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European Journal of Pharmacology 379 (1999) 211–218

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Characterization of the efflux of the organic cation MPP^+ in cultured rat hepatocytes

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Received 22 March 1999; received in revised form 6 July 1999; accepted 7 July 1999

Abstract

The aim of this study was to characterize the efflux of organic cations from primary cultured rat hepatocytes, using 1-methyl-4-phenylpyridinium (MPP^+) as a model compound. The efflux of [^{3}H]MPP^+ was temperature dependent, and pH and metabolic inhibition independent. It was either strongly reduced (verapamil, vinblastine and rhodamine123) or only moderately reduced (daunomycin) by other organic cations. The anti-*P*-glycoprotein antibody UIC2 (20 μg/ml) and the *P*-glycoprotein inhibitors vanadate and cyclosporine A had no effect on [^{3}H]MPP^+ efflux. Decynium22 and corticosterone, known inhibitors of rat Organic Cation Transporter 1 (rOCT1), markedly reduced [^{3}H]MPP^+ efflux. The uptake of [^{3}H]MPP^+ into hepatocytes, known to be mediated by rOCT1, was inhibited by verapamil and vinblastine (IC_{50}s of 2.6 and 34.4 μM, respectively). In conclusion, [^{3}H]MPP^+ efflux from primary cultured rat hepatocytes appears to be mediated by rOCT1, a polyspecific organic cation transporter. Moreover, our results do not support the involvement of *P*-glycoprotein or of an organic cation/proton antiporter in the efflux of [^{3}H]MPP^+.

Keywords: Hepatocytes, rat; MPP^+ (1-methyl-4-phenylpyridinium); Transport; rOCT1 (rat Organic Cation Transporter 1); *P*-glycoprotein

1. Introduction

The liver plays an essential role in the removal from the circulation and excretion of organic cations of both natural and synthetic origin (see Klaassen and Watkins, 1984; Meijer et al., 1990; Oude Elferink et al., 1995; Zhang et al., 1998a). Because organic cations are charged molecules at physiological pH, they must use more or less specific membrane-bound transport systems to be imported to or exported from cells or organisms. Thus, membrane-bound transporters largely affect the distribution and elimination of these compounds.

Current knowledge concerning the hepatic uptake of organic cations through the sinusoidal membrane of hepatocytes indicates that there are at least five different transport mechanisms: a Na^+-dependent thiamine and choline transporter, a *N*-methylnicotinamide/proton antiporter, adsorptive endocytosis and two Na^+-independent carriers named type I and II. The type I carrier system accepts small monovalent organic cations, whereas the type II carrier system accepts bulkier and/or multivalent organic cations. These hepatic organic cation transporters are multispecific and, therefore, possess widely overlapping substrate specificities (Oude Elferink et al., 1995; Zhang et al., 1998a).

1-Methyl-4-phenylpyridinium (MPP^+), a low-molecular-weight monovalent organic cation, is known to be avidly taken up and accumulated by rat primary cultured hepatocytes (Martel et al., 1996c, 1999) and freshly isolated hepatocytes (Martel et al., 1996a,b, 1998). We verified, using primary cultured rat hepatocytes, that the uptake of [^{3}H]MPP^+ is mediated by rat Organic Cation Transporter 1 (rOCT1), an organic cation transporter which most probably corresponds to the type I hepatic transporter of organic cations (Martel et al., 1996c). rOCT1 was first cloned and characterized by Gründemann et al. (1994) in the rat, and transporters homologous to rOCT1 were subsequently cloned and characterized in the mouse (Schweifer and Barlow, 1996), rabbit (Terashita et al., 1998), and man (Gorboulev et al., 1997; Zhang et al., 1997). This transporter was found to occur in the kidney (in proximal tubular cells), liver (in hepatocytes) and intestine (in ente-

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0014-2999/99/$ - see front matter
PII: S0014-2999(99)00501-4

rocytes), and was considered the first member of a new family of polyspecific transporters important for drug elimination (Gründemann et al., 1994).

The transport of organic cations across the canalicular membrane appears to involve at least two distinct mechanisms: an organic cation/proton antiporter that accepts both type I and type II organic cations, and an ATP-dependent process (*P*-glycoprotein-mediated transport) that transports bulky, amphiphilic cations (Oude Elferink et al., 1995; Zhang et al., 1998a).

In the liver, *P*-glycoprotein is confined to the apical (bile canalicular) membrane of hepatocytes, where it is known to be involved in the biliar excretion of organic cations (Kamimoto et al., 1989; Watanabe et al., 1992; Muller et al., 1994; Thalhammer et al., 1994; Smit et al., 1998a,b). It was recently demonstrated that *P*-glycoprotein expression in epithelial cells induces the directional transport of small (type I) and bulky (type II) organic cations (Smit et al., 1998c), and that this transporter contributes to the hepatobiliar elimination of both type I and II organic cations (Smit et al., 1998a,b). The transport of the organic cations tetraethylammonium and MPP^+ across the canalicular membrane of rat hepatocytes was found to occur through an organic cation/proton antiporter (Moseley et al., 1992, 1997).

