British Journal of Pharmacology (1996) 119, 1519–1524

Inward transport of [^{3}H]-1-methyl-4-phenylpyridinium in rat isolated hepatocytes: putative involvement of a P-glycoprotein transporter

[1]F. Martel, M.J. Martins, C. Hipólito-Reis & *I. Azevedo

Department of Biochemistry and *Institute of Pharmacology and Therapeutics, Faculty of Medicine, 4200 Porto, Portugal

1 The liver has an important role in the detoxification of organic cations from the circulation. [^{3}H]-1-methyl-4-phenylpyridinium ([^{3}H]-MPP$^+$), a low molecular weight organic cation, is efficiently taken up and accumulated by rat hepatocytes through mechanisms partially unknown.

2 The aim of the present work was to characterize further the uptake of MPP$^+$ by rat isolated hepatocytes. The putative interactions of a wide range of drugs, including inhibitors/substrates of P-glycoprotein, were studied.

3 The uptake of MPP$^+$ was investigated in rat freshly isolated hepatocytes (incubated in Krebs-Henseleit medium with 200 nM [^{3}H]-MPP$^+$ for 5 min) and in the rat liver *in situ* (perfused with Krebs-Henseleit/BSA medium with 200 nM [^{3}H]-MPP$^+$ for 30 min). [^{3}H]-MPP$^+$ accumulation in the cells and in tissue was determined by liquid scintillation counting.

4 Verapamil (100 μM), quinidine (100 μM), amiloride (1 mM), (+)-tubocurarine (100 μM), vecuronium (45 μM), bilirubin (200 μM), progesterone (200 μM), daunomycin (100 μM), vinblastine (100 μM), cyclosporin A (100 μM) and cimetidine (100 μM) had a significant inhibitory effect on the accumulation of [^{3}H]-MPP$^+$ in isolated hepatocytes. Tetraethylammonium (100 μM) had no effect.

5 In the rat perfused liver, both cyclosporin A (100 μM) and verapamil (100 μM) had much less marked inhibitory effects as compared to their effects on isolated hepatocytes (0% against 35% and 45% against 96% of inhibition, respectively).

6 Inhibition of alkaline phosphatase activity by increasing or decreasing the pH of the incubation medium or by the presence of vanadate (1 mM) or homoarginine (500 μM) led to a significant increase in the accumulation of [^{3}H]-MPP$^+$ in isolated hepatocytes.

7 It was concluded that, in addition to the type I organic cation hepatic transporter, [^{3}H]-MPP$^+$ is taken up by rat isolated hepatocytes through P-glycoprotein, a canalicular transport system that usually excretes endobiotics and xenobiotics. We proposed that the reversal of transport through P-glycoprotein may be related to the loss of efficiency of alkaline phosphatase in isolated hepatocytes.

Keywords: Rat hepatocytes; organic cations; 1-methyl-4-phenylpyridinium (MPP$^+$); inward transport; P-glycoprotein

Introduction

As charged compounds, organic cations cannot freely diffuse through biological membranes. To be imported into or exported from cells or organisms they use more or less specific membrane-bound transport systems. Detoxification of organic cations from the circulation of complex organisms is usually accomplished by the kidney (Rennick, 1981) and the liver (Klaassen & Watkins, 1984).

1-Methyl-4-phenylpyridinium (MPP$^+$), a low molecular weight organic cation and the neurotoxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), is efficiently taken up and accumulated by rat hepatocytes (Di Monte *et al.*, 1987; Singh *et al.*, 1988; Martel *et al.*, 1995). However, the mechanisms involved in the hepatic uptake of MPP$^+$ remain partially unknown. Recently, Martel *et al.* (1996b) performed a study on the characteristics of the inward transport of [^{3}H]-MPP$^+$ in rat cultured hepatocytes. It was shown that [^{3}H]-MPP$^+$ is efficiently taken up by cultured hepatocytes by a carrier-mediated mechanism with kinetic and pharmacological characteristics similar to those of a recently cloned renal transporter for organic cations (OCT1) (Gründemann *et al.*, 1994). Curiously enough, the kinetic and pharmacological characteristics of [^{3}H]-MPP$^+$ uptake by rat isolated hepatocytes (Martel *et al.*, 1996a) are not identical with those of cultured hepatocytes (higher K_m, lower cell:medium ratios, lower sensitivity to cyanine863 and decynium22).

When incubated as a cell suspension, hepatocytes contact with the medium through all their membrane extent, including the apical (bile canalicular) membrane. As this apical membrane is endowed with P-glycoprotein (Fojo *et al.*, 1987; Smit *et al.*, 1993), a polyspecific transporter (Gottesman & Pastan, 1993) which also accepts organic cations (Dutt *et al.*, 1994), the putative involvement of P-glycoprotein in the uptake of [^{3}H]-MPP$^+$ by isolated hepatocytes was considered.

The aim of the present work was thus to characterize further the uptake of [^{3}H]-MPP$^+$ by rat isolated hepatocytes, namely by investigating the putative interactions of a wide range of drugs, including inhibitors/substrates of P-glycoprotein.

Methods

Isolation of rat hepatocytes

Male Wistar rats (Biotério do Instituto Gulbenkian de Ciência, Oeiras) 50–70 days old and weighing 200–300 g were used in the experiments. Animals were kept two per cage under controlled environmental conditions (12 h light/dark cycle and room temperature 24°C). Food and tap water were allowed *ad libitum*. Hepatocytes were isolated according to a procedure described previously (Martel *et al.*, 1993) with a modification in the composition of the initial liver perfusion medium (buffer A) which contained in addition 0.6 mM EGTA. Isolated cells were finally collected in ice-cold Krebs-Henseleit medium

[1] Author for correspondence.

containing (in mM): NaCl 137, KCl 5.37, $NaHCO_3$ 25, (+)-glucose 11, KH_2PO_4 1.18, $MgSO_4$ 0.57, $CaCl_2$ 2.51, ascorbic acid 0.3 and Na_2EDTA 0.04, pH 7.4. Cell viability was determined by Trypan Blue exclusion (0.16%). Preparations with 70–75% viable cells were used.

