the TBH-induced increase in lactate dehydrogenase leakage by hepatocytes in the perfusion medium and increased oxygen consumption as it may be seen in Figures 5 and 6. Lipid peroxides production was reduced after diltiazem pretreatment as compared to the controls (1.9±0.3 vs. 4.5±0.4 nmol/10^6 cells).

Discussion

The aim of the present study was to compare the effects of some putative hepatoprotective agents, namely calcium channel blockers (CCB). Since some publications concerning the pharmacological properties of these compounds have obtained controversial results, a controlled *in vitro* study was performed using the hepatocyte bioreactor model of immobilized and perfused cells.

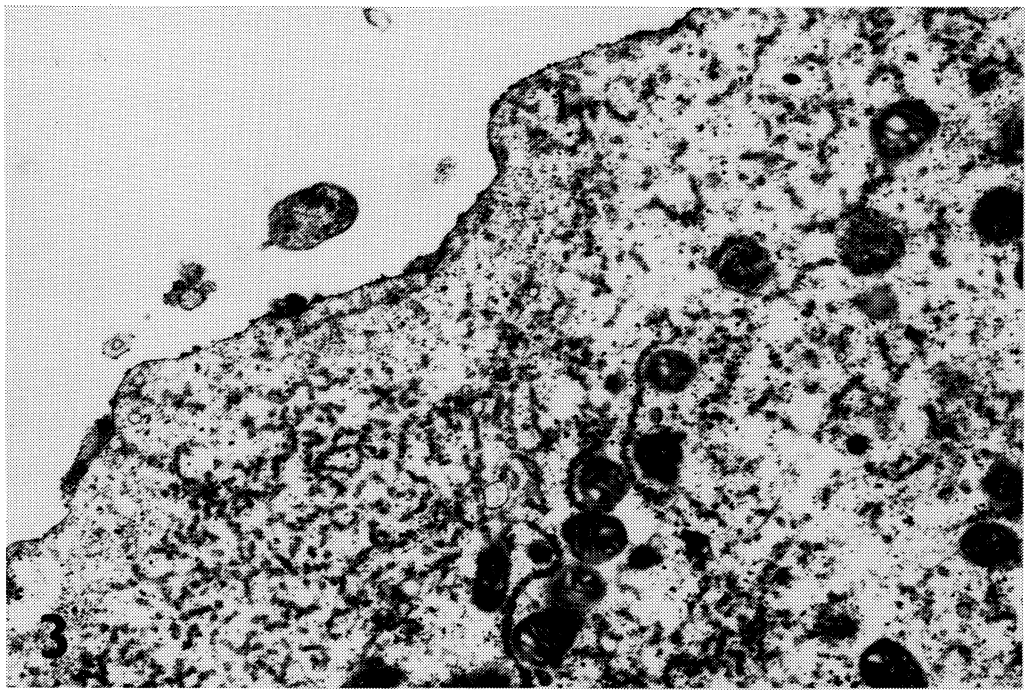

Fig. 3. *A control hepatocyte injured by THP has a substantially lower density of the cytoplasmic matrix. Many rounded electron optically empty regions without any membrane limitation and a reduced number of narrow formations of the granular endoplasmic reticulum can be identified. The mitochondria exhibit a relatively non-altered inner structure. An electron dense reaction product demonstrating the ATPase activity which can be seen at the surface of the cytoplasmic membrane. x 10 600.*