So, there are multiple transport mechanisms able to transport organic cations across both the sinusoidal and apical membranes of hepatocytes. Therefore, the aim of this work was the characterization of the mechanism(s) involved in the outward transport (efflux) of the organic cation MPP^+ in primary cultured rat hepatocytes.

2. Materials and methods

2.1. Primary culture of rat hepatocytes

Male Wistar rats (Biotério do Instituto Gulbenkian de Ciência, Oeiras) weighing 200–300 g were used. Animals were kept under controlled environmental conditions (12 h light/dark cycle and room temperature 24°C) and food and tap water were allowed ad libitum.

On the day of the experiment, animals were treated with heparin (1000 IU i.p.) and anaesthetized with pentobarbital (50 mg kg^{-1}, i.p.). Isolated hepatocytes were prepared by a collagenase-perfusion technique as described previously (Martel et al., 1996c, 1999). After cannulation of the portal vein, the liver was perfused (20 ml min^{-1}) at 37°C with 250 ml of buffer A containing (in mM): 137 NaCl, 5.37 KCl, 25 $NaHCO_3$, 12.1 D(+)-glucose, 1.18 KH_2PO_4, 50.4 HEPES, 0.6 EGTA (pH 7.4). Subsequently, the liver was perfused (12 ml min^{-1}) at 37°C with 200 ml of buffer B (containing, in mM: 137 NaCl, 5.37 KCl, 25 $NaHCO_3$, 12.1 D(+)-glucose, 1.18 KH_2PO_4, 0.57 $MgSO_4$, 2.51 $CaCl_2$, 50.4 HEPES, 0.05% (w/v) collagenase, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 0.25 μg ml^{-1} amphotericin B; pH 7.4). After perfusion with buffer B, the liver was removed and gently dissolved in buffer C (containing, in mM: 137 NaCl, 5.37 KCl, 25 $NaHCO_3$, 12.1 D(+)-glucose, 1.18 KH_2PO_4, 0.57 $MgSO_4$, 2.51 $CaCl_2$, 50.4 HEPES, 0.3 ascorbic acid, 0.045 Na_2EDTA, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 0.25 μg ml^{-1} amphotericin B; pH 7.4). Isolated cells were obtained by filtration through a nylon filter (100 μm pore size) followed by centrifugation 5 times in buffer C ($30 \times g$, 5 min, 4°C). Hepatocytes were finally suspended in L_{15} Leibovitz medium (Sigma, St. Louis, MO, USA) supplemented with 10% (v/v) fetal calf serum, 5 μg ml^{-1} insulin, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 0.25 μg ml^{-1} amphotericin B (all from Sigma) and seeded on plastic culture dishes (21 cm^2; Corning Costar, Cambridge, MA, USA) previously coated with collagen. After incubation for 3 h at 37°C in a humidified atmosphere (5% CO_2), the culture medium was replaced by Dulbecco's Modified Eagle's Medium:F-12 (1:1) medium (Sigma) supplemented with 5% (v/v) fetal calf serum, 0.1 μM dexamethasone, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 0.25 μg ml^{-1} amphotericin B (all from Sigma). The culture medium was changed daily. After 48–72 h the cells formed a confluent monolayer and each culture dish contained about 2 mg cell protein.

2.2. Transport studies

2.2.1. Study of [^{3}H]MPP^+ efflux

The cells were preincubated at 37°C for 20 min in 3 ml of buffer D (containing: 137 mM NaCl, 5.37 mM KCl, 25 mM $NaHCO_3$, 12.1 mM D(+)-glucose, 1.18 mM KH_2PO_4, 0.57 mM $MgSO_4$, 2.51 mM $CaCl_2$, 50.4 mM HEPES; pH 7.4). Subsequently, hepatocytes were incubated at 37°C for 15 min in 3 ml buffer D containing 200 nM [^{3}H]MPP^+. At the end of this incubation, the buffer was rapidly removed and replaced by 3 ml buffer D without [^{3}H]MPP^+. Cells were incubated at 37°C in this washout buffer for 5, 15, 30, 60 or 90 min. Control samples were samples that were not incubated with washout buffer (0 min of washout).

Incubation was stopped by rinsing the cells with 3 ml ice-cold buffer D. The cells were then solubilized with 0.1% (v/v) Triton X-100 (in 5 mM Tris HCl, pH 7.4) and placed at 37°C overnight. Radioactivity present in the cells was measured by liquid scintillation counting.

