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Rhodamine 123 accumulates extensively in the isolated perfused rat kidney and is secreted by the organic cation system

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Abstract

Rhodamine 123 has been shown to be a substrate for P-glycoprotein in multidrug resistant cells. In the present investigation the disposition of rhodamine 123 was studied in the isolated perfused rat kidney. After exposing the kidneys to perfusate concentrations ranging from 10 to 1000 ng/ml, the renal clearance was 4–1 times the clearance by glomerular filtration, respectively, indicating active and saturable secretion of rhodamine 123. The rate-limiting step in secretion was found to be membrane passage from cell to tubular lumen. Suprisingly, renal clearance was not influenced by the P-glycoprotein inhibitors cyclosporin A or digoxin. However, pretreatment of the kidneys with verapamil and quinidine (inhibitors of both P-glycoprotein and organic cation transport) or cimetidine (organic cation transport inhibitor) resulted in a significantly reduced rhodamine 123 clearance, indicating that the renal organic cation carrier may be involved in active secretion. Rhodamine 123 accumulated extensively in the isolated perfused rat kidney; tissue concentrations of 270–360 times the perfusate concentration were determined. Similar accumulation ratios at different perfusate concentrations were found, suggesting that the compound enters the tubular cells by (facilitated) diffusion. In conclusion, rhodamine 123 accumulated extensively in the isolated perfused rat kidney and active renal secretion appears to be preferentially mediated by the organic cation carrier and not by P-glycoprotein.

Keywords: Renal clearance; Active secretion; Drug interaction; P-glycoprotein; Multidrug resistance; Organic cation carrier

1. Introduction

Resistance to various chemotherapeutic drugs is a phenomenon often observed in patients and cell cultures. Drug resistance associated with the overexpression of the multidrug resistance-1 gene, which encodes the P-glycoprotein or multidrug transport protein, is characterized by a reduced intracellular drug accumulation and increased cellular drug efflux. P-glycoprotein is an ATP-dependent integral membrane protein, which is not only expressed in resistant tumor cells, but also in normal tissues such as liver, intestine, adrenal gland, placenta and kidney. The physiological function of P-glycoprotein is not clear, but its apical distribution in secretory epithelia and role in drug efflux suggest that P-glycoprotein may function in the excretion of endogenous compounds and naturally occurring toxins (Ford and Hait, 1990; Thibaut et al., 1987; Gottesman and Pastan, 1993; Leveille-Webster and Arias, 1995; Al-Awqati, 1995). Physiological substrates for P-glycoprotein are cortisol, dexamethasone and aldosterone, whereas progesterone is a potent inhibitor of P-glycoprotein-mediated transport (Ichikawa-Haraguchi et al., 1993; Ueda et al., 1992). Other substrates for P-glycoprotein are anthracylines, vinca alkaloids, epipodophyllotoxins, taxol, actinomycin D (Ford and Hait, 1990). Furthermore, the widely used cardiac glycoside, digoxin, is actively secreted into urine by P-glycoprotein in the kidney (De Lannoy et al., 1992; Hori et al., 1993).

The ability to excrete these substrates can be reduced by treatment with chemosensitizers, compounds that reverse or antagonize multidrug resistance. The group of inhibitors consists of a wide variety of structurally unrelated drugs, including calcium channel blockers, hormones and steroids, hydrophobic peptides, cyclosporins, calmodulin antagonists, and miscellaneous hydrophobic cationic drugs. It is suggested that the interaction between substrates and inhibitors is related to a common binding site on P-glycoprotein. Resistance reversal by cyclosporin A may in part

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also be due to competition for transport. ATP binding is not blocked by any of the reversal agents, indicating separate ATP and drug binding sites on P-glycoprotein (Ford and Hait, 1990; Gottesman and Pastan, 1993; Leveille-Webster and Arias, 1995).

The proximal tubule of the kidney is the most important site in active renal drug elimination, and proximal tubular cells contain separate systems for organic anions and cations. The active step in the secretion of organic anions is located at the basolateral membrane, and that of organic cations at the luminal membrane (Pritchard and Miller, 1993). In addition, P-glycoprotein is also present at the luminal membrane (Lieberman et al., 1989). Several hydrophobic amines known to interact with the classical organic cation transport system, for example verapamil, quinine, quinidine, and quinacrine, inhibit P-glycoprotein-mediated transport. For this reason P-glycoprotein was suggested to be an unidentified component of the renal organic cation carrier, however, photoaffinity labeling studies implied that separate carrier systems exist (Holohan et al., 1992). More recently, it was shown in a functional expression system in *Xenopus laevis* oocytes, that P-glycoprotein and the organic cation carrier are indeed different transport proteins (Nelson et al., 1995).

