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Transport functions of the liver. Lack of correlation between hepatocellular ouabain uptake and binding to $(Na^+ + K^+)$-ATPase

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Ouabain uptake was studied on isolated rat hepatocytes. Hepatocellular uptake of the glycoside is saturable ($K_m = 348$ μmol/l, $V_{max} = 1.4$ nmol/mg cell protein per min), energy dependent and accumulative. Concentrative ouabain uptake is not present on permeable hepatocytes, Ehrlich ascites tumor cells and AS-30D ascites hepatoma cells. There is no correlation between ouabain binding to rat liver $(Na^+ + K^+)$ATPase and ouabain uptake into isolated rat hepatocytes. While ouabain uptake is competitively inhibited by cevadine, binding to $(Na^+ + K^+)$-ATPase is not affected by the alkaloid. Although the affinities of digitoxin and ouabain to $(Na^+ + K^+)$-ATPase are similar, digitoxin is 10000-times more potent in inhibiting [^{3}H]ouabain uptake as compared to ouabain. That binding to $(Na^+ + K^+)$-ATPase appears to be no precondition for ouabain uptake was also found in experiments with plasmamembranes derived from Ehrlich ascites tumor cells and AS-30D hepatoma cells. While tumor cell $(Na^+ + K^+)$-ATPase is ouabain sensitive, the intact cells are transport deficient. Hepatic ouabain uptake might be related to bile acid transport. Several inhibitors of the bile acid uptake system also inhibit ouabain uptake.

Introduction

Since the early observation of high concentration of water-soluble digitalis glycosides in rat bile [1] and an enterohepatic circulation [2] it became evident that ouabain is excreted into bile by active transport [3,4]. It was assumed that the active excretion system was localized in the canalicular membrane of hepatocytes.

Later, however, carrier-mediated uptake of ouabain into isolated rat liver cells was found [5] indicating that transport properties of the sinusoidal site contribute to hepatic glycoside clearance. The uptake of ouabain into hepatocytes was competitively blocked by taurocholate [6]. This gave evidence that in addition to phalloidin [7,8] and iodipamide [9] the digitalis glycosides might be substrates of the multispecific bile acid transport system of rat hepatocytes.

Recently, another uptake mechanism of ouabain was found on HeLa cells [10,11]. Accumulation of the drug within lysosomes suggested an endocytotic internalization. It was proposed that $(Na^+ + K^+)$-ATPase is responsible for receptor mediated vesicular ouabain uptake in HeLa cells. A similar conclusion derived from clearance studies performed with isolated perfused rat livers [12]. Again uptake of an ATPase-ouabain complex was also claimed for liver cells. The studies so far published on carrier-mediated ouabain uptake [5,6] do not exclude the possibility of an endocytotic uptake mechanism on liver cells. In the present study we compared hepatocellular ouabain uptake with binding to $(Na^+ + K^+)$-ATPase to find out whether $(Na^+ + K^+)$-ATPase internalization is an additional uptake process for ouabain in the liver.

Methods and Materials

Preparation of rat hepatocytes. Hepatocytes from Wistar rats were prepared by perfusion of the liver with 0.05% collagenase [13] in Krebs-Henseleit buffer in the absence of Ca^{2+}. Details of the isolation procedure are given elsewhere [14]. Hepatocytes were equilibrated for 30 min in Tyrode buffer (pH 7.4) at 37°C in O_2/CO_2 (95%/5%) atmosphere and used within 2 h. One ml cell suspension contained $2 \cdot 10^6$ hepatocytes corresponding to 3.8 mg cell protein. Most of the isolated hepatocytes (80–90%) were viable as judged by 0.2% Trypan blue.

AS-30D ascites hepatoma cells, grown in the abdomen of Sprague-Dawley rats, were harvested 8 days after inocculation. Cells were washed in Tyrode buffer and incubated at identical conditions as described for isolated hepatocytes.

Ehrlich ascites tumor cells of white mouse were harvested 10 days after inocculation. The washing and incubation conditions were the same as for AS 30D cells [15].