When the effect of drugs was tested, these compounds were present during the washout period. To test the effect of medium pH, cells were incubated in washout buffer pH 6.2 or pH 8.2 (the pH was changed by dropwise addition of HCl or Tris/EDTA buffer (1.5 M; pH 9.7). To test the effect of metabolic inhibition, cells were incubated in washout buffer containing 2,4-dinitrophenol (1 mM) and without glucose (glucose being isotonically replaced by 2-deoxy-D-glucose). To test the temperature dependence of [^{3}H]MPP^+ washout, cells were incubated in washout buffer at 4°C.

In order to study the possible involvement of *P*-glycoprotein in the efflux of [^{3}H]MPP$^+$ from hepatocytes, the effect of the anti-*P*-glycoprotein antibody UIC2 (20 μg/ml) was studied. In these experiments, washout of [^{3}H]MPP$^+$ from the cells was performed at 37°C in 1.5 ml of washout buffer containing, or not containing, UIC2. Cells were incubated with washout buffer for 90 min, and aliquots of the washout buffer were collected at 30 and 90 min of washout. At the end of the washout period (90 min), the incubation was stopped (see above) and the radioactivity present in the cells and in the washout buffer samples was determined by liquid scintillation counting. Control samples (0 min of washout) were also assessed.

2.2.2. Study of [^{3}H]MPP$^+$ uptake

The cells were preincubated at 37°C for 20 min in 3 ml of buffer D (see above). Subsequently, hepatocytes were incubated at 37°C for 2 min with 3 ml buffer D containing 200 nM [^{3}H]MPP$^+$. The incubation was stopped by rinsing the cells with 3 ml ice-cold buffer D. The cells were then solubilized with 0.1% (v/v) Triton X-100 (in 5 mM Tris HCl, pH 7.4) and placed at 37°C overnight. When used, inhibitors of transport were present during both the preincubation and incubation periods. Radioactivity was measured by liquid scintillation counting.

2.3. Protein determination

The protein content of cell monolayers was determined as described by Bradford (1976), with human serum albumin as standard.

2.4. Calculations and statistics

For the calculation of IC_{50}s, the parameters of the Hill equation for multisite inhibition were fitted to the experimental data by nonlinear regression analysis, using a computer-assisted method (Motulsky et al., 1994).

Arithmetic means are given with S.E.M. and geometric means are given with 95% confidence limits; *n* indicates the number of experiments. The statistical significance of the difference 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 $P < 0.05$.

2.5. Materials

[^{3}H]MPP$^+$ (*N*-[methyl-^{3}H]-4-phenylpyridinium acetate; specific activity 82 Ci mmol^{-1}) (New England Nuclear Chemicals, Dreieich, Germany); cyclosporine A, MPP$^+$ (1-methyl-4-phenylpyridinium iodide) (Research Biochemicals International, Natick, MA, USA); ammonium vanadate (May and Baker, Dagenham, UK); rhodamine123 hydrate (Aldrich Chem., Milwaukee, WI, USA); DMSO (dimethylsulphoxide), Triton X-100 (Merck, Darmstadt, Germany); verapamil hydrochloride (Knoll, Ludwigshafen, Germany); collagen type I (from rat tail); collagenase type I; corticosterone; daunomycin hydrochloride; decynium22 (1,1′-diethyl-2,2′-cyanine iodide); 2-deoxy-D-glucose; 2,4-dinitrophenol; EGTA (ethyleneglycol-bis-(β-amino ethyl ether) *N,N,N′,N′*-tetraacetic acid); HEPES (*N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid); penicillin/streptomycin/amphotericin B solution; Tris (tris-(hydroxymethyl)-aminomethane hydrochloride); vinblastine sulphate (Sigma); UIC2 (Immunotech, Marseille, France).

In experiments using cyclosporine A, the final concentration of DMSO and fetal calf serum in the buffer was 1% and 0.5% (v/v), respectively; controls for this drug were run in the presence of these solvents. In experiments using UIC2, the washout buffer was diluted with 10% H_2O; controls for this substance were run under the same conditions. Decynium22 and corticosterone were dissolved in ethanol, and 2,4-dinitrophenol was dissolved in DMSO. The final concentration of these solvents in the washout buffer was 1%.

3. Results

In previous experiments (Martel et al., 1996c, 1999) it was found that the uptake of [^{3}H]MPP$^+$ by rat primary cultured hepatocytes increased linearly with time for about 10 min, and that after a 15-min incubation period a steady-state accumulation of this compound was reached.

3.1. Time course of [^{3}H]MPP$^+$ efflux from hepatocytes

For studies on the efflux of [^{3}H]MPP$^+$, cells were incubated with [^{3}H]MPP$^+$ for 15 min in order to obtain a steady-state accumulation of [^{3}H]MPP$^+$ in the cells.