In the present investigation the transport mechanisms of rhodamine 123 and the interaction with various cationic drugs were studied in the isolated perfused rat kidney. This in vitro technique has been shown useful for the investigation of renal organic anionic and cationic drug excretion (Cox et al., 1990; Boom et al., 1994). Previous investigations in various carcinoma cells showed that the mitochondrial dye with anticarcinoma activity, rhodamine 123 (Bernal et al., 1983), is effectively pumped out of these cells via P-glycoprotein (Neyfakh et al., 1989; Lee et al., 1994; Denis-Gay et al., 1995). More recently, rhodamine 123 resistance was found to be related to the expression of the mdr1 gene in a monkey kidney epithelial cell line, although direct evidence that rhodamine 123 is transported by P-glycoprotein in this cell line was not obtained (Brouty-Boyé et al., 1995). Rhodamine 123 is not in use as a drug, but because of its excellent fluorescent properties and transport characteristics it is a potential model compound for studying P-glycoprotein-mediated transport in the kidney and other organs. Aim of our study was to investigate the role of P-glycoprotein as a drug transport system in the isolated perfused rat kidney.

2. Materials and methods

2.1. Methods

2.1.1. Kidney isolation and perfusion

The isolation and perfusion of the rat kidney and stability of the perfused kidney system were described in detail previously (Cox et al., 1990). Briefly, male Wistar-Hannover rats (225–275 g) were anesthetized intraperitoneally with pentobarbital (6 mg/100 g) and furosemide was injected intraperitoneally (1 mg/100 g) to prevent deterioration of the distal nephron. Heparin (125 I.U./100 g) was injected via the femoral vein. The ureter of the right kidney was cannulated and the renal artery via the mesenteric artery without interruption of the blood flow. The kidney was then excised and placed in a fluid bath with a constant temperature of 37.5°C. The perfusion fluid had the following composition (mM, except where indicated otherwise): NaCl 114.0, KCl 5.2, $CaCl_2$ 1.8, $MgCl_2$ 1.0, $NaHCO_3$ 22.5, Na_2HPO_4 0.84, KH_2PO_4 0.28, glucose 5.0, urea 4.0, pluronic F108 25.0 g/l, glutathione 0.33, inositol 0.083, cysteine 0.50, glycine 2.3, Na-pyruvate 2.0, Na-acetate 1.22, Na-propionate 0.21, inosine 1.0, alanine 5.0, glutamine 0.11, L-glutamic acid 2.0, α-ketoglutarate 1.15, ascorbic acid 0.01, Na-lactate 1.0, choline chloride 1.0 mg/l, insulin 4 I.U./l, aldosterone 2.0 μg/l, lysine-vasopressin 0.01 I.U./l, and angiotensin II 15.0 ng/l. To this solution 1.0% Synthamin 14, a mixture of 15 amino acids, was added. Pluronic F-108 was used as oncotic agent in the albumin-free perfusion fluid. For the determination of glomerular filtration rate, inulin was added to the perfusion fluid (100 μg/ml).

2.1.2. Clearance experiments

In our perfused kidney preparation, perfusion medium was recirculated at a constant flow rate with a perfusate pressure of approximately 90 mmHg. During the first 5 min of perfusion, the venous effluent was discarded and after this period the perfusion fluid was recirculated and the kidney was allowed to stabilize for 30 min. Then the experiment was started with a 30 min baseline period, after which rhodamine 123 was administered. During the baseline period the perfusate volume was 500 ml from which a sample of 5 ml was drawn. After the baseline period, the experimental fluid was connected to the kidney, with a total volume of 150 ml in which rhodamine 123 was already dissolved. The experimental period was 120 min. Doses added to the perfused kidneys were 0 (time controls), 1.5, 15, 52,5 and 150 μg of rhodamine 123, resulting in initial perfusate concentrations of 0, 10, 100, 350 and 1000 ng/ml. The influence on rhodamine 123 clearance of the following compounds were studied: cyclosporin A and digoxin (P-glycoprotein-inhibitors), verapamil and quinidine (organic cation transport and P-glycoprotein inhibitors), and cimetidine (organic cation transport inhibitor). All inhibitory agents were added to the isolated perfused rat kidney at the start of the baseline period, and remained in perfusion fluid during the experimental period. Urine samples were collected during control and experimental periods over 10 min intervals. Perfusate samples (500 μl) were drawn at the midpoint of each urine collection interval. Two additional perfusate samples were taken, one at the beginning of the experimental period ($t = 0$), and one at the end of the experiment. At the end of the

experiment the kidney was removed from the system, blotted, weighed, and frozen until analysis. Urine and perfusate samples were stored at −20°C until analysis. Perfusion fluid during experimental period, and perfusion and urine samples were protected from light.