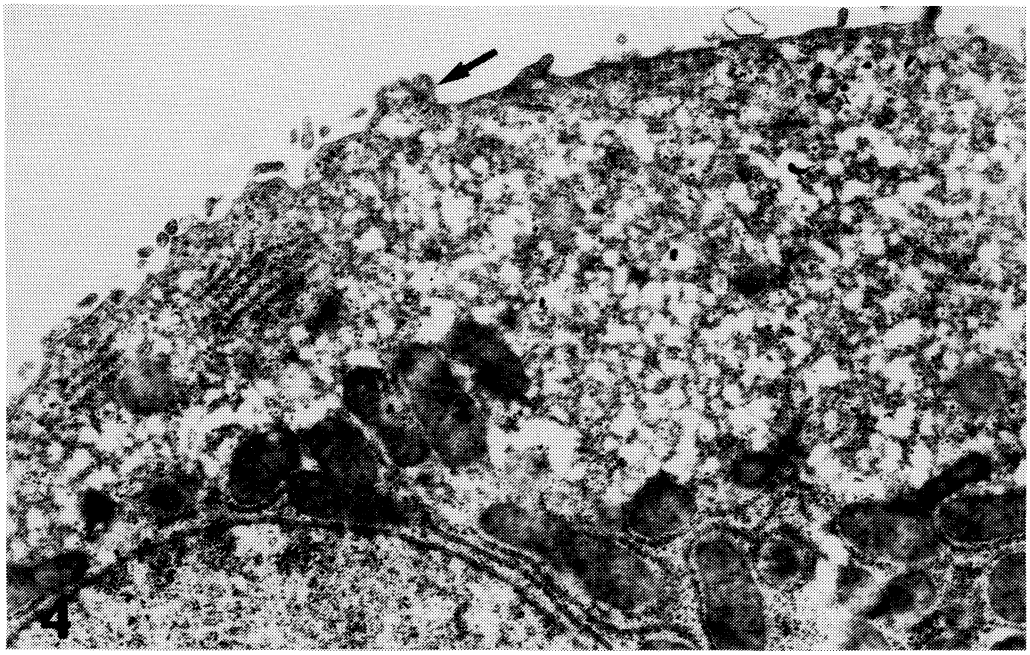

Fig. 4. *Verapamil-pretreated hepatocyte demonstrates a relatively well-preserved structure of the cytoplasm. Smooth endoplasmic reticulum vesicles and sacculi are also visible. In the cytoplasm an accumulation of polysomes and components of the granular endoplasmic reticulum are recognizable. The reaction product of ATPase catalytic histochemical reaction can be rarely found at the cytoplasmic membrane in comparison with control material (arrow). x 11 500.*

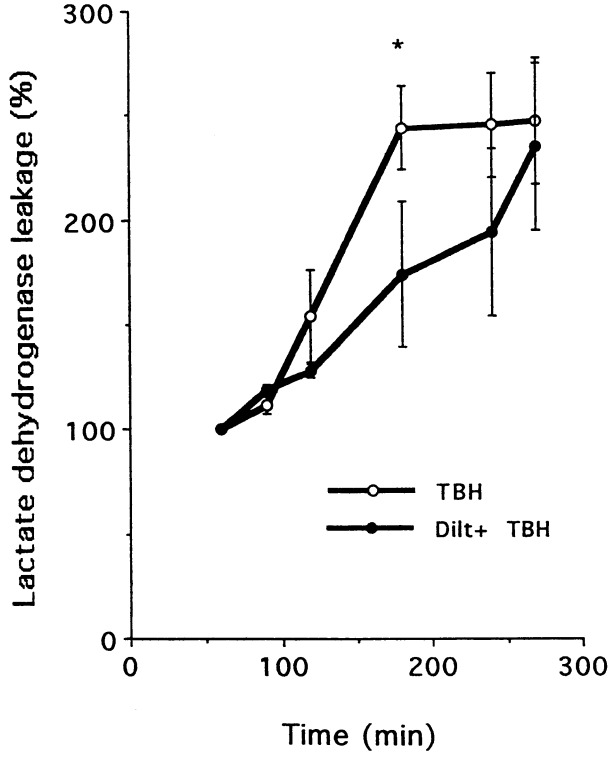

Fig. 5. *The time course of lactate dehydrogenase leakage into the perfusion medium of immobilized hepatocytes treated with TBH alone (control) and those pretreated with diltiazem (Dilt) that was followed by TBH (mean ± S.E.M, n=5-9). The asterisks indicate significant difference (P<0.05).*

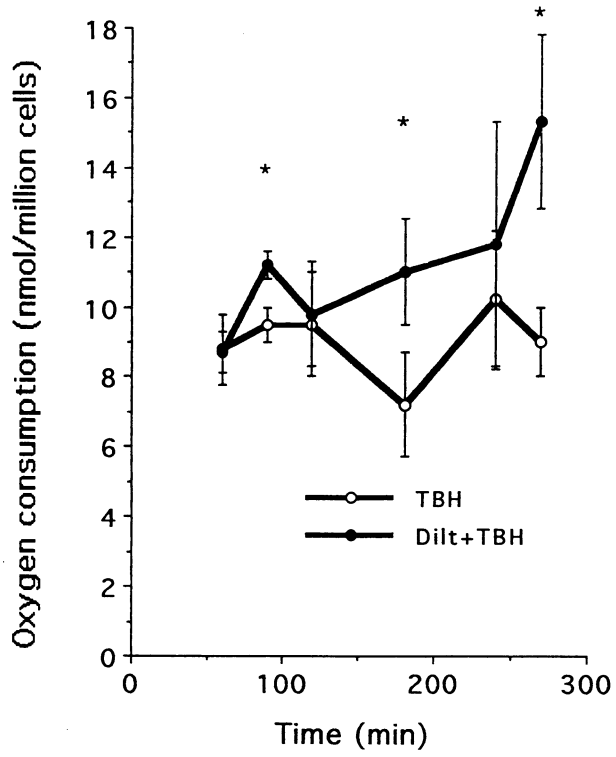

Fig. 6. *The time course of oxygen consumption by hepatocytes treated with TBH (control) and those pretreated with diltiazem (Dilt), which was followed by TBH (means ± S.E.M, n=5-8).*