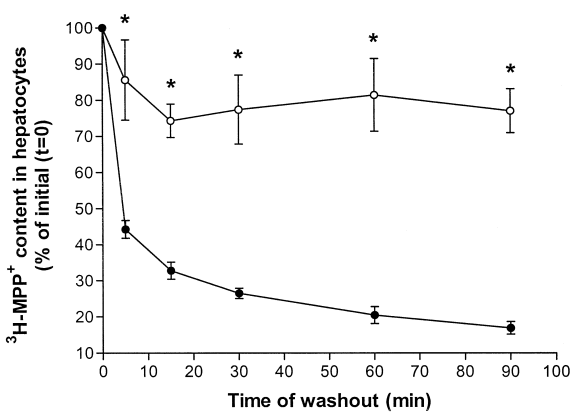

Fig. 1. Time course of [^{3}H]MPP$^+$ efflux in primary cultured rat hepatocytes. The cells were incubated at 37°C in buffer containing 200 nM [^{3}H]MPP$^+$ for 15 min, and were subsequently incubated at 37°C (●; $n = 11$) or 4°C (○; $n = 4$) in washout buffer for 5, 15, 30, 60 or 90 min. Shown is the [^{3}H]MPP$^+$ present in the cells as a percentage of the initial (washout time = 0) cellular content of this compound (8.1 ± 0.7 pmol/mg protein; $n = 11$) (arithmetic means ± S.E.M). * Significantly different from control at 37°C ($P < 0.05$).

Primary cultures of hepatocytes incubated for 15 min with 200 nM [^{3}H]MPP$^+$ accumulated this compound (8.09 ± 0.68 pmol/mg protein, $n = 11$). After this incubation period, cells were incubated in washout buffer for 5, 15, 30, 60 or 90 min, in order to study the efflux of [^{3}H]MPP$^+$ from the cells. As shown in Fig. 1, the efflux of [^{3}H]MPP$^+$ from the cells was rapid. After 5 min of washout, [^{3}H]MPP$^+$ present in the cells represented only 44.3 ± 2.5% ($n = 11$) of the initial cell content, and only 16.9 ± 1.7% ($n = 11$) of the initial [^{3}H]MPP$^+$ remained in the cells after 90 min of washout.

3.2. Effect of temperature

To test the temperature dependence of [^{3}H]MPP$^+$ efflux from hepatocytes, washout of this compound from the cells was studied at 4°C. As can be seen in Fig. 1, lowering of the incubation temperature from 37°C to 4°C produced a very strong decrease in [^{3}H]MPP$^+$ efflux. Non-specific efflux of [^{3}H]MPP$^+$ (by diffusion) was minimal, indicating that most [^{3}H]MPP$^+$ is extruded from cells by a transport-mediated mechanism.

3.3. Effect of metabolic inhibition

In order to determine the energy dependence of [^{3}H]MPP$^+$ efflux, the effect of metabolic inhibition of hepatocytes was studied. The presence, in the washout buffer, of the oxidative phosphorylation uncoupler 2,4-dinitrophenol (1 mM) in the absence of glucose (which was isotonically replaced by 2-deoxy-D-glucose) did not affect [^{3}H]MPP$^+$ efflux (Fig. 2).

3.4. Effect of extracellular pH

The effect of extracellular pH on the efflux of [^{3}H]MPP$^+$ by rat hepatocytes was studied by incubating the cells in washout buffer with a pH of 6.2 or 8.2. As can be seen in Fig. 3, the efflux of [^{3}H]MPP$^+$ from the cells was not significantly changed when the pH was either lower or higher than in control experiments (pH 7.4). However, there was a tendency to an increase in the efflux when the extracellular pH was decreased to 6.2, which was statistically significant only at 15 min of washout. Overall, these results show that a pH-dependent mechanism is not involved in the efflux of [^{3}H]MPP$^+$.

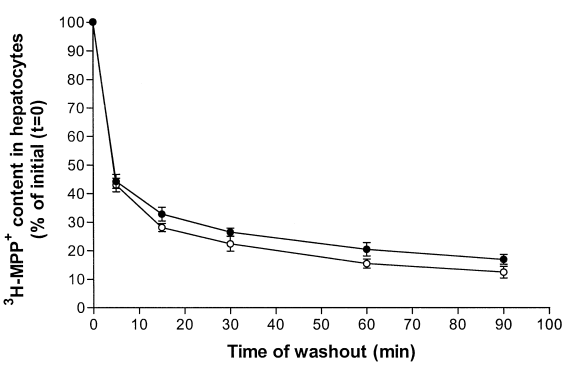

Fig. 2. Effect of metabolic inhibition on [^{3}H]MPP$^+$ efflux in primary cultured rat hepatocytes. The cells were incubated at 37°C in buffer containing 200 nM [^{3}H]MPP$^+$ for 15 min, and were subsequently incubated at 37°C in washout buffer for 5, 15, 30, 60 or 90 min, in the absence (●; $n = 11$) or presence of 2,4-dinitrophenol plus 2-deoxy-D-glucose (replacing glucose) (○; $n = 4$). Shown is the [^{3}H]MPP$^+$ present in the cells as a percentage of the initial (washout time = 0) cellular content of this compound (8.1 ± 0.7 pmol/mg protein; $n = 11$) (arithmetic means ± S.E.M).