2.1.3. Preload experiments

Kidneys were isolated and perfused in a similar way as described above for the clearance experiments, except for the experimental period. The 30 min baseline period was followed by a 60 min preload period, in which 150 μg rhodamine 123 was added to perfusion medium (150 ml). Subsequently an efflux period was studied for 90 min, at which kidneys were perfused with clean perfusion fluid (1000 ml) without recirculation. After 40 min of efflux a urinary flow change from 60 to 16 μl/min was induced by changing the perfusate flow rate from 16 to 11 ml/min. Whenever verapamil was used to inhibit rhodamine 123 transport, this agent was added to perfusion fluid at the start of the baseline period and remained in perfusion fluid during the preload and efflux period. Urine samples were collected during all periods over 10 min intervals. Perfusate samples (400 μl) were drawn at the beginning and after 5, 15, 35, 55 and 60 min of the preload period. During the single-pass efflux period, samples of 600 μl were drawn from the perfusion fluid every 5 min, and one at the end of the efflux period. Perfusate and urine samples, and kidneys were handled as described for the clearance experiments.

2.1.4. Analysis

Urine and perfusate samples were analyzed for glucose and various electrolytes as described previously (Cox et al., 1990). Inulin was determined according to a previously published method (Heyrovski, 1956). The concentration of rhodamine 123 in perfusate, urine and kidney samples was determined by means of fluorescence spectrophotometry. To this end, an aliquot of 2–200 μl of the perfusate sample or 0.5–20 μl of the urine samples was taken and adjusted to 600 μl with analysis buffer (0.01 M KH_2PO_4, pH 2.6), and fluorescence was measured using a Perkin Elmer LS50 luminescence spectrophotometer (Perkin Elmer, Beaconsfield, Buckinghamshire, UK). The excitation wavelength was set to 501 nm, the emission wavelength to 531 nm and for both wavelengths a slit width of 5 nm was used. Concentrations were calculated by comparing fluorescence intensity (in photomultiplier units) with a calibration curve of spiked samples of blank perfusion fluid with different concentrations of rhodamine 123. The concentration of rhodamine 123 in kidney tissue was determined similarly. The kidneys were homogenized in 5 ml distilled water with a Polytron homogenizer (Braun Melsungen, Germany) on setting 10 for 2 times 60 s. Subsequently, to 50 μl of the kidney homogenate 250 μl acetonitrile was added, vortexed and centrifuged for 10 min at 2000 × *g*. Of the supernatant, an aliquot of 200 μl was added to ice-cold glass tubes and evaporated until dryness. The residue was dissolved in 600 μl analysis buffer, either undiluted or 10–50 times diluted (dependent on the expected concentration), and fluorescence intensities of triplicate samples was measured and averaged. Concentrations were calculated by comparing fluorescence intensity with a calibration curve of spiked samples of blank kidney homogenates with various concentrations of rhodamine 123. Linear calibration curves were obtained in all cases ($r^2 > 0.998$). Rhodamine 123 did not bind to pluronic F-108. This was checked by means of ultrafiltration using YM-10 membranes with a molecular weight cut-off of 10 000 in the MPS-1 micropartition system (Amicon, Grace B.V., Dronten, Netherlands).

2.1.5. Data analysis

All data are expressed as means ± S.D. Statistical differences between means were determined with one-way analysis of variance followed by the least significant difference test for comparison of multiple means. Differences were considered significant if $P < 0.05$. The concentration of rhodamine 123 in kidney tissue (C_T) was expressed as the amount of drug per weight of tissue.

2.2. Materials

Sodium pentobarbital was obtained from Apharmo (Arnhem, Netherlands). Heparin, aldosterone and inulin were from Organon (Oss, Netherlands). Rhodamine 123 was purchased from ICN (Costa Mesa, CA, USA), lysine-vasopressin from Sandoz Pharma (Basel, Switzerland), angiotensin II from Beckman (Palo Alto, CA, USA) and Synthamin 14 was from Travenol (Thetford, Norfolk, UK). Cyclosporin A was kindly provided by Sandoz Pharma (Basel, Switzerland) and Pluronic F-108 was obtained from BASF (Arnhem, Netherlands). All other chemicals were of analytical grade and obtained either from Sigma (St. Louis, MO, USA) or Merck AG (Darmstadt, Germany). A stock solution of cyclosporin A was prepared in DMSO (20 mg/ml).

