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Membrane transport of conjugated and unconjugated bile acids into hepatocytes is susceptible to SH-blocking reagents

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The present study indicates that SH-groups are essential for the uptake of [^{3}H]taurocholate and [^{14}C]cholate into isolated rat hepatocytes. Several sulfhydryl-modifying reagents viz. *p*-chloromercuribenzenesulfonate (PCMBS), *N*-ethylmaleimide (NEM), dithio-bis(5-nitropyridine) (DTNP), bromosuccinimide and $HgCl_2$ inhibited uptake of bile acids in a concentration-dependent manner. PCMBS was the most effective inhibitor in the uptake of taurocholate, while NEM is preferentially blocking the cholate uptake. PCMBS inhibited both the sodium- dependent and the sodium-independent bile acid uptake. Two different moieties of SH-groups seemed to be important for bile acid transport. One group was susceptible to DTNP and NEM, whereas PCMBS was able to block another type of SH-groups in addition. Cell viability was altered by SH-blockers, except by PCMBS. Efflux studies with $^{86}Rb^+$ demonstrated that the transmembrane potential of hepatocytes was less effected by 100 μM PCMBS in contrast to 100 μM $HgCl_2$. Efflux of tetra[^{3}H]phenylphosphonium and of [^{3}H]aflatoxin in PCMBS-treated hepatocytes documented membrane integrity during at least 10 min. PCMBS did not reduce cellular ATP levels significantly (minus 7%) nor did it markedly increase the amount of the Trypan-blue stained hepatocytes (plus 8.5%). The blocking effect of PCMBS was immediate and was completely reversed by the addition of 500 μM dithiothreitol (DTT), indicating a specific interaction with sulfhydryl-groups. This antagonizing effect of DTT depends on the concentration and exposure time of PCMBS. Six other thiols viz. 2-mercaptoethanol, 1,2-dimercaptoethane, 1,4-dimercaptobutane, 1,6-dimercaptohexane, L-cysteine and L-glutathione were less effective. The results suggest that free SH-groups on the outer surface of hepatocytes play an important role in the uptake process for conjugated and unconjugated bile acids.

Introduction

Conjugated and unconjugated bile acids are transfered into hepatocytes by membrane proteins. Two proteins of 48–49 and 52–54 kDa have been identified by affinity labeling techniques [1–3]. Taurocholate is transported by the 48–49 kDa protein [4], whereas cholate is assumed to be the main substrate for the 52–54 kDa protein [3,5].

Another important approach to characterize the membrane transport of bile acids was the determination of the driving forces. Taurocholate uptake into hepatocytes is nearly exclusively sodium dependent [6–8], while for cholate an additional sodium independent saturable uptake component was described [6,9]. This component was identified in plasma membrane vesicles to be a carrier with characteristics of a hydroxyl/cholate exchange mechanism [10].

Recently, a great deal of interest has been focused on the role of SH-groups in membrane carrier proteins and their functional importance in the regulation of substrate transport across biological membranes. The role of essential sulfhydryl groups has been demonstrated in the system A-mediated transport of amino acids in primary rat hepatocytes cultures [11,12]. Furthermore the uptake of sulfobromophthalein into rat liver plasma membrane vesicles was susceptible to SH-group blocking reagents [13]. Additional examples for transport systems on non-liver cells with functional SH-groups were documented by several authors [14–17].

In order to get information about the importance of SH-groups in the bile acid uptake and to elucidate a possible difference between the taurocholate and cholate carrier proteins, we investigated the effects of SH-blocking reagents on the uptake of [^{3}H]taurocholate and

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[^{14}C]cholate into isolated rat hepatocytes. The results demonstrate that SH-groups are essential for both, sodium-dependent and sodium-independent uptake of conjugated as well as of unconjugated bile acids.

Methods and Materials

Cell preparation

Male Wistar rats of 260–300 g body weight were kept on a standard diet and water ad libitum. Hepatocytes were prepared by using a modified protocol of Berry and Friend [18,19]. In brief, the rats were anesthetized with urethane by intraperitoneal injection (1 g/kg body weight) and 1000 U heparin (Liquemin^R) were administered via the femoral vein. The portal vein and the thoracic inferior of the vena cava were cannulated and the liver was flushed in situ with 37°C, Ca^{2+}-free Krebs-Henseleit buffer. A recirculating perfusion was started with 0.05% collagenase in Krebs-Henseleit solution for 15 min. The liver was then sliced and replaced in collagenase buffer. Within two min cells were dissociated by bubbling O_2/CO_2 into the suspension. The hepatocytes were separated from cell debris and non-parenchymal cells by centrifugation at $40 \times g$ (twice) for 5 min. The isolated hepatocytes were equilibrated for 30 min in Tyrode buffer (pH 7.4) at 37°C in O_2/CO_2 (95 : 5) atmosphere and were used within 2 h in a concentration of $2 \cdot 10^6$ hepatocytes/ml (3.8 mg cell protein/ml). 85–90% of the isolated cells were viable as jugded by the Trypan-blue test. Protein concentration was analysed with Bio-Rad reagent according to the method of Bradford [20].

Uptake experiments

Uptake studies with taurocholate were initiated by adding a mixture of [^{3}H]taurocholate/taurocholate at a final concentration of either 11 nM/5 μM or 11 nM/70 μM. [^{14}C]cholate/cholate was used in a final concentration of 1.25 μM/5 μM. Aliquots of 100 μl cell suspension were withdrawn at 15, 45, 75, 105, 135 s and as indicated in the figures. The cells were pelleted by rapid centrifugation through silicon oil according to Ref. 21. The radioactivity of the sedimented cells was measured in Lipoluma/Lumasolve/water mixture (100 : 10 : 2, v/v, Baker Chemicals, Phillipsburg, NJ, U.S.A.) with a Packard counter 2660.