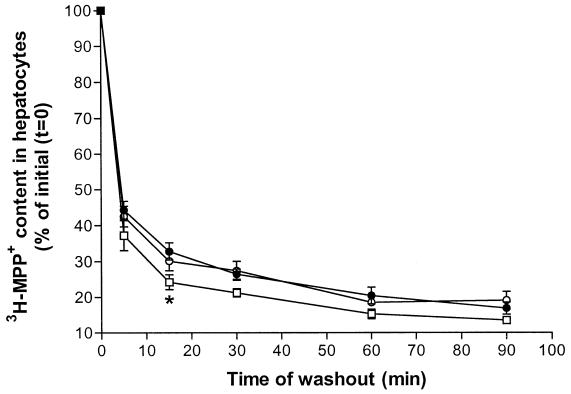

Fig. 3. Effect of extracellular pH on [^{3}H]MPP$^+$ efflux in primary cultured rat hepatocytes. The cells were incubated at 37°C in buffer containing 200 nM [^{3}H]MPP$^+$ for 15 min, and were subsequently incubated at 37°C in washout buffer for 5, 15, 30, 60 or 90 min. The pH of the washout buffer was 7.4 (●; $n = 11$), 6.2 (□; $n = 4$) or 8.2 (○; $n = 3$). Shown is the [^{3}H]MPP$^+$ present in the cells as a percentage of the initial (washout time = 0) cellular content of this compound (8.1 ± 0.7 pmol/mg protein; $n = 11$) (arithmetic means ± S.E.M). * Significantly different from control ($P < 0.05$).

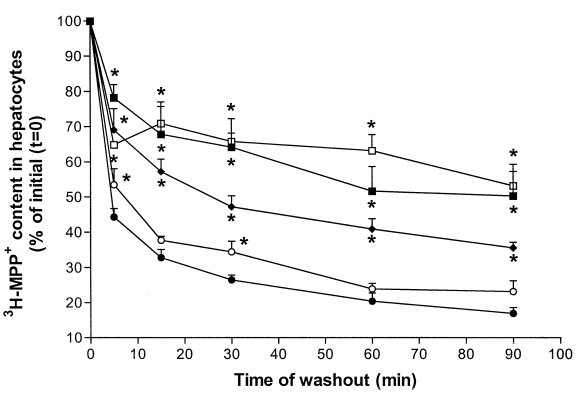

Fig. 4. Effect of organic cations on [^{3}H]MPP$^+$ efflux in primary cultured rat hepatocytes. The cells were incubated at 37°C in buffer containing 200 nM [^{3}H]MPP$^+$ for 15 min. Subsequently, they were incubated in washout buffer at 37°C for 5, 15, 30, 60 or 90 min in the absence (●; $n = 11$) or presence of 100 μM vinblastine (♦; $n = 4$), 100 μM daunomycin (○; $n = 4$), 10 μM rhodamine123 (■; $n = 3$) or 100 μM verapamil (□; $n = 3$). Shown is the [^{3}H]MPP$^+$ present in the cells as a percentage of the initial (washout time = 0) cellular content of this compound (8.1 ± 0.7 pmol/mg protein; $n = 11$) (arithmetic means ± S.E.M). * Significantly different from control ($P < 0.05$).

3.5. Effects of organic cations and various drugs

To investigate the nature of the mechanism involved in [^{3}H]MPP$^+$ efflux from primary cultured rat hepatocytes, the effect of several different compounds (present during the washout period only) was studied.

The effect of several compounds on [^{3}H]MPP$^+$ efflux is shown in Figs. 4 and 5. The organic cations rhodamine123 (10 μM), verapamil (100 μM) and vinblastine (100 μM) produced very marked reductions in [^{3}H]MPP$^+$ efflux (Fig. 4). The rank order of inhibitory potency was found to be rhodamine123 > verapamil > vinblastine. The organic cation daunomycin (100 μM) produced only a small inhibition of [^{3}H]MPP$^+$ efflux which was significant only at 5 and 30 min of washout (Fig. 4). Vanadate, used in a concentration (100 μM) known to inhibit the ATPase activity of *P*-glycoprotein, was found to be devoid of effect on [^{3}H]MPP$^+$ efflux (results not shown) and the peptide cyclosporine A (25 μM), which is a good inhibitor of *P*-glycoprotein, showed no effect (Fig. 5).