3. Results

3.1. Effect on kidney function

The mean values for the functional parameters of baseline period (−30–0 min) of all isolated perfused rat kidney experiments are presented in Table 1. The control values (first data column) represent mean values of all experiments in which rhodamine 123 was added solely to the perfused kidney system. The other columns show data of the functional parameters after pretreatment of the kidneys with verapamil, cimetidine, cyclosporin A, quinidine or digoxin. Although some parameters were slightly changed after pretreatment with one of the inhibitory

Table 1
Experimental conditions of the isolated perfused rat kidney in baseline period, without or with pretreatment with verapamil [a]

Functional parameter	Control	Verapamil		Cimetidine	Cyclosporin A	Quinidine	Digoxin
		10 μM	50 μM	10 μM	5 μg/ml	6 μM	10 μM
$FE_{glucose}$ (%)	0.84 ± 0.31	0.46 ± 0.13 [b]	0.87 ± 0.74	1.5 ± 0.8	1.3 ± 0.6	1.4 ± 0.2 [b]	1.5 ± 1.0
FE_{sodium} (%)	1.8 ± 0.4	1.8 ± 0.8	2.6 ± 1.9	2.2 ± 0.7	2.5 ± 0.1 [b]	2.1 ± 0.8	2.5 ± 0.9
GFR (μl/min)	530 ± 80	540 ± 100	520 ± 70	468 ± 34	422 ± 44	445 ± 53	458 ± 128
Urinary flow (μl/min)	25 ± 4	25 ± 2	25 ± 4	26 ± 1	24 ± 3	23 ± 2	24 ± 3
Urinary pH	5.5 ± 0.2	5.6 ± 0.3	5.5 ± 0.2	5.8 ± 0.4	5.5 ± 0.1	5.5 ± 0.1	5.8 ± 0.2 [b]
RPP (mmHg)	94 ± 11	95 ± 7	90 ± 10	105 ± 10	105 ± 7	89 ± 2	100 ± 10
FR_{water} (%)	95 ± 1	95 ± 1	95 ± 2	94 ± 1	94.2 ± 0.1	95 ± 1	95 ± 1
Perfusate flow (ml/min)	13 ± 3	14 ± 2	12 ± 2	12 ± 2	16 ± 5	13 ± 2	13 ± 2
n	16	8	8	6	3	3	4

[a] Means ± S.D. FE = fractional excretion, FR = fractional reabsorption, RPP = renal perfusion pressure. [b] $P < 0.05$: significantly different from control value.

agents, no severe effects on kidney functioning could be determined. In agreement with previous investigations (Cox et al., 1990), the system remained stable and viable during the whole experimental period. Addition of rhodamine 123 slightly increased renal functioning as compared to control experiments, which could be concluded from a slightly decreased fractional excretion of sodium, calcium and glucose throughout the whole experimental period ($P < 0.05$).

3.2. Renal handling

Rhodamine 123 was cleared very rapidly from perfusate. Fig. 1 shows the perfusate concentration and corresponding excretion rate data as a function of time. Biphasic perfusate concentration-time curves were obtained, with initially a rapid decline (50% of the amount rhodamine 123 was cleared after 35 min of perfusion), and subsequently a virtual infinite terminal phase. The urinary excretion rate increased slowly after addition of rhodamine 123, a maximum rate was achieved after 40–50 min followed by a gradual decrease. Within the perfusate concentration range, the renal clearance was 1–4 times higher than the clearance by glomerular filtration (renal fractional clearance or $Cl_R/GF > 1$), indicating active secretion of rhodamine 123 (Fig. 2A). Surprisingly, the renal clearance of rhodamine 123 (at a dose of 1.5 μg) was not affected by the P-glycoprotein inhibitors, cyclosporin A (5 μg/ml) and digoxin (10 μM) (Fig. 2B). However, pretreatment of the kidneys with cimetidine (10 μM) and quinidine (6 μM) resulted in a significant reduction in renal rhodamine 123 clearance. Treatment with 10 or 50 μM verapamil also resulted in a significantly decreased renal clearance of rhodamine 123 (Fig. 2C). None of the inhibitors had an effect on the perfusate concentration-time profiles of rhodamine 123, suggesting that transport from perfusion fluid into the tubular cells was unaffected. Treatment with 50 μM verapamil resulted in a $Cl_R/GF < 1$, suggesting that a

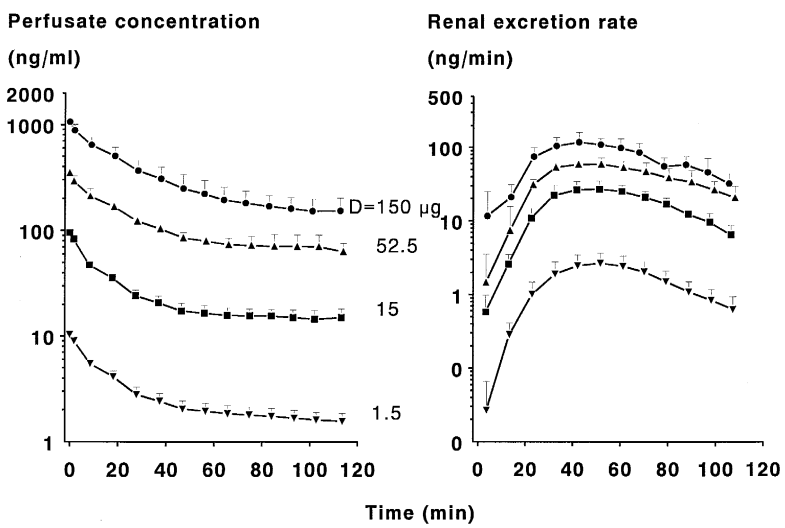

Fig. 1. Rhodamine 123 perfusate concentration (left panel) and urinary excretion rate (right panel), as a function of time. Doses added to the perfused kidneys were 1.5 (▼); 15 (■); 52.5 (▲) and 150 μg (●). All data points are means ± S.D. of four experiments.