Uptake of [^{3}H]-MPP$^+$ into hepatocytes

Cells were diluted in Krebs-Henseleit medium to a final concentration of $2-3 \times 10^6$ cells ml^{-1}. Cell suspension (1 ml) was incubated at 37°C under continuous shaking and gassing with 95% O_2 and 5% CO_2. After a 5 min preincubation period, incubation with [^{3}H]-MPP$^+$ was started by adding 1 ml Krebs-Henseleit medium containing [^{3}H]-MPP$^+$ to the suspension. The final concentration of [^{3}H]-MPP$^+$ in the incubation medium was 200 nM. Incubation was stopped after 5 min (to measure initial rates of uptake–see Martel *et al.*, 1996a) by rapid filtration through Whatman cellulose nitrate membrane filters (25 mm diameter, 0.8 μm pore size), resulting in separation of the cells (retained in the filters) from the incubation medium. The filters were washed twice with 4 ml ice-cold Krebs-Henseleit medium, and placed in 2 ml perchloric acid 0.2 M. Radioactivity remaining on the filters was determined by liquid scintillation counting. Blanks were submitted to the same conditions but contained no hepatocytes. The final concentration of viable hepatocytes and the protein content in one sample of the cell suspension carried throughout the experiment were used to express the results. When the effect of drugs was tested these compounds were present during both the preincubation and incubation periods.

Effect of ionic composition of the incubation medium To study the effect of sodium on [^{3}H]-MPP$^+$ uptake, cells were preincubated and incubated in NaCl-free Krebs-Henseleit medium. The composition of the NaCl-free Krebs-Henseleit medium was the same as the Krebs-Henseleit medium except that NaCl (corresponding to 137 mM) was replaced isotonically either with lithium chloride or choline chloride.

Inhibition of alkaline phosphatase Alkaline phosphatase was inhibited by increasing or decreasing the pH of the incubation medium or by the presence of homoarginine or vanadate. To determine the effect of various pHs of the extracellular medium on the uptake of [^{3}H]-MPP$^+$, cells were preincubated and incubated in Krebs-Henseleit medium in which the pH was varied by a dropwise addition of HCl 1 M or TRIS/EDTA buffer (1.5 M, pH 9.7).

Effect of metabolic inhibitors The effect of 2,4-dinitrophenol (500 μM) was studied in the presence and in the absence of glucose (glucose being isotonically replaced by sucrose).

Protein determination

Proteins were determined as described by Lowry *et al.* (1951), with human serum albumin as a standard.

Uptake of [^{3}H]-MPP$^+$ into the rat perfused liver

Male Wistar rats weighing 200–300 g (Biotério do Instituto Gulbenkian de Ciência, Oeiras) were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.) and given 1000 iu heparin, i.p.. The portal vein and vena cava were cannulated, and the liver was then perfused *in situ* (via the portal vein), in a non-recirculating system at 37°C, with Krebs-Henseleit medium pH 7.4 with 0.25% (w/v) bovine serum albumin. The flow rate was kept constant at 10 ml min^{-1} throughout the experiment by means of a peristaltic pump (Cole-Parmer Instruments Co., Niles, Illinois, U.S.A.). All areas of the liver were perfused as verified by visual inspection.

The livers were initially equilibrated with [^{3}H]-MPP$^+$-free Krebs-Henseleit/BSA medium for 10 min. [^{3}H]-MPP$^+$ (200 nM) was then added to the perfusion medium for 30 min. At the end of the perfusion with [^{3}H]-MPP$^+$, the liver was rapidly perfused (±15 s) with 20 ml of Krebs-Henseleit medium at 4°C and subsequently tissues samples from 4 different regions of the liver were removed, cut into small pieces, weighed and placed in 4 ml perchloric acid 0.2 M. Radioactivity remaining in the tissue was measured by liquid scintillation counting.

When the effect of drugs was tested, the compounds were present throughout the experiment.

Calculations and statistics

Results are expressed as arithmetic means (±s.e.mean); *n* indicates the number of experiments.

The cell:medium ratio (C/M) for [^{3}H]-MPP$^+$ at the end of the incubation period was calculated on the basis of [^{3}H]-MPP$^+$ accumulation in hepatocytes, the hepatocyte weight (4.5 mg per 10^6 cells) and the concentration of [^{3}H]-MPP$^+$ in the incubation medium, as described by Martel *et al.* (1993).

Statistical significance of the differences between various groups was evaluated by one-way analysis of variance (ANOVA test) followed by the Newman-Keuls test. For comparison between two groups, Student's *t* test was used. Differences were considered to be significant when $P<0.05$.

Drugs used

[^{3}H]-MPP$^+$ (N-[methyl-^{3}H]-phenylpyridinium acetate; specific activity 84.0 Ci mmol^{-1}) (New England Nuclear Chemicals, Dreieich, Germany); bilirubin, DMSO (dimethylsulphoxide) (Merck, Darmstadt, Germany); cyclosporin A (Sandimmun, Sandoz Farma SA, Basel, Switzerland); MPP$^+$ (1-methyl-4-phenylpyridinium iodide) (Research Biochemicals International, Natick, MA, U.S.A.); (+)-tubocurarine chloride (Koch-Light Laboratories Ltd., Colnbrook, Bucks, U.K.); verapamil hydrochloride (Knoll AG, Ludwigshafen, Germany); ammonium vanadate (May & Baker Ltd, Dagenham, U.K.); vecuronium bromide (Organon Teknika B.V., Boxtel, Holland); amiloride hydrochloride, bovine serum albumin, cimetidine, collagenase type I, daunomycin hydrochloride, 2,4-dinitrophenol, EGTA (ethyleneglycol-bis-(β-amino ethyl ether) N,N,N′,N′-tetraacetic acid), homoarginine hydrochloride, ouabain octahydrate, progesterone, quinidine sulphate, sodium taurocholate, tetraethyl-ammonium bromide, trypan blue, vinblastine sulphate (Sigma, St. Louis, MO, U.S.A.). Amiloride, 2,4-dinitrophenol and ouabain were dissolved in DMSO and progesterone was dissolved in ethanol, the final concentration of these agents in the incubation media was 70 and 86 mmol l^{-1}, respectively. Cyclosporin A was dissolved in olive oil. Controls for each drug were run in the presence of its correspondent solvent.