In order to clarify the putative involvement of *P*-glycoprotein in the efflux of [^{3}H]MPP$^+$ from hepatocytes, some experiments were performed using an anti-*P*-glycoprotein antibody (UIC2; 20 μg/ml). The amount of [^{3}H]MPP$^+$ present in the washout buffer after 30 and 90 min of washout in the absence or presence of UIC2 was not different (at 30 min: 26.4 ± 3.1 pmol/mg protein ($n = 3$) vs. 26.3 ± 0.9 pmol/mg protein ($n = 3$); at 90 min: 31.3 ± 3.2 pmol/mg protein ($n = 3$) vs. 29.4 ± 1.2 pmol/mg protein ($n = 3$)). Moreover, UIC2 also did not change the cell content of [^{3}H]MPP$^+$ after 90 min of washout (28.8 ± 2.5% vs. 27.0 ± 1.1% of initial content of [^{3}H]MPP$^+$, $n = 3$).

The effects of inhibitors of the organic cation transporter rOCT1 were also investigated (Fig. 6). Decynium22

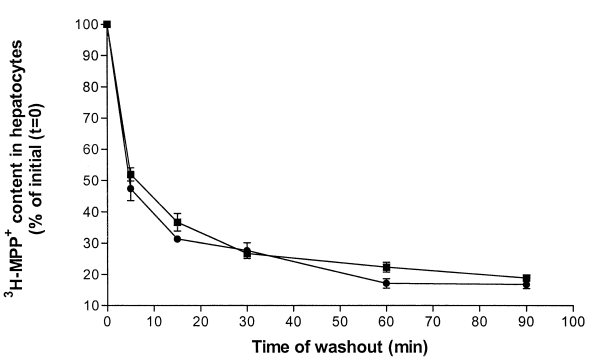

Fig. 5. Effect of cyclosporine A on [^{3}H]MPP$^+$ efflux in primary cultured rat hepatocytes. The cells were incubated at 37°C in buffer containing 200 nM [^{3}H]MPP$^+$ for 15 min. Subsequently, they were incubated in washout buffer at 37°C for 5, 15, 30, 60 or 90 min in the absence (●; $n = 3$) or presence of 25 μM cyclosporine A (■; $n = 4$). Shown is the [^{3}H]MPP$^+$ present in the cells as a percentage of the initial (washout time = 0) cellular content of this compound (6.6 ± 0.02 pmol/mg protein; $n = 3$) (arithmetic means ± S.E.M). The presence of 1% DMSO + 0.5% fetal calf serum did not affect [^{3}H]MPP$^+$ efflux from hepatocytes (results not shown).

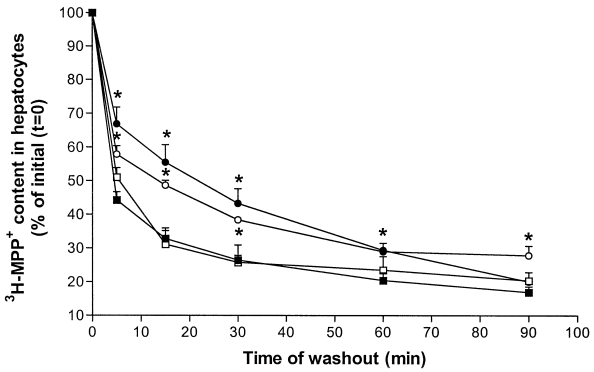

Fig. 6. Effect of corticosterone and decynium22 on [^{3}H]MPP$^+$ efflux in primary cultured rat hepatocytes. The cells were incubated at 37°C in buffer containing 200 nM [^{3}H]MPP$^+$ for 15 min. Subsequently, they were incubated in washout buffer at 37°C for 5, 15, 30, 60 or 90 min in the absence (■; $n = 11$) or presence of 1 μM corticosterone (□; $n = 3$), 100 μM corticosterone (●; $n = 3$) or 2 μM decynium22 (○; $n = 4$). Shown is the [^{3}H]MPP$^+$ present in the cells as a percentage of the initial (washout time = 0) cellular content of this compound (8.1 ± 0.7 pmol/mg protein; $n = 11$) (arithmetic means ± S.E.M). * Significantly different from control ($P < 0.05$).

(2 μM) and corticosterone (100 μM), used in concentrations known to strongly inhibit rOCT1-mediated transport, produced a significant reduction in [^{3}H]MPP$^+$ efflux from the cells. This inhibitory effect was more marked during the first minutes of the washout period. When 1 μM of corticosterone was used, which is a concentration known to inhibit the extraneuronal catecholamine transporter (uptake$_2$), but not rOCT1, there was no modification of [^{3}H]MPP$^+$ efflux (Fig. 6).