Measurement of the ouabain uptake into isolated cells. Uptake of [^{3}H]ouabain was measured either in 2 ml suspension of isolated rat hepatocytes ($2 \cdot 10^6$ cells/ml corresponding to 3.8 mg cell protein) or in 2 ml of tumor cells ($14 \cdot 10^6$ cells/ml corresponding to 4.0 mg cell protein) during 30 min by taking off 100 µl aliquots at 15, 45, 75, 105, 135, 180, 300 s and 5, 10, 15, 20 and 30 min. Separation of cells from extracellular fluid was achieved by centrifugation through silicon oil according to [16,17]. Radioactivity was measured in Lipoluma/Lumasolve/water scintillation cocktail (100 : 10 : 2, v/v) in Packard Tri-Carb 2660. Permeabilization of hepatocytes was achieved by rapid freezing in N_2 liquid and thawing at 37°C.

Preparation of plasma membranes from rat liver, AS-30D hepatoma cells and Ehrlich ascites tumor cells. Plasma membranes from rat liver were prepared according to Ref. 18. Standard enzyme characterization (5'-nucleotidase EC 3.1.3.5, glucose-6-phosphatase EC 3.1.3.9 and ATPase EC 3.6.1.3) indicated an enrichment of the plasma-membrane fraction by a factor of 10 to 40. Ehrlich ascites tumor cell plasma membranes were prepared according to Ref. 19. Enzyme characterization (NADH-dehydrogenase EC 1.6.99.3, ATPase EC 3.6.1.3, succinate dehydrogenase EC 1.3.99.1, glucose-6-phosphate dehydrogenase EC 1.1.1.49, DNA) gave a purification factor of 10. Plasma membranes from AS-30D hepatoma cells were prepared according to Ref. 18 from cells which were first subjected to a cavitation-excavitation procedure in a French press. Details of the procedure are given by Ref. 15. The membrane marker enzyme nucleotide pyrophosphatase EC 3.1.4.1 was enriched 15-fold when compared to glucose-6-phosphatase EC 3.1.3.9 activity.

Purification of ox brain ($Na^+ + K^+$)-ATPase. Ox brain ($Na^+ + K^+$)-ATPase was prepared essentially according to Ref. 20. The purity of the enzyme was 96–98% as was measured by the degree of inhibition in presence of 1 mM ouabain. The specific activity of the enzyme was 2.2 U/mg protein. Enzyme samples were stored at −70°C and used for ($Na^+ + K^+$)-ATPase assay immediately after thawing.

($Na^+ + K^+$)-ATPase assay. Determination of ($Na^+ + K^+$)-ATPase activity was performed by a spectrophotometric method described by Ref. 21. Activity was measured at 334 nm by adding 50–100 µg membrane protein (20 µl) to 945 µl reaction buffer (consisting of 130.9 mM Tris, 1.05 mM EGTA, 125.6 mM NaCl, 13.9 mM KCl, 5.24 mM NaN_3 (pH 7.4)) 25 µl Mg^{2+}-ATP (200 mM ATP in 200 mM $MgCl_2$) and 10 µl lactate dehydrogenase/pyruvate kinase with 1000–2000 units of each enzyme in 1 ml reaction buffer.

Protein determination. Protein determination was performed by the biuret method with bovine serum albumin as a standard.

Materials. Collagenase type II, ATP and NADH was from Boehringer Mannheim, lactate dehydrogenase and pyruvate kinase from Sigma Munich. Ouabain was from Serva Heidelberg and Cevadine was from Roth Karlsruhe. Cassaine was a gift of Professor Dr. Wassermann, Kiel. [^{3}H]Ouabain, spec. act. 1.18 TBq/mmol and ^{86}Rb spec. act. 37–296 MBq/mg was purchased from Amersham Buchler Braunschweig.

Results

Saturability of ouabain uptake into intact hepatocytes

Isolated rat hepatocytes accumulate ouabain by

TABLE I

INHIBITION OF [^{3}H]OUABAIN UPTAKE BY METABOLIC INHIBITORS

$2 \cdot 10^6$ hepatocytes/ml were incubated 30 s prior to the addition of 1 ng [^{3}H]ouabain/10 μg ouabain in the presence of 10 μg antimycin A, 4 μg carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 0.1 μg valinomycin. Oligomycin, 10 μg/ml, was added 10 min prior to ouabain. [^{3}H]Ouabain uptake was measured during 30 min. V_i was calculated from the initial linear slope of the uptake curves between 15 s and 5 min. Values are given in percent of uptake of controls which received 10 μl ethanol/ml ($n = 3$ or 4, $\bar{x} \pm$S.D.). Corresponding kinetic values for controls are: $V_i = 61.7 \pm 17.8$ pmol/mg cell protein per min; Δ 15-min uptake $= 794 \pm 143$ pmol/mg cell protein; Δ 30-min uptake $= 1154 \pm 197$ pmol/mg cell protein.