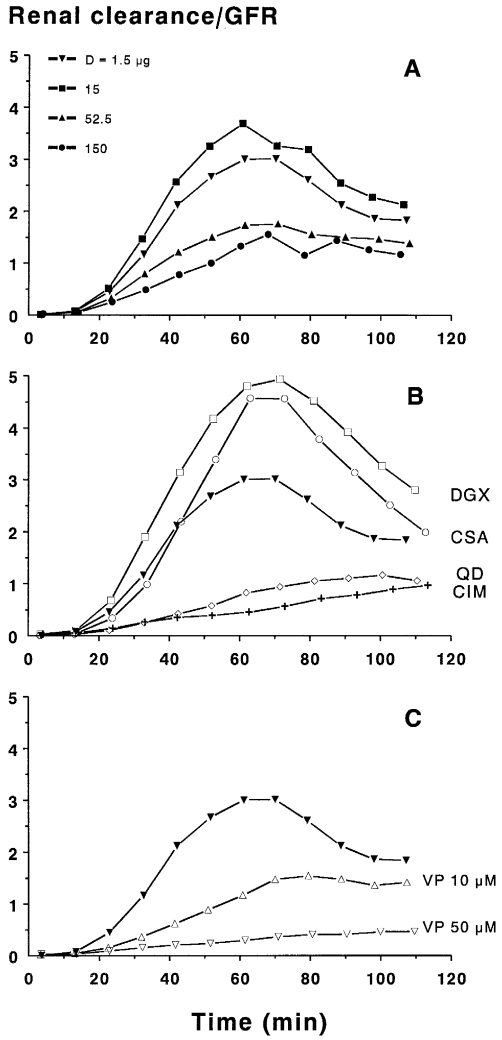

Fig. 2. Renal fractional clearance (Cl_R /GF) as a function of time. (Panel A) Four different doses of rhodamine 123 were studied ($n = 4$ for each dose). Doses added to the perfused kidneys were 1.5 (▼); 15 (■); 52.5 (▲) and 150 µg (●). (Panel B) A dose of 1.5 µg was studied (▼, $n = 4$), and after preincubation with 10 µM cimetidine, CM (+, $n = 6$), 5 µg/ml cyclosporin A, CSA (○, $n = 3$), 10 µM digoxin, DGX (□, N = 4) and 5 µg/ml quinidine, QD (△, $n = 3$). (Panel C) A dose of 1.5 µg rhodamine was used alone (▼, $n = 4$), and after preincubation with 10 (▽, $n = 4$) or 50 µM verapamil, VP (▽, $n = 4$). Mean values are presented. For the sake of clarity standard deviations were omitted from this figure, but varied between 8 and 40% for the different doses rhodamine. For the pretreated kidneys standard deviations varied between 14 and 38% for cimetidine and quinidine, between 38 and 67% for cyclosporin A and digoxin, and between 4 and 26% for verapamil.

reabsorptive mechanism is also involved in the overall renal clearance of rhodamine 123.

To further investigate renal excretion and reabsorption of rhodamine 123, preload experiments were performed. In these experiments, the perfused kidneys were exposed to 150 µg rhodamine 123 for 60 min, after which the kidneys were connected to clean perfusion fluid. Functional parameters of the perfused kidneys in preload experiments were not different from the parameters in normal clearance experiments (data not shown). In order to determine the mechanism of reabsorption for rhodamine 123 found after pretreatment with 50 µM verapamil, urine flow was changed after preloading the kidneys. Fig. 3 presents the curves of the perfusate rate and renal excretion rate of rhodamine 123 as a function of time, with and without pretreatment of the kidneys with 50 µM verapamil. Evidently, a certain amount of rhodamine 123 is transported back from tubular cells to the perfusion fluid (upper panel), and a urinary flow change from 60 to 16 µl/min hardly affected this transport (A versus B). Verapamil (50 µM) significantly reduced rhodamine 123 transport back into the perfusion medium. The renal excretion rate after preloading the kidneys with rhodamine 123 decreased with the urine flow, indicating that passive back-diffusion probably accounts for the reabsorptive mechanism found earlier. At a urinary flow of 16 µl/min, a renal excretion rate of 118 ± 18 ng/min was found (Fig. 3, lower panel, B), which is very similar to the renal excretion rate of rhodamine 123 after addition of 150 µg in the normal clearance experiments (103 ± 12 ng/min, Fig. 1). This suggests that in the overall renal clearance of rhodamine 123 membrane passage from cell to tubular lumen is the rate-limiting step in secretion. Pretreatment of the perfused