3.6. Effect of compounds on [^{3}H]MPP$^+$ uptake

For studies on [^{3}H]MPP$^+$ uptake, cells were incubated with [^{3}H]MPP$^+$ for 2 min, in order to measure initial rates of uptake. The effect of some compounds on initial rates of [^{3}H]MPP$^+$ transport into rat hepatocytes was investigated. As can be seen in Fig. 7, verapamil and vinblastine

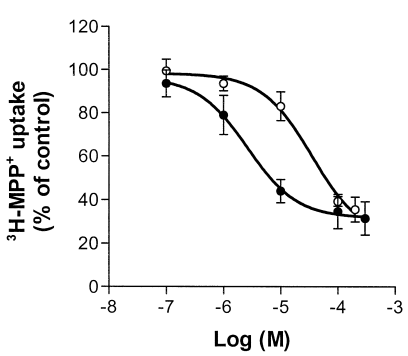

Fig. 7. Inhibition of [^{3}H]MPP$^+$ uptake in primary cultured rat hepatocytes. Initial rates of [^{3}H]MPP$^+$ (200 nM) transport were determined in the presence of verapamil (●; $n = 4$) or vinblastine (○; $n = 4$). Shown are arithmetic means ± S.E.M of the uptake in the presence of the inhibitor relative to the control.

concentration dependently reduced [^{3}H]MPP$^+$ uptake. Based on the analysis of the inhibition curves, IC_{50}s were calculated to be 2.6 (1.5–4.5) μM and 34.4 (7.2–165.0) μM for verapamil and vinblastine, respectively.

4. Discussion

The aim of this study was the characterization of the mechanism(s) involved in the outward transport of organic cations in the liver. For this purpose, we investigated the characteristics of the efflux of a model organic cation, MPP$^+$, in primary cultured rat hepatocytes. MPP$^+$ was chosen because it is known to be avidly taken up and accumulated in cultured rat hepatocytes, and the characteristics of MPP$^+$ uptake by these cells have been investigated in the past (Martel et al., 1996c, 1999). It is also of interest that this compound is not markedly metabolized in vivo (Sayre, 1989).

Cells were incubated with [^{3}H]MPP$^+$ for 15 min, because in previous studies we verified that with this incubation time, a steady-state accumulation of this compound in the cells is attained (Martel et al., 1996c, 1999). The efflux of [^{3}H]MPP$^+$ from the cells was a rapid, temperature dependent process: only about 20% of the compound remained in the cells after 90 min of washout. The observation that non-specific efflux of [^{3}H]MPP$^+$ (by diffusion) was minimal indicates that most of [^{3}H]MPP$^+$ is extruded from cells by a transport-mediated mechanism.

Because *P*-glycoprotein is expressed in the canalicular membrane of hepatocytes and is known to be involved in the hepatobiliar excretion of organic cations (Kamimoto et al., 1989; Watanabe et al., 1992; Muller et al., 1994; Thalhammer et al., 1994; Smit et al., 1998a,b), we first decided to study the possible involvement of this transporter in the efflux of [^{3}H]MPP$^+$. For this purpose, we tested the effect of substrates/inhibitors of this transporter, and the effect of an anti-*P*-glycoprotein antibody (UIC2). The results obtained, listed below, led us to the conclusion that *P*-glycoprotein is not involved in the efflux of MPP$^+$ from cultured hepatocytes.

(1) The efflux of [^{3}H]MPP$^+$ was not modified by metabolic inhibition: the combination of an uncoupler of oxidative phosphorylation with glucose removal from the medium did not change the efflux of [^{3}H]MPP$^+$. However, *P*-glycoprotein is an ATP-dependent transporter (see Gatmaitan and Arias, 1995; Zhang et al., 1998a) and it is known to be strongly inhibited under this experimental condition (Tsuji et al., 1993).

(2) The efflux of [^{3}H]MPP$^+$ was strongly reduced by organic cations that are substrates/inhibitors of *P*-glycoprotein (rhodamine123, verapamil, vinblastine), but was only modestly affected by another one (daunomycin) and was not affected by the undecapeptide cyclosporine A, an inhibitor of this transporter.

(3) The efflux of [^{3}H]MPP$^+$ was not changed by vanadate, which is known to strongly inhibit the ATPase activity of *P*-glycoprotein (Kamimoto et al., 1989; Miller, 1995).

(4) The efflux of [^{3}H]MPP$^+$ was not influenced by UIC2, an anti-*P*-glycoprotein monoclonal antibody effective in inhibiting *P*-glycoprotein-mediated transport. UIC2 recognizes and inhibits human *P*-glycoprotein (Mechetner and Roninson, 1992; Schinkel et al., 1993; Cavet et al., 1997), but it is known to be effective also in rat (Jakob et al., 1998) and pig (Soares-da-Silva et al., 1998) tissues.