Compound	%inhibition		
	v_i	Δ 15-min uptake	Δ 30-min uptake
Antimycin	53± 4	58±5	73±9
Oligomycin	89± 3	87±3	91±3
CCCP	44±12	55±6	75±3
Valinomycin	35± 8	41±4	68±3

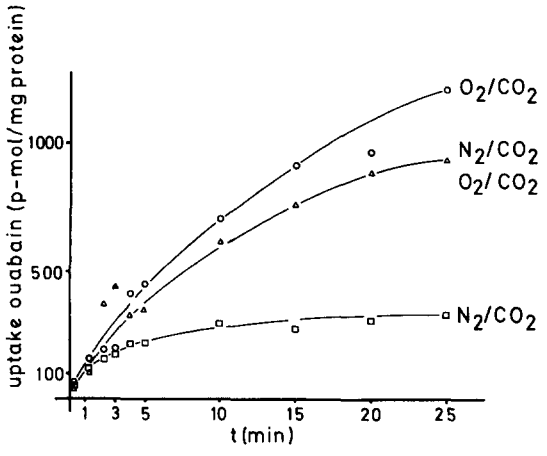

Fig. 1. Modification of ouabain uptake by anaerobic conditions. $2 \cdot 10^6$ isolated hepatocytes/ml were incubated 30 min under O_2/CO_2 (○) or N_2/CO_2 (□) atmosphere before adding 1.5 ng [^{3}H]ouabain/10 μg ouabain. At the time indicated 100-μl aliquots of cell suspension were withdrawn by a syringe through a rubber diaphragma. △———△, uptake of ouabain into liver cells which were gassed 30 min by N_2/CO_2 followed by reoxygenation for 30 min with O_2/CO_2. Data are from a representative experiment out of four.

saturable uptake. The kinetic constants are $K_m =$ 348 μmol/l and $V_{max} = 1.4$ nmol/mg cell protein per min for rat hepatocytes. Ouabain is concentrated within rat liver cells. At 10 μM extracellular ouabain the intracellular concentration reached within 60 min was calculated to be 267 μM,

TABLE II

INHIBITION OF [^{3}H]OUABAIN UPTAKE BY INHIBITORS OF THE BILE ACID TRANSPORT SYSTEM IN ISOLATED RAT HEPATOCYTES

Isolated rat hepatocytes were incubated in the presence of inhibitors of bile acid uptake 30 s prior [^{3}H]ouabain. DIDS was added 20 min (cholate, ouabain) and 30 min (BSP) prior to the uptake experiments. Uptake of bromo[^{35}S]sulfophthalein was measured because this compound is not taken up by the sodium dependent bile acid transport system [9,34]. Experiments were performed with three or four different cell preparations. Large standard deviation of bromo[^{35}S]sulfophthalein uptake was due to negative values (stimulated uptake) in some experiments. The absolute values for controls are:
v_i ouabain (determined from Δ15 s–5 min uptake) $= 62.5 \pm 11.4$ pmol/mg protein per min,
v_i cholate (determined from Δ15 s–75 s uptake) $= 47.7 \pm 8.6$ pmol/mg protein per min,
v_i bromosulfophthalein (from Δ15 s–75 s uptake) $= 146 \pm 22$ pmol/mg protein per min.

Inhibitors [a] (μM)		%Inhibition of the initial rate of uptake v_i ($\bar{x} \pm$S.D.)		
		[^{14}C]Cholate (1 μM)	[^{3}H]Ouabain (15 μM)	Bromo[^{35}S]sulfophthalein (24 μM)
Cevadine,	1000	81±8	93± 5	13±15
DIDS,	50	44±9	29± 7	8± 8
Probenecid,	35	11±5	7± 5	2± 2
Probenecid,	350	55±2	48± 5	16± 7
Furosemide,	100	39±3	5± 4	16±14
Furosemide,	250	68±8	27± 7	27±18
p-AH,	1000	7±7	14±10	8± 8

[a] DIDS, diisothiocyanostilbenedisulfonic acid; *p*-AH, *p*-aminohippuric acid.

which corresponds to a 26-fold accumulation. Only intact hepatocytes accumulated ouabain. Permeable liver cells contained only trace amounts (Fig. 6).