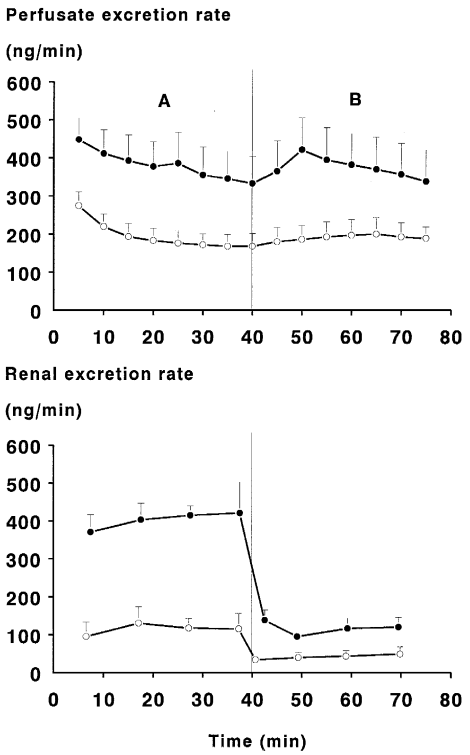

Fig. 3. Rate of transport back into the perfusate (perfusate excretion rate) and renal excretion rate after preloading perfused kidneys with 150 µg rhodamine 123. Preload experiments were done as described in detail in Section 2. Closed circles represent data with rhodamine 123 alone, open circles are data after pretreatment with 50 µM verapamil. During the first 40 min of the efflux period, the urinary flow was 60 µl/min (A), whereafter the flow was reduced to 16 µl/min (B). All data points are means ± S.D. of three experiments.

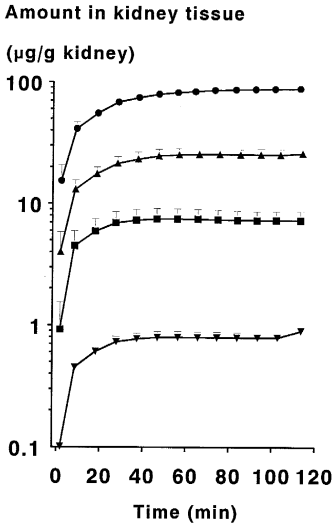

Fig. 4. Calculated amount of rhodamine 123 per weight of kidney as a function of time. Four different doses were added to the perfused rat kidneys: 1.5 μg (▼); 15 μg (■); 52.5 μg (▲) and 150 μg (●). All data points are means ± S.D. of four experiments.

kidneys with verapamil resulted in significantly lower excretion rates, which were also similar to the rates obtained in the normal clearance experiments in presence of 50 μM verapamil. Rhodamine 123 excretion rates were 41 ± 6 and 27 ± 6 for preload and normal clearance experiments, respectively, which corresponded with a reduction in renal clearance of approximately 70%.

3.3. Renal accumulation

The rapid disappearance from perfusion fluid and the delayed appearance in urine of rhodamine 123, indicate that the compound is retained by the tubular cells. In Fig. 4, the concentration of rhodamine 123 in the kidney, expressed as the amount of drug per weight of tissue, is plotted as a function of time. For every time point, the concentration was estimated by subtracting the amount in perfusate and the amount excreted in urine at time t from the total amount added to perfusate at time 0. Actual kidney tissue concentrations were determined at the end of the experiments and presented in Tables 2 and 3. For every rhodamine 123 dose, the tissue concentration is 30–40% lower than the estimated concentration after 120 min of perfusion. Probably because of an extensive intracellular binding we were not able to recover 100% of the theoretically calculated concentration in kidney tissue (Fig. 4). Furthermore, the accumulation ratios are given, which were obtained by dividing the concentration in kidney tissue by the concentration at the end of the experiment (Tables 2 and 3). Between the doses, similar accumulation ratios were found. Treatment with cimetidine and quinidine significantly enhanced the cellular rhodamine 123 uptake.

Table 3
Dose-dependent accumulation of rhodamine 123 in the isolated perfused rat kidney [a]

Dose (μg)	Amount in kidney (μg/g)	Kidney/perfusate ratio	*n*
15	5.0 ± 1.2	350 ± 140	4
52.5	17 ± 1	270 ± 60	4
150	52 ± 8	360 ± 100	4
150 + VP10 [b]	64 ± 28	350 ± 180	4
150 + VP50 [c]	57 ± 5	350 ± 50	4

[a] Mean data ± S.D. are presented. Measurements as described in Section 2. In the presence of [b] 10 μM verapamil and [c] 50 μM verapamil; *n* represents the number of perfused kidneys used.