It is well recognized that a number of hepatocyte injuries are associated with an elevation of intracellular calcium and that calcium signaling plays a pivotal role in the cascade of events that lead to cell injury (Nicotera *et al.* 1992, Gasbarrini *et al.* 1992). A protective effect of calcium channel blockers against hepatotoxins has been reported in some *in vitro* studies (Schanne *et al.* 1979, Deakin *et al.* 1991, Matsuda 1991). These data indicated that both verapamil and diltiazem play a hepatoprotective role in acute TBH intoxication. This hepatoprotective effect of calcium channel blockers in oxidative stress injury is confirmed by a number of observations at the biochemical, physiological, morphological or histochemical levels. The mechanisms responsible for oxidative damage by organic hydroperoxides have been considered to be due to either lipid peroxidation (Kyle *et al.* 1989, Masaki *et al.* 1989, Miguez *et al.* 1994) or alkylation of cellular macromolecules or oxidation of thiol groups in cellular proteins (Furuno and Sugihara 1994). Even the role of lipid peroxidation in the toxicity of organic hydroperoxides has been questioned (Furuno and Sugihara 1994, Rush *et al.* 1985). Whatever the role is played by lipid peroxides in prooxidant cell injury, the degree of lipid peroxidation, which was increased by TBH above levels encountered in normal controls, was significantly reduced by calcium channel blockers. The CCB protective effect was also reflected in preserving the integrity of the plasma membrane. This was demonstrated biochemically by decreased lactate dehydrogenase leakage into the perfusion medium. The elevated oxygen consumption by TBH-treated hepatocytes under CCB pretreatment indicates an improved function of the cell respiratory apparatus. The effect of CCB pretreatment on the energy status of liver cells was not directly investigated in the present work. However, perfused hepatocytes obtained from CCB-pretreated cells did not exhibit significant changes in carrying out urea synthesis from various amino acids precursors in the media. This highly energy demanding process requires four ATP equivalents for the synthesis of one mole of urea (Sierra-Santoyo *et al.* 1994). Indeed, the pretreatment of hepatocytes with calcium channel blockers only slightly increased the rate of urea synthesis in the perfusion medium at the end of the perfusion period. The histochemical findings demonstrate that the amount of the reaction product in ATPase processed samples is decreased due to CCB pretreatment as compared to TBH control treatment. It is well established that the Ca^{2+}-ATPase, known as the calcium pump, which is a prototype of P-class ion pump, maintains a low cytosolic Ca^{2+} concentration. The plasma membrane contains Ca^{2+}-ATPase that extrudes calcium ions from the cytosol into the extracellular medium. The enhanced activity of this enzyme is stimulated by a rise in intracellular calcium (Lodish *et al.* 1995). In contrast to the high membrane ATPase activity achieved by TBH control treatment, the lower activity of membrane ATPase, which was demonstrated in this study after CCB pretreatment in TBH-intoxicated hepatocytes, could serve as indirect evidence of the normalization of intracellular calcium levels by calcium channel blockers. This is supported by the study of Striggo and Bohnensack (1993) who demonstrated that the L-type calcium channel blocker inhibits receptor-operated calcium channels in rat hepatocytes. Moreover, it was reported that TBH induces the formation of reactive oxygen species as was demonstrated by confocal microscopy with the consequent induction of mitochondrial permeability transition (MPT) (Nieminen *et al.* 1997). The role of MPT in apoptosis as a result of the increased intramitochondrial calcium load is now well recognized and MPT is implicated as a critical-rate-limiting event in apoptosis and cell death (Lemasters *et al.* 1998). TBH may alter the pump-leak relationship for calcium across the plasma and intracellular membranes so that a gradual rise in Ca^{2+}_i and a mitochondrial calcium overload occur. Increased Ca^{2+}_i is one of the signals in a cascade of events that may lead to cell death during TBH intoxication and that calcium channel blockers, under the present experimental conditions, maintain Ca^{2+}_i below the threshold concentration.

In conclusion, the present results offer an opportunity to gain new insights as to the potential hepatoprotective action of some calcium channel blockers and their effect on liver pathobiology. In the present study, an approach using a combination of perfused hepatocyte as *in vitro* liver injury model with putative hepatoprotective compounds is likely to be successful in the clarification of beneficial hepatic effects. In hepatocyte injury, namely oxidative stress, calcium channel blockers exhibited hepatoprotective properties. This CCB hepatoprotective effect was accompanied by a decrease in ATPase activity, which may implicate a normalization of Ca^{2+}_i after TBH intoxication.

Acknowledgements

This work was supported by research grants from the Grant Agency of the Czech Republic (307/97/0474), Internal Grant Agency of the Ministry of Health (4180-3) and Charles University (UK 95/96)

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Reprint requests

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