Results

Uptake of [^{3}H]-MPP$^+$ by rat isolated hepatocytes

The accumulation of [^{3}H]-MPP$^+$ in rat hepatocytes incubated at 37°C for 5 min with 200 nM [^{3}H]-MPP$^+$ amounted to 10.57±1.32 pmol mg^{-1} protein ($n=21$). This quantity of [^{3}H]-MPP$^+$ in cells corresponded to a cell/medium (C/M) ratio of 17.4 ($n=21$). When cells were incubated with [^{3}H]-MPP$^+$ (200 nM) at 4°C, the accumulation of [^{3}H]-MPP$^+$ was almost abolished (reduced to 0.55±0.55% of control at 37°C; $P<0.05$; $n=3$).

Effect of drugs The effect of several substrates/inhibitors of hepatic transporters for cationic drugs (type I and type II) and of P-glycoprotein on [^{3}H]-MPP$^+$ uptake was studied.

Verapamil, quinidine (100 μM) and amiloride (1 mM) markedly reduced the accumulation of [^{3}H]-MPP$^+$ (to 4, 0 and

0% of control, respectively). The monovalent low molecular weight cation, tetraethylammonium (TEA) (100 μM), had no significant effect on [^{3}H]-MPP$^+$ accumulation, whereas the bulky bivalent cations (+)-tubocurarine (100 μM) and vecuronium (45 μM), significantly reduced [^{3}H]-MPP$^+$ content in hepatocytes (to 8 and 45% of control, respectively) (Figure 1).

Bilirubin, progesterone (200 μM) and daunomycin (100 μM) caused a large reduction in the accumulation of [^{3}H]-MPP$^+$ (to 19, 7% and 1% of control, respectively). Likewise, vinblastine, cyclosporin A and cimetidine (100 μM) reduced [^{3}H]-MPP$^+$ accumulation significantly (to 47, 65 and 50% of control, respectively) (Figure 2). The solvent of cyclosporin A (olive oil) had no effect on the uptake of [^{3}H]-MPP$^+$ (data not shown). On the other hand, the bile salt, taurocholate (100 μM), or ouabain (10 μM) did not affect the inward transport of [^{3}H]-MPP$^+$ (Figure 2).

Effect of ionic composition of the incubation medium The effect of sodium ions in the incubation medium was studied, in experiments where sodium chloride (in the preincubation and incubation media) was replaced isotonically with either choline chloride or lithium chloride. When sodium was replaced by choline, the accumulation of [^{3}H]-MPP$^+$ was strongly reduced (to 20% of control). However, when sodium was replaced by lithium, no reduction was observed (Figure 3).

Effect of inhibition of alkaline phosphatase Alkaline phosphatase was inhibited by increasing or decreasing the pH of the incubation medium. As shown in Figure 4, [^{3}H]-MPP$^+$ content in the cells was significantly increased when the pH of the preincubation and incubation media was either lower (pH 6.2) or higher (pH 7.8 or 8.2) than in control experiments (pH 7.4). When the hepatocytes were incubated in the presence of homoarginine (500 μM) or vanadate (1 mM), inhibitors of alkaline phosphatase, [^{3}H]-MPP$^+$ accumulation was significantly increased (Figure 5). The viability of hepatocytes was not affected by pH changes.

Effect of metabolic inhibitors In order to determine the energy-dependence of the inward transport of [^{3}H]-MPP$^+$ into hepatocytes, the effect of 2,4-dinitrophenol (500 μM) was studied. When glucose was present in Krebs-Henseleit medium, 2,4-dinitrophenol showed no effect on the accumulation of [^{3}H]-MPP$^+$. However, in glucose-free Krebs-Henseleit medium (glucose being isotonically replaced by sucrose), 2,4-dinitrophenol significantly reduced [^{3}H]-MPP$^+$ accumulation (to 45% of control; $P<0.05$; $n=5$; control 11.22 ± 3.68 pmol mg^{-1} protein, $n=4$).

Uptake of [^{3}H]-MPP$^+$ by the rat perfused liver

The accumulation of [^{3}H]-MPP$^+$ in rat livers perfused at 37°C for 30 min with 200 nM [^{3}H]-MPP$^+$ amounted to 1122.0 ± 97.9 pmol g^{-1} ($n=4$). Verapamil (100 μM) reduced the accumulation of [^{3}H]-MPP$^+$ in the liver to 55% of control. On the other hand, cyclosporin A (100 μM) had no effect on the accumulation of [^{3}H]-MPP$^+$ (Figure 6).