Energy dependence of hepatocellular ouabain uptake

Accumulation of ouabain within liver cells could either result from intracellular binding or energy-dependent transport. The effect of metabolic inhibitors as well as of anaerobiosis indicates that energy dependent uptake causes ouabain accumulation. Metabolic inhibitors (Table I) inhibited ouabain uptake at concentrations sufficient to reduce hepatocellular ATP content to 1–5% of controls [22]. Nitrogen gassing also reduced ouabain uptake as was previously shown for energy-dependent cholate uptake [22]. The reduced transport was restored by reoxygenation (Fig. 1) indicating that inhibition was not the result of cell destruction.

Inhibition of ouabain uptake by cevadine and organic anions

The results so far demonstrated are accepted criteria for carrier-mediated transport but do not exclude the possibility of receptor-mediated vesicular uptake by $(Na^+ + K^+)$-ATPase internalization. To distinguish both uptake mechanisms transport inhibitors were tested. Cevadine is an inhibitor of bile acid transport without inhibiting bromosulfophthalein uptake (Ref. 23 and Table II). 1 mM of the steroidal alkaloid completely blocked ouabain uptake into isolated rat hepatocytes.

The type of inhibition was competitive (Fig. 2); $K_i = 13$ μM. Further inhibitors of the bile acid transport system were tested (Table II). A parallism of inhibition of ouabain uptake and cholate

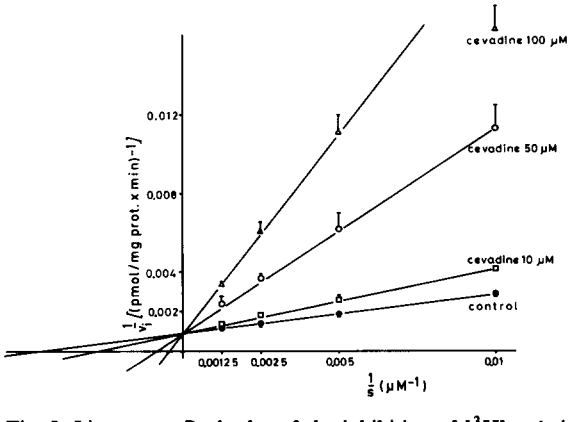

Fig. 2. Lineweaver-Burk plot of the inhibition of [^{3}H]ouabain uptake by cevadine. [^{3}H]Ouabain uptake by $2 \cdot 10^6$ hepatocytes/ml was measured in the presence of 10 μM (□), 50 μM (○) and 100 μM (△) cevadine. Cevadine was added 30 s prior to ouabain. 2 ng [^{3}H]ouabain was diluted with 100, 200, 400 and 800 μM nonlabelled ouabain (●). Experiments were performed with three or four different cell preparations. K_m in these experiments was 210 μM, $V_{max} = 1111$ pmol/mg cell protein per min. $K_i = 13 \pm 2$ μM was determined according to $K_i = K_m \cdot [I]/(K'_m - K_m)$. For several data, error bars are within the curve symbols.