So, as *P*-glycoprotein did not appear to be involved in the transport of [^{3}H]MPP$^+$ out of hepatocytes, we tried to further characterize the efflux of this compound. A previous study with rat canalicular membrane vesicles suggested that MPP$^+$ is transported through an MPP$^+$/proton antiporter, a transporter distinct from *P*-glycoprotein (Moseley et al., 1997). We found [^{3}H]MPP$^+$ efflux from primary cultures hepatocytes to be a pH-independent process and thus concluded that an MPP$^+$/proton antiporter mechanism is also not involved.

Considering that we previously showed that the uptake of [^{3}H]MPP$^+$ into rat primary cultured hepatocytes is mediated by rOCT1 (Martel et al., 1996c, 1999) and that others have shown that rOCT1 is a pH-independent, potential-dependent transporter (Gründemann et al., 1994; Busch et al., 1996) and is able to mediate efflux of MPP$^+$ in oocytes (Busch et al., 1996), we tested the possibility that rOCT1 is involved in the efflux of [^{3}H]MPP$^+$ out of primary cultured hepatocytes.

The results obtained are compatible with the involvement of rOCT1 in the efflux of [^{3}H]MPP$^+$ from primary cultured hepatocytes. First, we verified that the efflux of [^{3}H]MPP$^+$ was inhibited by 100 μM corticosterone and 2 μM decynium22, concentrations at which these compounds are potent inhibitors of rOCT1 (Gründemann et al., 1994; Martel et al., 1996c, 1999). Second, the efflux of [^{3}H]MPP$^+$ was potently reduced by verapamil, at a concentration (100 μM) shown to significantly inhibit hOCT1 (Zhang et al., 1998b). Third, the efflux of [^{3}H]MPP$^+$ was not inhibited by 1 μM corticosterone, a concentration at which corticosterone inhibits the extraneuronal catecholamine transporter ($uptake_2$), but not rOCT1 (Trendelenburg, 1988; Gründemann et al., 1994; Martel et al., 1996c, 1999). Fourth, the efflux of [^{3}H]MPP$^+$ from cultured rat hepatocytes was found to be pH independent.

rOCT1 is an organic cation transporter that accepts as substrates organic cations such as MPP$^+$ and tetraethylammonium (Gründemann et al., 1994; Martel et al., 1996c; see Zhang et al., 1998a). However, organic cations are also substrates of *P*-glycoprotein (see Gottesman and Pastan, 1993; Gatmaitan and Arias, 1995; Zhang et al., 1998a). So, it is possible that organic cations such as verapamil, vinblastine, daunomycin and rhodamine123 are inhibitors of both rOCT1 and *P*-glycoprotein. If this happens, it is probable that the inhibitory effect of these compounds on [^{3}H]MPP$^+$ efflux reflects inhibition of rOCT1. In this context, it is important to note that vinblastine and vera-

pamil were able to inhibit [^{3}H]MPP$^+$ uptake by rat cultured hepatocytes, a mechanism previously characterized as rOCT1 mediated (Martel et al., 1996c, 1999). In contrast, cyclosporine A, a peptide that does not interact with the renal organic cation transport system (Schramm et al., 1995), showed no effect on [^{3}H]MPP$^+$ efflux.

In conclusion, our results suggest that [^{3}H]MPP$^+$ efflux from primary cultured rat hepatocytes is mediated by rOCT1, and that there is no involvement of *P*-glycoprotein or of an organic cation/proton antiporter in this process. To our knowledge, this is the first report to show that rOCT1 is able to mediate the efflux of organic cations from cultured cells. The involvement of rOCT1 in [^{3}H]MPP$^+$ efflux from cultured hepatocytes, together with the fact that rOCT1 is located in the sinusoidal membrane of hepatocytes (Martel et al., 1996c), suggests that this transporter is able, in vivo, to transport organic cations from hepatocytes into the blood stream. This rOCT1-mediated transport of organic cations from hepatocytes into the blood will occur, especially if there is a high intracellular concentration of these compounds in the cells (e.g., following inhibition of metabolism and/or biliary excretion of these compounds).

Finally, we think that because *P*-glycoprotein and rOCT1 share a number of substrates and inhibitors, i.e., they have an overlapping substrate specificity, care must be taken when characterizing transport mechanisms for organic cations based on the use of substrates/inhibitors only. This is especially important when studies are performed with epithelial cells such as hepatocytes, kidney tubular cells or enterocytes, which possess both *P*-glycoprotein and multiple transporters for organic cations in their cell membranes.

Acknowledgements

This work was supported by Fundação para a Ciência e a Tecnologia (PRAXIS XXI 2/2.1/SAU/1251/95).

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