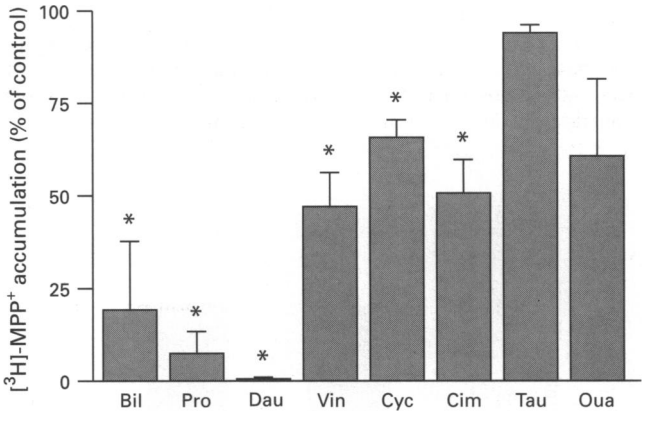

Figure 2 Effect of bilirubin 200 μM (Bil; $n=4$), progesterone 200 μM (Pro; $n=3$), daunomycin 100 μM (Dau; $n=3$), vinblastine 100 μM (Vin; $n=3$), cyclosporin A 100 μM (Cyc; $n=3$), cimetidine 100 μM (Cim; $n=4$), sodium taurocholate 100 μM (Tau; $n=3$) and ouabain 10 μM (Oua; $n=5$) on [^{3}H]-MPP$^+$ accumulation in rat hepatocytes. Cells were incubated at 37°C with 200 nM [^{3}H]-MPP$^+$ for 5 min, in the absence or presence of drugs. Results are shown as percentage of control (100%) value (arithmetic means ± s.e.mean) (control 11.33 ± 1.21 pmol mg^{-1} protein for bilirubin, daunomycin, vinblastine, cimetidine and sodium taurocholate, $n=5$; 8.68 ± 1.19 pmol mg^{-1} protein for progesterone, $n=3$; 16.43 ± 3.17 pmol mg^{-1} protein for ouabain, $n=5$; 7.46 ± 0.68 pmol mg^{-1} protein for cyclosporin A, $n=3$). *Significantly different from control ($P<0.05$).

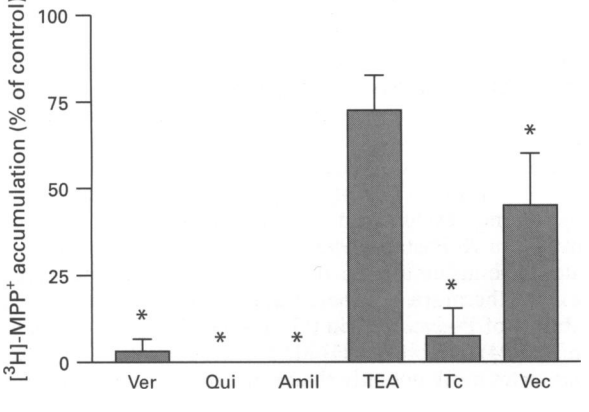

Figure 1 Effect of verapamil 100 μM (Ver; $n=4$), quinidine 100 μM (Qui; $n=3$), amiloride 1 mM (Amil; $n=4$), tetraethylammonium 100 μM (TEA; $n=5$), (+)-tubocurarine 100 μM (Tc; $n=3$) and vecuronium 45 μM (Vec, $n=5$) on [^{3}H]-MPP$^+$ accumulation in rat hepatocytes. Cells were incubated at 37°C with 200 nM [^{3}H]-MPP$^+$ for 5 min, in the absence or presence of drugs. Results are shown as percentage of control (100%) value (arithmetic means ± s.e.mean) (control 11.33 ± 1.21 pmol mg^{-1} protein; $n=5$). *Significantly different from control ($P<0.05$).

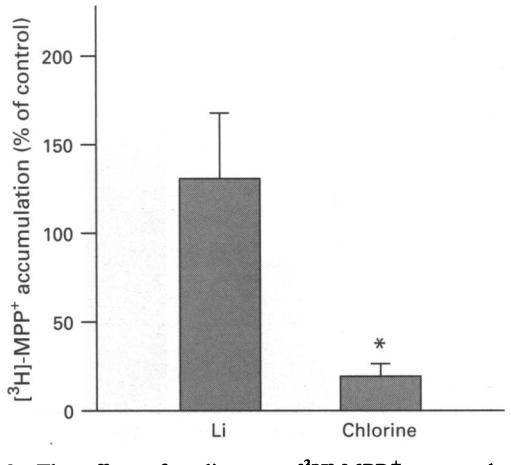

Figure 3 The effect of sodium on [^{3}H]-MPP$^+$ accumulation in rat hepatocytes. Cells were incubated at 37°C with 200 nM [^{3}H]-MPP$^+$ for 5 min. Sodium chloride in the Krebs-Henseleit medium was replaced by either lithium chloride (Li; $n=6$) or choline chloride (Choline; $n=4$). Results are shown as percentage of control (100%) value (arithmetic means ± s.e.mean) (control 12.60 ± 1.69 pmol mg^{-1} protein, $n=6$). *Significantly different from control ($P<0.05$).

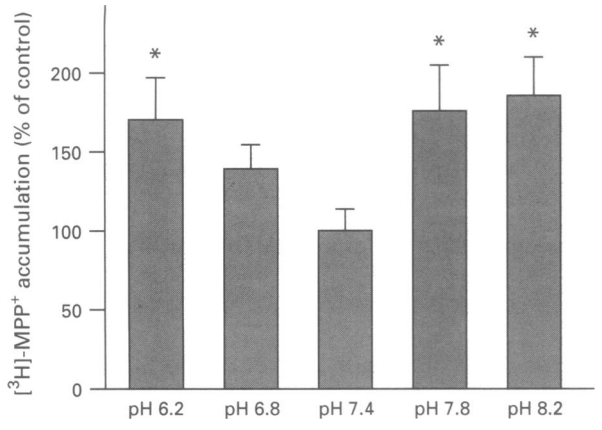

Figure 4 Effect of the pH of the Krebs-Henseleit medium on [^{3}H]-MPP$^+$ accumulation in rat hepatocytes. Cells were incubated at 37°C with 200 nM [^{3}H]-MPP$^+$ for 5 min. Experiments were performed at pH 6.2 ($n=4$), pH 6.8 ($n=5$), pH 7.8 ($n=4$) or pH 8.2 ($n=5$). Results are shown as percentage of control (100%) value (arithmetic means ± s.e.mean) (control 7.93 ± 1.10 pmol mg^{-1} protein, $n=5$). *Significantly different from control ($P<0.05$).