4. Discussion

The kidneys used in this study showed a good renal functioning, and were stable for at least 2 h. Surprisingly, the fractional excretion (%) of sodium, calcium and glucose decreased after addition of rhodamine 123, indicating that kidney viability increased as compared to control experiments. This is in contrast with several in vitro investigations showing that rhodamine 123 possesses a cytotoxic potential, including uncoupling of oxidative phosphorylation and mitochondrial swelling (Bernal et al., 1983; Emaus et al., 1986; Lampidis et al., 1983). The reason that we did not find a decrease in renal function is

Table 2
Effect of various drugs on the accumulation of rhodamine 123 in the isolated perfused rat kidney [a]

Treatment	Amount in kidney (μg/g)	Kidney/perfusate ratio	*n*
Control (dose = 1.5 μg)	0.53 ± 0.06	350 ± 50	4
+ VP10 [b]	0.57 ± 0.05	340 ± 50	4
+ VP50 [c]	0.60 ± 0.03	390 ± 80	4
+ CM10 [d]	0.85 ± 0.13 [h]	470 ± 160	6
+ DGX10 [e]	0.56 ± 0.05	490 ± 270	4
+ CSA6 [f]	0.41 ± 0.30	330 ± 170	3
+ QD6 [g]	0.67 ± 0.07	750 ± 70 [h]	3

[a] Mean data ± S.D. are presented. Measurements as described in Section 2. In the presence of [b] 10 μM verapamil, [c] 50 μM verapamil, [d] 10 μM cimetidine, [e] 10 μM digoxin, [f] 6 μM cyclosporin A and [g] 6 μM quinidine. [h] $P < 0.05$: significantly different from the same dose without treatment; *n* represents the number of perfused kidneys used.

most likely a result of the low concentrations we used; perfusate concentrations were between 25 nM and 2.5 μM. At these concentrations other investigators also found little or no toxic effects for rhodamine 123 (Emaus et al., 1986). However, we have no explanation for the slight increase in proximal tubular function observed in the present study. Kidney functional parameters were hardly changed due to the presence of verapamil, cimetidine, cyclosporin A, quinidine or digoxin in perfusion fluid. In contrast with the known renal effects of verapamil (Chan and Schrier, 1990), the fractional excretion of calcium and glomerular filtration rate were not enhanced in the isolated perfused rat kidney, which can be explained by the fact that blood vessels are maximally dilated in this preparation.

Our clearance experiments demonstrate that the cationic drug, rhodamine 123, is efficiently secreted by the perfused kidney in a dose-dependent manner. Fig. 2 shows that the Cl_R/GF of the lowest dose is significantly higher than that for the highest dose (3.1 ± 0.5 vs. 1.6 ± 0.2; $P < 0.01$), indicating active, saturable secretion. However, it is unlikely that P-glycoprotein is involved. Cyclosporin A, a potent P-glycoprotein inhibitor, which has been shown to inhibit rhodamine 123 efflux in multidrug resistant cells (Lee et al., 1994) and in the isolated perfused rat liver (Stapf et al., 1994), failed to reduce the renal excretion of the compound in the isolated perfused rat kidney. Furthermore, digoxin, a substrate for P-glycoprotein, was not able to affect rhodamine 123 transport either. Two other P-glycoprotein inhibitors, verapamil and quinidine, significantly reduced renal rhodamine 123 clearance, however, this effect is probably related to the organic cation carrier of which both compounds are also known to be good inhibitors (Pritchard and Miller, 1993). Supporting evidence is given by cimetidine, an effective inhibitor of the organic cation carrier (Somogoyi, 1987; Boom et al., 1992), which reduced the renal rhodamine 123 clearance by as much as 70%. It was recently speculated that cimetidine might also be a substrate for P-glycoprotein, because in a distal tubule derived epithelial cell line (Madin-Darby canine kidney cells) transfected with human P-glycoprotein an increased capacity for cimetidine transport from basolateral to luminal membrane was observed (Pan et al., 1994). However, in the same model it was also shown that cimetidine was not able to inhibit transport of P-glycoprotein substrates. Additionally, cimetidine had no effect on the P-glycoprotein-mediated biliary excretion and disposition of cyclosporin A (Hughes et al., 1995), an inhibitor as well as a substrate for P-glycoprotein (Saeki et al., 1993). Therefore, we believe that the inhibitory potency of cimetidine is related to inhibition of the organic cation system, although the involvement of P-glycoprotein-mediated transport in overall renal rhodamine 123 excretion can not be ruled out completely, because both transport systems exhibit overlapping substrate specificities (Ford and Hait, 1990). A recent study with daunomycin in renal proximal tubules of killifish revealed that P-glycoprotein and the organic cation system may provide parallel excretory pathways for drugs in the kidney (Miller, 1995).