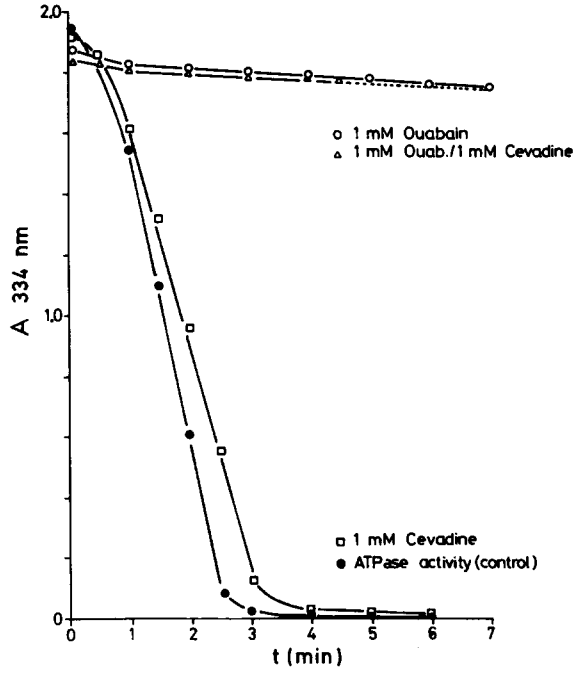

Fig. 3. Fig. 3. Effect of cevadine on the activity of purified ox brain $(Na^+ + K^+)$-ATPase. Enzyme activity was measured either in the presence of 1 mM ouabain (○), 1 mM cevadine (□) and 1 mM ouabain plus 1 mM cevadine (△). From the slope of the curves ATPase activity was calculated according to Ref. 21. The figure shows a representative experiment out of four. In this experiment control activity (●) was 110 μmol P_i/mg protein per h corresponding to 100%. Plus 1 mM ouabain activity was reduced to 4.6 μmol P_i/mg per h (4.2% of control), plus cevadine and ouabain to 5.1 μmol P_i/mg per h (4.6%). In the presence of 1 mM cevadine enzyme activity was 97 μmol P_i/mg per h (88%). The mean data out of the four experiments are ($\bar{x} \pm$ S.D.): control = 145 ± 31 μmol P_i/mg per h; 1 mM ouabain = 4.2 ± 1.2; 1 mM ouabain plus 1 mM cevadine = 4.2 ± 1.3; 1 mM cevadine = 120 ± 26 μmol P_i/mg protein per min.

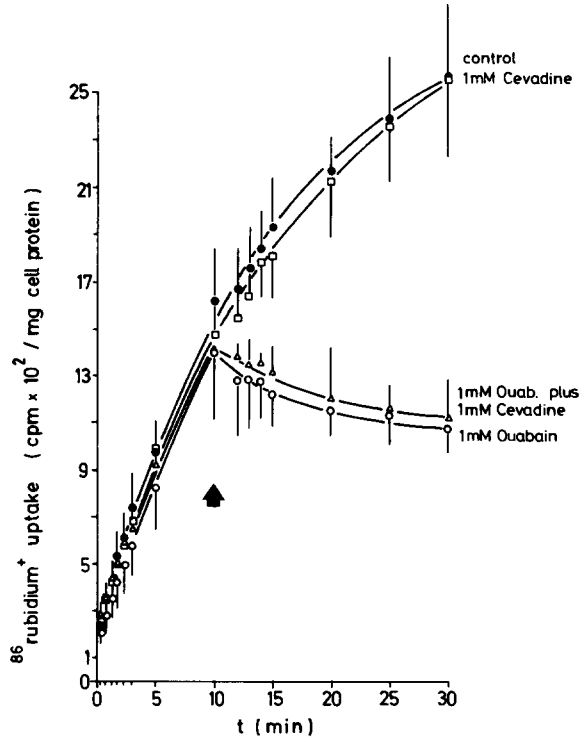

Fig. 4. Effect of cevadine on $^{86}Rb^+$ uptake into isolated rat liver cells. $2 \cdot 10^6$ hepatocytes/ml were incubated in the presence of 1–5 μCi $^{86}Rb^+$ for 30 min at 37°C under O_2/CO_2 (95/5) atmosphere in Tyrode buffer containing 2.7 mM KCl. 1 mM ouabain (○), 1 mM cevadine (□) and 1 mM ouabain plus 1 mM cevadine (△) were added after 10 min (arrow). Data are the mean out of experiments with three different cell preparations ($n = 3$, $\bar{x} \pm$ S.D.).

uptake could be demonstrated. Probenecid, DIDS and furosemide were inhibitors of bile acid and ouabain uptake whereas *p*-aminohippurate was ineffective on both transports.