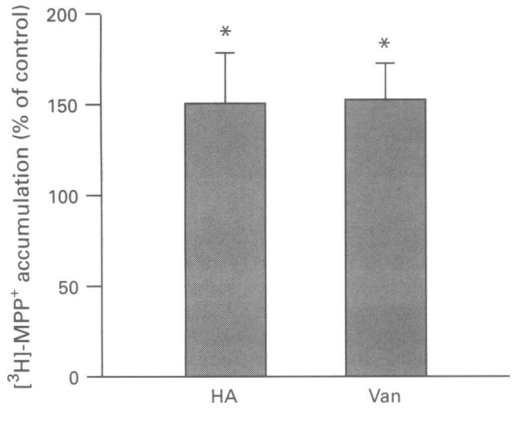

Figure 5 Effect of homoarginine 500 μM (HA; $n=5$) and vanadate 1 mM (Van; $n=5$) on [^{3}H]-MPP$^+$ accumulation in rat hepatocytes. Cells were incubated at 37°C with 200 nM [^{3}H]-MPP$^+$ for 5 min, at pH 7.4, in the absence or presence of drugs. Results are shown as percentage of control (100%) value (arithmetic means ± s.e.mean) (control 10.42 ± 1.48 pmol mg^{-1} protein, $n=5$). *Significantly different from control ($P<0.05$).

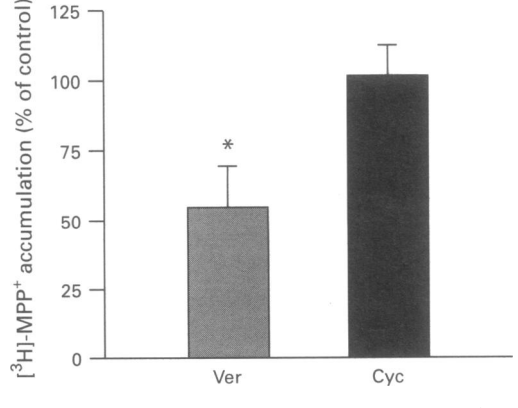

Figure 6 Effect of verapamil 100 μM (Ver; $n=4$) or cyclosporin A 100 μM (Cyc; $n=4$) on [^{3}H]-MPP$^+$ accumulation in rat perfused liver. Livers were perfused with 200 nM [^{3}H]-MPP$^+$ at 37°C for 30 min. Results are shown as percentage of control (100%) value (arithmetic means ± s.e.mean) (control 1122.0 ± 97.9 pmol g^{-1} for verapamil, $n=4$; 1038.6 ± 138.6 pmol g^{-1} for cyclosporin A, $n=3$). *Significantly different from control ($P<0.05$).

Discussion

The liver plays a critical role in the elimination of a number of cationic endobiotics and xenobiotics. A variety of recent studies on the hepatic handling of organic cations suggest that multiple mechanisms, with overlapping substrate specificies, are responsible for the basolateral uptake of organic cations. Meijer *et al.* (1990) and Oude Elferink *et al.* (1995) proposed at least 6 different mechanisms: sodium-driven choline and thiamine transporters, a N-methyl-nicotinamide/proton antiport, adsorptive endocytosis and two sodium-independent carrier-mediated processes (type I and type II). The type I uptake system for cationic drugs accepts relatively small monovalent organic cations, such as procainamide ethobromide and tetraethylammonium. The type II system accepts mostly bivalent organic cations with bulky ring structures, such as vecuronium and (+)-tubocurarine. The uptake of compounds via the type II system is greatly inhibited by K-strophanthoside and taurocholate, whereas the type I uptake is not affected by such compounds. Furthermore, the uptake into hepatocytes of type I compounds is inhibited by type II compounds, but the reverse does not occur (Meijer *et al.*, 1990).

Rat hepatocytes are known to take up and accumulate [^{3}H]-MPP$^+$ through a system which transports small organic cations, identical with the renal basolateral transporter for organic cations (OCT1) (Gründemann *et al.*, 1994; Martel *et al.*, 1996b). This system seems to be identical with the transport mechanism that has been referred to as the type I hepatic transport system for cationic drugs (Martel *et al.*, 1996b).

The results presented here show that [^{3}H]-MPP$^+$ uptake by rat isolated hepatocytes is sodium-independent and inhibited by a quantity of other cations, such as cimetidine, verapamil, quinidine, amiloride, including bulky ring molecules as (+)-tubocurarine and vecuronium. These large organic cations ((+)-tubocurarine and vecuronium) are not taken up by the type I hepatic transporter, but are substrates of the hepatic transporter type II, and are known to inhibit the type I transporter of organic cations (Meijer, 1989; Meijer *et al.*, 1990). Moreover, taurocholate and ouabain, inhibitors of the hepatic type II system (Meijer *et al.*, 1990), had no significant effect on the uptake of [^{3}H]-MPP$^+$ by rat isolated hepatocytes. Furthermore, [^{3}H]-MPP$^+$ was inhibited by a rather high concentration (137 mM) of the endogenous cation, choline. The hepatic uptake of procainamide ethobromide (type I) was also inhibited by choline (Eaton & Klaassen, 1978), whereas that of vecuronium (type II) was not affected by choline (Mola *et al.*, 1988). Together, these results support the hypothesis of [^{3}H]-MPP$^+$ being taken up by rat hepatocytes through the type I system.

An energy requirement for transport of [^{3}H]-MPP$^+$ into hepatocytes is likely as we have shown that the combination of an uncoupler of oxidative phosphorylation with glucose removal from the incubation medium significantly decreased the uptake of [^{3}H]-MPP$^+$.