Organic cation transport in renal proximal tubules involves entry into the tubular cell by (facilitated) diffusion, for which the potential difference over the basolateral membrane of -60 to -80 mV is the driving force (Pritchard and Miller, 1993). Although the contribution of a nonsaturable active uptake mechanism for rhodamine 123 into proximal tubular cells can not be ruled out completely, the similar accumulation ratios at different perfusate concentrations and the lack of an effect of any of the inhibitory agents on the perfusate concentration-time curves suggest that the compound enters the cells by (facilitated) diffusion. After preloading the kidneys with a high dose of rhodamine 123, a substantial amount of the drug was transported back into the perfusion fluid, suggesting that the basolateral organic cation carrier is bidirectional and driven by the concentration gradient. Verapamil significantly reduced rhodamine 123 back-flux by about 50%, indicating that this transport is specific. These two properties are in agreement with the characteristics found for facilitated diffusion, as has been described for the basolateral organic cation system.

The next step in proximal tubular secretion is transport into the luminal compartment. For organic cations this step is active and in exchange for protons (Pritchard and Miller, 1993). Since neither one of the inhibitors affected the plasma concentration-time curves, active secretion may be considered to take place at the luminal membrane. An important finding in the characterization of the overall excretion of rhodamine 123 is that the rate-limiting step in secretion is transport from tubular cells into the luminal compartment. Pietruck and Ullrich (1995) showed previously that rhodamine 123 exhibits affinity for the renal organic cation system, but to a higher extent for the luminal carrier in comparison to the basolateral carrier. Furthermore, they also observed a net reabsorption in their in situ perfused rat kidney preparation, but in contrast to our results they suggested that reabsorption is an active mechanism. The preload experiments in our study revealed that reabsorption is a passive process.

Because perfusate clearance was initially very rapid and urinary excretion delayed, a substantial amount of rhodamine 123 must be retained by the tubular cells. We were able to estimate the amount accumulated in kidney tissue accurately in the course of the experiment. The extensive accumulation found for rhodamine 123 in the isolated perfused rat kidney, with tissue-to-medium concentrations up to 360, can not be simply explained by uptake driven by the electrochemical gradient, which is the mechanism for basolateral entry of organic cations. The similar accumulation ratios at different perfusate concentrations may signify that uptake of rhodamine 123 is not mediated by an active mechanism, which is able to transport the organic cation to levels above its electrochemical gradient. There-

fore, the drug is most likely subject to intracellular sequestration. In addition, the biphasic profile of the perfusate concentration-time curves suggests the existence of an intracellular compartment in the renal handling of rhodamine 123. Earlier reports showed that rhodamine 123 accumulates specifically in mitochondria (Bernal et al., 1983; Emaus et al., 1986), probably driven by the inside negative membrane potential of these organelles (−220 mV). Preliminary uptake experiments in isolated proximal tubular cells of the rat kidney using confocal microscopy, demonstrated that intracellular accumulation may include more than just mitochondrial compartmentation (Masereeuw et al., unpublished findings). Additional organellar compartments that sequester this cationic dye may be endosomes, lysosomes and Golgi vesicles. These organelles are more acidic than the surrounding cytoplasm, and the inside negative pH gradient may drive organic cation uptake (Mellman et al., 1986). A recent study on tetra-ethylammonium accumulation in rat kidney cortex endosomal vesicles showed that uptake is specific, ATP-dependent, and driven by a pH-gradient (Pritchard et al., 1994). Whether or not such a specific sequestration mechanism is involved in rhodamine 123 accumulation remains to be determined.

Significant enhancement of rhodamine 123 accumulation in the perfused kidney was found for cimetidine and quinidine, probably due to inhibition of the cation system at the luminal membrane. For cimetidine it has been reported that this cationic drug exhibits a higher affinity for the luminal transporter than for the basolateral system (Pietruck and Ullrich, 1995), and possesses also a higher inhibitory potency against the luminal system (Boom et al., 1992). Less clear is why verapamil did not reduce the renal accumulation of rhodamine 123. An explanation may be that verapamil reduced both influx and efflux of the organic cation. Supporting evidence is given by the preload experiments in which verapamil was able to affect the back-flux of rhodamine 123 into perfusion medium by inhibition of the basolateral system.

In summary, rhodamine 123 is actively secreted in the isolated perfused rat kidney. Transport across the luminal membrane into the tubular urine was found to be the rate-limiting step in overall excretion. Renal clearance was susceptible to inhibition by cimetidine, quinidine and verapamil, but not to cyclosporin A and digoxin. These observations suggest that rhodamine 123 is predominantly secreted by the organic cation transport system in the isolated perfused rat kidney, and that P-glycoprotein transport plays a minor role.

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