Lack of effect of cevadine on ($Na^+ + K^+$)-ATPase

Cevadine was tested for inhibition of ($Na^+ + K^+$)-ATPase. Although hepatocellular ouabain uptake was blocked completely, 1 mM cevadine inhibited ($Na^+ + K^+$)-ATPase purified from ox brain only slightly to 12%. Inhibition by 1 mM ouabain which was for comparison 97% lasted even in the presence of 1 mM cevadine (Fig. 3). In addition, cevadine did not effect ($Na^+ + K^+$)-ATPase activity of vital isolated rat hepatocytes. This was checked indirectly by measuring fluxes of ^{86}Rb through the liver cell membrane in the presence of ouabain and cevadine (Fig. 4). In contrast to ouabain which inhibited ^{86}Rb influx and enhanced ^{86}Rb efflux, cevadine was without any effect (Fig. 4).

Inhibition of ouabain uptake by inhibitors of ($Na^+ + K^+$)-ATPase

Digoxin, digitoxin and cassaine inhibit ($Na^+ + K^+$)-ATPase in addition to ouabain. The IC_{50} concentration for enzyme inhibition are comparable with ouabain [24–27]. The effect on ouabain uptake was, however, markedly different. Cassaine was 10-times, digoxin 1000-times and digitoxin

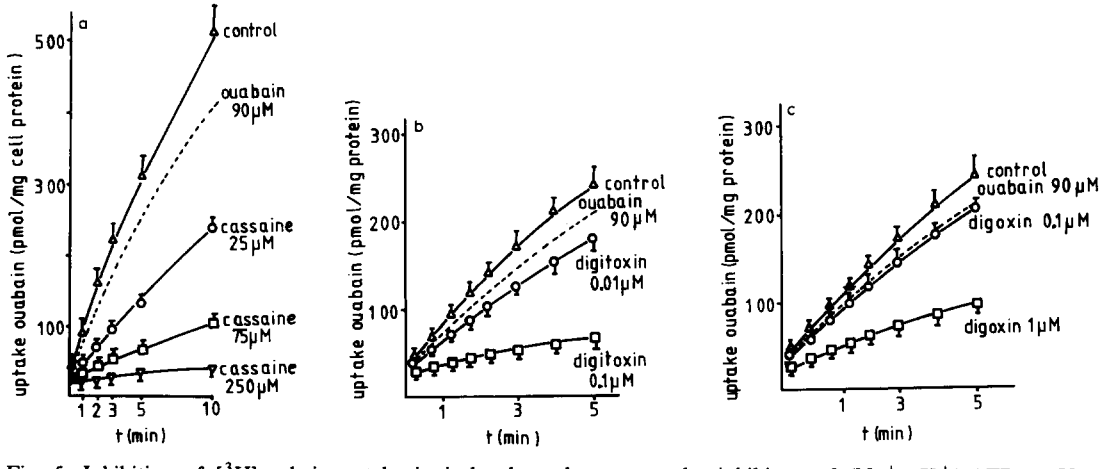

Fig. 5. Inhibition of [^{3}H]ouabain uptake in isolated rat hepatocytes by inhibitors of ($Na^+ + K^+$)-ATPase. Uptake of 2 ng [^{3}H]ouabain/10 μg ouabain by $2 \cdot 10^6$ hepatocytes/ml was measured in the presence of 25, 75, and 250 μM cassaine (a), 0.1 and 0.01 μM digitoxin (b), 1.0 and 0.1 μM digoxin (c) and 90 μM ouabain for comparison (dotted line). Data are out of experiments with four different cell preparations, $\bar{x} \pm$ S.D.

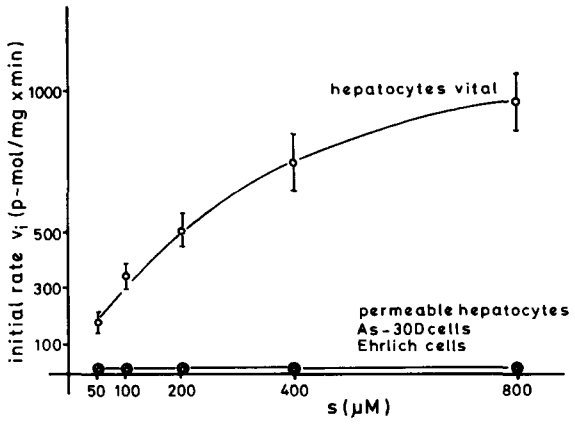

Fig. 6. Cell specificity of saturable ouabain uptake. [^{3}H]Ouabain uptake in the presence of 50, 100, 200, 400 and 800 μM ouabain was measured in vital isolated rat hepatocytes, AS-30D ascites hepatoma cells, Ehrlich ascites tumor cells and hepatocytes which were made permeable by freezing and thawing. The initial rate of uptake of 0.2 μCi [^{3}H]ouabain/ml was determined at 37°C within 135 s. $n = 3$, $\bar{x} \pm$S.D.