On the other hand, a variety of drugs which are known to be inhibitors or substrates of P-glycoprotein, such as bilirubin, progesterone, cyclosporin A, daunomycin and vinblastine (Gottesman & Pastan, 1993; Sikic *et al.*, 1994), significantly reduced the accumulation of [^{3}H]-MPP$^+$ by isolated hepatocytes. Furthermore, verapamil and quinidine, which are also inhibitors of P-glycoprotein (Gottesman & Pastan, 1993; Sikic *et al.*, 1994) inhibited [^{3}H]-MPP$^+$ accumulation in isolated hepatocytes more potently than some other cations (see Figure 1) and taurocholate, known to be devoid of effects on P-glycoprotein (Hsing *et al.*, 1992; Mazzanti *et al.*, 1994), had no effect.

P-glycoprotein, or the multi-drug resistance gene (MDR) product, was first described as a transmembrane protein in tumour cells conferring on them resistance to a broad range of cytotoxic compounds (Gottesman & Pastan, 1993). Subsequent investigation showed that P-glycoprotein is not a unique entity but instead a family of related transporters, existing

also in normal tissues such as the kidney, intestine and liver (Fojo *et al.*, 1987; Buschman *et al.*, 1994). In the liver, the P-glycoprotein is confined to the apical (bile canalicular) membrane, where it is known to excrete phospholipids into the bile (Smit *et al.*, 1993).

Our results, showing a reduction in the accumulation of [^{3}H]-MPP$^+$ by such a wide variety of drugs which have in common their ability to inhibit or to be transported by P-glycoprotein, are strongly suggestive of the involvement of this transport protein in the uptake of [^{3}H]-MPP$^+$ by isolated hepatocytes. Indeed, inhibition of transport by verapamil, cyclosporin A, daunomycin and vinblastine, is usually considered to indicate the participation of P-glycoprotein in that transport (Horio *et al.*, 1989; Kamimoto *et al.*, 1989; Karlsson *et al.*, 1993; Thalhammer *et al.*, 1994; Pan *et al.*, 1994). Furthermore, secretion of vincristine, daunomyin and acridine orange into the bile through P-glycoprotein has been demonstrated previously (Kamimoto *et al.*, 1989; Watanabe *et al.*, 1992; Thalhammer *et al.*, 1994).

In the perfused liver [^{3}H]-MPP$^+$ is offered to the hepatocytes only through the baso-lateral membrane. It is interesting that under these conditions (liver perfusion) verapamil was much less efficient in reducing [^{3}H]-MPP$^+$ uptake (to 55% of control) than in the isolated hepatocytes (where it reduced uptake to 4% of control). On the other hand, cyclosporin A which significantly reduced uptake in the isolated hepatocytes (to 65% of control) had no effect in the perfused liver. These results add strong support to our hypothesis of P-glycoprotein activity in the apical membrane of isolated hepatocytes. Then, how can it be explained that a transport protein which usually exports drugs from cells is actually taking up [^{3}H]-MPP$^+$?

Although some published results with cultured cells could be interpreted as indicating bidirectional transport through P-glycoprotein (Horio *et al.*, 1989; Karlsson *et al.*, 1993), that fact has not been discussed by the authors.

As P-glycoprotein activity can be regulated through phosphorylation (Mellado & Horwitz, 1987; Chambers *et al.*, 1990; Staats *et al.*, 1990; Ma *et al.*, 1991; Bates *et al.*, 1992), and liver alkaline phosphatase has been shown to dephosphorylate phosphoproteins of the liver plasma membranes (Chan & Stinson, 1986), the hypothesis that the alkaline phosphatase, for dephosphorylating P-glycoprotein in isolated hepatocytes, is impaired has been raised.

Alkaline phosphatase is an enzyme anchored in the apical membrane of cells that possess P-glycoprotein (Moss, 1982; 1992; Robinson & Karnovsky, 1983; Fojo *et al.*, 1987; Vorbrodt & Trowbridge, 1991). All these cells – intestinal epithelium, kidney proximal tubules, hepatocytes, blood-brain barrier, placenta – are lining surfaces endowed with intense transport activities (secretion and absorption). Alkaline phosphatase could thus impart directionality to the transport activity of P-glycoprotein. Isolated hepatocytes obviously lose their polarity, and it seems plausible that diffusion of proteins along a larger membrane extent could lead to a decrease of efficiency of alkaline phosphatase toward dephosphorylation of P-glycoprotein. In agreement with our hypothesis, we verified that uptake of [^{3}H]-MPP$^+$ was significantly increased in the presence of inhibitors of alkaline phosphatase, homoarginine (Lin & Fishman, 1972) and vanadate (Chan & Stinson, 1986; Becq *et al.*, 1994; Bonet *et al.*, 1994). Moreover, increasing or decreasing the pH of the incubation medium resulted also in an increase in the cellular accumulation of [^{3}H]-MPP$^+$. It is known that P-glycoprotein-mediated transport is not dependent on extracellular pH (Altenberg *et al.*, 1993; Dutt *et al*, 1994; Zacherl *et al.*, 1994), the same applying to OCT1 (type I)-mediated transport (Gründemann *et al.*, 1994). On the other hand, the activity of alkaline phosphatase is pH-dependent, with optimal activity of human purified liver alkaline phosphatase having been observed at a pH of 7.8 (Chan & Stinson, 1986). It seems possible that in isolated hepatocytes alkaline phosphatase may dephosphorylate P-glycoprotein with lesser efficiency than in *in vivo* conditions. In line with this hypothesis, the results obtained with increased or decreased pH would be due to further impairment of alkaline phosphatase activity, reinforcing the inward direction of [^{3}H]-MPP$^+$ transport through P-glycoprotein.

Hence, we propose that [^{3}H]-MPP$^+$ is taken up by isolated hepatocytes through P-glycoprotein as well as the type I cation hepatic transporter. That canalicular transport system usually excretes endobiotics and xenobiotics but may, under pathological circumstances, take up the same type of substances.

The authors would like to express their thanks to Ms. Manuela Moura for technical assistance. Engº Eusébio Carretero (Organon Portuguesa Lda.) is also gratefully acknowledged for the generous supply of vecuronium bromide. This research was supported by PRAXIS XXI and FEDER (2/2.1/SAU/1251/95).