10000-times more effective in uptake inhibition than ouabain (Fig. 5).

Cell specificity of ouabain uptake

Energy-dependent ouabain uptake was cell specific. Ouabain was neither taken up by AS-30D hepatoma cells nor by Ehrlich ascites tumor cells (Fig. 6). Small amounts of radioactivity were trapped by the cells probably by adsorption. Provided that $(Na^+ + K^+)$-ATPase is essential in ouabain uptake, lack of ouabain uptake in these tumor cells could be due to enzyme defect. It was tested whether AS-30D cells and Ehrlich tumor cells lack ouabain-sensitive $(Na^+ + K^+)$-ATPase. However, in plasma membranes prepared from both cell lines ouabain-sensitive ATPase activity was found (not shown). The results indicated that these tumor cells are inactive with respect to ouabain uptake but not with respect to binding to $(Na^+ + K^+)$-ATPase.

Discussion

Ouabain is taken up into HeLa cells by internalization of $(Na^+ + K^+)$-ATPase [11,28], the rate of uptake being identical to the rate of $(Na^+ + K^+)$-ATPase turnover in plasma membranes [29]. Sequestration of ouabain within lysosomes which follows uptake, was regarded to be essential for recovery of blocked $(Na^+ + K^+)$-ATPase. Our results, on the contrary, clearly demonstrate that uptake of ouabain by $(Na^+ + K^+)$-ATPase internalization is not responsible for hepatic glycoside clearance and do not support findings by Ref. 12 who suggested the uptake of an ATPase-ouabain complex by the isolated perfused rat liver. The results strongly support the presence of additional ouabain recognizing proteins on rat liver plasma membranes with properties for glycoside translocation. Cevadine seems to be a tool for discrimination of both processes since uptake but not binding to $(Na^+ + K^+)$-ATPase was inhibited exclusively (Fig. 2, 3). In addition the $(Na^+ + K^+)$-ATPase inhibitors cassaine, digoxin and digitoxin were by far stronger inhibitors of [^{3}H]ouabain uptake than ouabain itself. Provided that these compounds share the same transmembrane pathway, strong inhibition may be due to their lipophilicity. These inhibitors may dissociate more slowly apart from the transport system and therefore block ouabain uptake more effectively. Actually the strong inhibition of [^{3}H]ouabain uptake by digitoxin which exceeds that by ouabain by 10000-fold points out that digitoxin uptake, at least partially, may not be a simple diffusion as has been suggested [30].

Ouabain uptake, as described for hepatocytes, was not present in tumor cells. Ehrlich ascites tumor cells and AS-30D hepatoma cells were transport deficient but not deficient with respect to binding to $(Na^+ + K^+)$-ATPase. This is a further argument that glycoside binding to ATPase is no prerequisite for cellular ouabain uptake.

The question is now, what is the nature of the ouabain transport system in rat liver. Our results indicate that the bile acid uptake system might transport ouabain. The arguments are: Common inhibitors of cholate and ouabain uptake are cevadine, probenecid, DIDS and furosemide (Table II); Ehrlich ascites tumor cells (unpublished) as well as AS-30D hepatoma cells [7,31] lack bile acid as well as ouabain uptake (Fig. 6); ouabain uptake in isolated rat hepatocytes is competitively blocked by taurocholate [6].

In former studies we have shown that the uptake of the cyclic peptides phalloidin and antamanide [8,9] and of the organic anion

iodipamide [9] is competitively blocked by bile acids. We propose that steroidal compounds like ouabain and cevadine might be further substrates of that multispecific uptake system.

Originally ouabain was regarded a model compound for the neutral transportsystem at the canalicular pole of hepatocytes [4,32]. This excretory system for noncharged xenobiotics was postulated in addition to the excretion systems for organic cations and bile acids [33]. At the hepatocyte's sinusoidal membrane, however, where uptake mechanisms are located, either an overlapping substrate specificity between the bile acid and a neutral transport system exists or both systems are components of a common one.

Acknowledgements

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