References

ALTENBERG, G.A., YOUNG, G., HORTON, J.K., GLASS, D., BELLI, J.A. & REUSS, L. (1993). Changes in intra- or extracellular pH do not mediate P-glycoprotein-dependent multidrug resistance. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 9735–9738.

BATES, S.E., CURRIER, S.J., ALVAREZ, M. & FOJO, A.T. (1992). Modulation of P-glycoprotein phosphorylation and drug transport by sodium butyrate. *Biochemistry*, **31**, 6366–6372.

BECQ, F., JENSEN, T.J., CHANG, X.B., SAVOIA, A., ROMMENS, J.M., TSUI, L.C., BUCHWALD, M., RIORDAN, J.R. & HANRAHAN, J.W. (1994). Phosphatase inhibitors activate normal and defective CFTR chloride channels. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 9160–9164.

BONET, M.L., LLORCA, F.I. & CADENAS, E. (1994). Kinetic mechanism of *Halobacterium halobium* Mn(2+)-activated alkaline phosphatase. *Biochem. Mol. Biol. Int.*, **34**, 1109–1120.

BUSCHMAN, E., LEPAGE, P. & GROS, P. (1994). P-glycoprotein homologues. In *Anticancer Drug Resistance: Advances in Molecular and Clinical Research*. ed. Goldstein, L.J. & Ozols, R.F. pp. 17–39. London: Kluwer Academic Publishers.

CHAMBERS, T.C., MCAVOY, E.M., JACOBS, J.W. & EILON, G. (1990). Protein kinase C phosphorylates P-glycoprotein in multidrug resistant human KB carcinoma cells. *J. Biol. Chem.*, **265**, 7679–7686.

CHAN, J.R.A. & STINSON, R.A. (1986). Dephosphorylation of phosphoproteins of human liver plasma membranes by endogenous and purified liver alkaline phosphatases. *J. Biol. Chem.*, **261**, 7635–7639.

DI MONTE, D., EKSTROM, G., SHINKA, T., SMITH, M., TREVOR, A. & CASTAGNOLI, Jr, N. (1987). Role of 1-methyl-4-phenylpyridinium ion formation and accumulation in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine toxicity to isolated hepatocytes. *Chem. Biol. Interact.*, **62**, 105–116.

DUTT, A., HEATH, L.A. & NELSON, J.A. (1994). P-glycoprotein and organic cation secretion by the mammalian kidney. *J. Pharmacol. Exp. Ther.*, **269**, 1254–1260.

EATON, D.L. & KLAASSEN, C.D. (1978). Carrier-mediated transport of the organic cation procaine amide ethobromide by isolated rat liver parenchymal cells. *J. Pharmacol. Exp. Ther.*, **206**, 595–606.

FOJO, A.T., UEDA, K., SLAMON, D.J., POPLACK, D.G., GOTTESMAN, M.M. & PASTAN, I. (1987). Expression of a multidrug-resistance gene in human tumors and tissues. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 265–269.

GOTTESMAN, M.M. & PASTAN, I. (1993). Biochemistry of multidrug resistance mediated by the multidrug transporter. *Ann. Rev. Biochem.*, **62**, 385–427.

GRÜNDEMANN, D., GORBOULEV, V., GAMBARYAN, S., VEYHL, M. & KOEPSELL, H. (1994). Drug excretion mediated by a new prototype of polyspecific transporter. *Nature*, **372,** 549–552.

HORIO, M., CHIN, K.-V., CURRIER, S.J., GOLDENBERG, S., WILLIAMS, C., PASTAN, I., GOTTESMAN, M.M. & HANDLER, J. (1989). Transepithelial transport of drugs by the multidrug transporter in cultured Madin-Darby canine kidney cell epithelia. *J. Biol. Chem.*, **264,** 14880–14884.

HSING, S., GATMAITAN, Z. & ARIAS, I.M. (1992). The function of Gp170, the multidrug-resistance gene product, in the brush border of rat intestinal mucosa. *Gastroenterology*, **102,** 879–885.

KAMIMOTO, Y., GATMAITAN, Z., HSU, J. & ARIAS, I.M. (1989). The function of Gp170, the multidrug resistance gene product, in rat liver canalicular membrane vesicles. *J. Biol. Chem.*, **264,** 11693–11698.

KARLSSON, J., KUO, S.-M., ZIEMNIAK, J. & ARTURSSON, P. (1993). Transport of celiprolol across human intestinal epithelial (Caco-2) cells: mediation of secretion by multiple transporters including P-glycoprotein. *Br. J. Pharmacol.*, **110,** 1009–1016.

KLAASSEN, C. & WATKINS, III, J. (1984). Mechanisms of bile formation, hepatic uptake, and biliary excretion. *Pharmacol. Rev.*, **36,** 1–67.

LIN, C.-W. & FISHMAN, W.H. (1972). L-homo-arginine. An organ-specific uncompetitive inhibitor of human liver and bone alkaline phosphohydrolases. *J. Biol. Chem.*, **247,** 3082–3087.

LOWRY, O.H., ROSENBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193,** 265–275.

MA, L., MARQUARDT, D., TAKEMOTO, L. & CENTER, M.S. (1991). Analysis of P-glycoprotein phosphorylation in HL60 cells isolated for resistance to vincristine. *J. Biol. Chem.*, **266,** 5593–5599.

MARTEL, F., AZEVEDO, I. & OSSWALD, W. (1993). Uptake and metabolism of ^{3}H-adrenaline and ^{3}H-noradrenaline by isolated hepatocytes and liver slices of the rat. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **348,** 350–357.

MARTEL, F., MARTINS, M.J. & AZEVEDO, I. (1996a). Inward transport of ^{3}H-MPP$^+$ in isolated rat hepatocytes: evidence for interaction with catecholamines. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **354,** 305–311.

MARTEL, F., RUSS, H., AZEVEDO, I. & SCHÖMIG, E. (1995). Uptake and accumulation of N-methyl-4-phenylpyridinium (MPP$^+$) by rat hepatocytes. *Br. J. Pharmacol.*, **114** (Suppl), 262P.

MARTEL, F., VETTER, T., RUSS, H., GRÜNDEMANN, D., AZEVEDO, I., KOEPSELL, H. & SCHÖMIG, E. (1996b). Transport of small organic cations in the rat liver. The role of the organic cation transporter OCT1. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **354,** 320–326.

MAZZANTI, R., FANTAPPIE, O., KAMIMOTO, Y., GATMAITAN, Z., GENTILINI, P. & ARIAS, I.M. (1994). Bile acid inhibition of P-glycoprotein-mediated transport in multidrug-resistant cells and rat liver canalicular membrane vesicles. *Hepatology*, **20,** 170–176.

MEIJER, D. (1989). Transport and metabolism in the hepatobiliary system. In *The Gastrointestinal System*, vol. 3, ed. Schulz, S., Forte, J. & BB, R., pp. 717–758. Oxford: University Press.

MEIJER, D., MOLA, W., MULLER, M. & KURZ, G. (1990). Carrier-mediated transport in the hepatic distribution and elimination of drugs, with special reference to the category of organic cations. *J. Pharmacokinet. Biopharm.*, **18,** 35–70.

MELLADO, W. & HORWITZ, S.B. (1987). Phosphorylation of the multidrug resistance associated glycoprotein. *Biochemistry*, **26,** 6900–6904.

MOLA, W.E.M., FOKKEMA, G.N., WEERT, B. & MEIJER, D.K.F. (1988). Mechanisms of the hepatic uptake of organic cations. Studies with the muscle relaxant vecuronium in isolated rat hepatocytes. *J. Pharmacol. Exp. Ther.*, **244,** 268–275.

MOSS, D.W. (1982). Alkaline phosphatase isoenzymes. *Clin. Chem.*, **28,** 2007–2016.

MOSS, D.W. (1992). Perspectives in alkaline phosphatase research. *Clin. Chem.*, **38,** 2486–2492.

OUDE ELFERINK, R., MEIJER, D., KUIPERS, F., JANSEN, P., GROEN, A. & GROOTHUIS, G. (1995). Hepatobiliary secretion of organic compounds; molecular mechanism of membrane transport. *Biochem. Biophys. Acta*, **1241,** 215–268.

PAN, B.-F., DUTT, A. & NELSON, J.A. (1994). Enhanced transepithelial flux of cimetidine by Madin-Darby canine kidney cells overexpressing human P-glycoprotein. *J. Pharmacol. Exp. Ther.*, **270,** 1–7.

RENNICK, B. (1981). Renal tubule transport of organic cations. *Am. J. Physiol.*, **240,** F83–F89.

ROBINSON, J.M. & KARNOVSKY, M.J. (1983). Ultrastructural localization of several phosphatases with cerium. *J. Histochem. Cytochem.*, **31,** 1197–1208.

SIKIC, B.I., FISCHER, G.A., LUM, B.L., BROPHY, N.A., YAHANDA, A.M., ADLER, K.M. & HALSEY, J. (1994). Clinical reversal of multidrug resistance. In *Anticancer Drug Resistance: Advances in Molecular and Clinical Research*, ed. Goldstein, L.J. & Ozols, R.F., pp. 149–165. London: Kluwer Academic Publishers.

SINGH, Y., SWANSON, E., SOKOLOSKI, E., KUTTY, R. & KRISHNA, G. (1988). MPTP and MPTP analogs induce cell death in cultured rat hepatocytes involving the formation of pyridinium metabolites. *Toxicol. Appl. Pharmacol.*, **96,** 347–359.

SMIT, J.J.M., SCHINKEL, A.H., OUDE ELFERINK, R.P.J., GROEN, A.K., WAGENAAR, E., VAN DEEMTER, L., MOL, C.A.A.M., OTTERHOF, R., VAN DER LUGT, N.M.T., VAN ROON, M.A., VAN DER VALK, M.A., OFFERHAUS, G.J.A., BERNS, A.J.M. & BORST, P. (1993). Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. *Cell*, **75,** 451–462.

STAATS, J.M., MARQUARDT, D. & CENTER, M.S. (1990). Characterization of a membrane-associated protein kinase of multidrug-resistant HL60 cells which phosphorylates P-glycoprotein. *J. Biol. Chem.*, **265,** 4084–4090.

THALHAMMER, T., STAPF, V., GAJDZIK, L. & GRAF, J. (1994). Bile canalicular cationic dye secretion as a model for P-glycoprotein mediated transport. *Eur. J. Pharmacol.* (Environmental Toxicology and Pharmacology Section), **270,** 213–220.

VORBRODT, A.W. & TROWBRIDGE, R.S. (1991). Ultracytochemical characteristics of cultured sheep brain microvascular endothelial cells. *J. Histochem. Cytochem.*, **39,** 1555–1563.

WATANABE, T., MIYAUCHI, S., SAWADA, Y., IGA, T., HANANO, M., INABA, M. & SUGIYAMA, Y. (1992). Kinetic analysis of hepatobiliary transport of vincristine in perfused rat liver. Possible roles of P-glycoprotein in biliary excretion of vincristine. *J. Hepatol.*, **16,** 77–88.

ZACHERL, J., HAMILTON, G., THALHAMMER, T., RIEGLER, M., COSENTINI, E.P., ELLINGER, A., BISCHOF, G., SCHWEITZER, M., TELEKY, B., KOPERNA, T. & WENZYL, E. (1994). Inhibition of P-glycoprotein-mediated vinblastine transport across HCT-8 intestinal carcinoma monolayers by verapamil, cyclosporin A and SDZ 833 in dependence of extracellular pH. *Cancer Chemother. Pharmacol.*, **34,** 125–132.

(Received April 22, 1996
Revised August 7, 1996
Accepted September 9, 1996)