Experiments with sulfhydryl-reacting reagents

p-Chloromercuribenzenesulfonate (PCMBS) and $HgCl_2$ were dissolved in Tyrode buffer. The organic SH-blockers, bromosuccinimide and *N*-ethylmaleimide were used in a 96%-ethanol solution, whereas DMSO was necessary for 2,2′-dithio-bis(5-nitropyridine) (DTNP). None of the solvents extended a concentration of 1–2% in the hepatocyte suspension and had no measurable effect on the uptake of each bile acid. Any particular solution was prepared just immediately before each uptake study. In experiments with PCMBS an equimolar amount of Na_2EDTA was added simultaneously to chelate free mercury that might has been released from the organic mercurial [12]. Na_2EDTA in a concentration up to 1 mM was without an effect on the bile acid uptake. Cell suspensions were incubated with these sulfhydryl reagents for 5 min and bile acid uptake was performed in the presence of these reagents.

In order to document the covalent binding, the sulfhydryl reagents were washed out of the labeled cell suspension. For this purpose hepatocytes were centrifuged within 1–2 s in an Eppendorf 5415 centrifuge and resuspended in Tyrode buffer. This washing procedure was repeated twice. Subsequently uptake experiments were initiated after a 5-min equilibration period.

Mercaptocompound experiments

Seven mercaptocompounds with either one or two SH-groups in different positions at an alkane chain were used. 4 ml hepatocyte suspension was incubated for 5 min with the particular sulfhydryl blocking reagent (dissolved in 96% ethanol). The radioactive labeled bile acids were added and the uptake was measured for 3 min. Then the cell suspension was splitted and placed into two separate flasks, one of them recieved the mercaptocompound in order to reverse the induced inhibition by the particular SH-reagent. The other flask was used as a control.

Choline buffer experiments

Uptake experiments in the absence of Na^+ were performed by substitution of Tyrode buffer A (in mM: 137 NaCl, 2.7 KCl, 1.05 $MgCl_2$, 1.8 $CaCl_2$, 12 $NaHCO_3$, 0.4 $NaH_2PO_4$and 5.6 glucose) with sodium-free buffer B (in mM: 137 choline chloride, 1.05 $MgCl_2$, 1.8 $CaCl_2$, 12 choline bicarbonate, 0.4 KH_2PO_4 and 5.6 glucose). In these experiments hepatocytes, which were maintained in a stock suspension in Tyrode buffer A, were centrifuged and resuspended in buffer B. The washing procedure was repeated twice. Thereafter, cells were kept in shaking Erlenmeyer flasks for 5 min at 37°C before addition of the radioactive compounds.

Efflux studies with $^{86}Rb^+$, tetra[^{3}H]phenylphosphonium and [^{3}H]aflatoxin B_1

Efflux measurements were made by using $^{86}Rb^+$ as a tracer for K^+ [22]. The cells were preloaded with a concentration of 7.4 kBq/ml $^{86}Rb^+$ for 45 min [23]. These cells were centrifuged and the obtained cell pellet was resuspended in $^{86}Rb^+$-free Tyrode buffer. After 5 min equilibration, the hepatocyte suspension recieved the particular sulfhydryl reagent. Aliquots of the suspension were sampled and radioactivity was measured.

Comparable efflux studies were performed with the lipophilic organic cation tetraphenylphosphonium

(TPP^+), which was offered to the liver cells at a concentration of 18.4 kBq [^{3}H]TPP^+ per ml/10 μM TPP^+ for 30 min. TPP^+ is regarded to represent an indicator for transmembrane potential [24,25]. For comparison also the efflux of an uncharged compound, [^{3}H]aflatoxin B_1, was measured. The concentration of [^{3}H]AFB_1 was 125 nM and the incubation time lasted 5 min.

Measurement of ATP

ATP was measured by the luciferin-luciferase method with intact hepatocytes which were preciptated by 10 mM $HClO_4$ [27]. In order to test for an effect of SH-group reagents, the cells were incubated for 15 min in the presence of 100 μM PCMBS or 100 μM $HgCl_2$ at 37°C. ATP content was measured according to the manufactors advice (medical applications, Lumac, Basel) in a Biolumat LB 9505 (Berthold, Wildbad) with Lumit^R standard solution (Lumac, Basel).

Materials

New England Nuclear, Dreieich, F.R.G., supplied [^{3}H]taurocholic acid (specific activity 77.7 GBq/mmol) and [*carboxyl*-^{14}C]cholic acid (specific activity 2.07 GBq/mmol), [^{3}H]aflatoxin B_1 (specific activity 1.44 TBq/mmol), $^{86}Rb^+$ (specific activity 37–296 GBq/mmol) and tetra[^{3}H]phenylphosphonium bromide (specific activity 962 GBq/mmol) were obtained from Amersham, Braunschweig, F.R.G. Unlabeled taurocholic acid was purchased by Carl Roth KG, Karlsruhe, F.R.G. The collagenase was delivered by Boehringer, Mannheim, F.R.G. and the Bio-Rad Protein Assay was from Bio-Rad Labaratories, München. PCMBS was obtaind from Sigma, St. Louis, while all the other chemicals were either from Merck, Darmstadt, F.R.G. or from Serva, Heidelberg, F.R.G.

Calculation and statistics

The initial rates of [^{3}H]taurocholate/(^{14}C)- cholate uptake are determined by the 15, 45, 75 and 105 s values and are expressed as pmol · min^{-1} · mg cell protein^{-1}. All data are presented as $x \pm$ S.D. of at least $n = 3$ experiments, where n gives the number of different cell preparations. The degree of inhibition is calculated by:

TABLE I

The effects of the organic mecurial PCMBS and $HgCl_2$ on bile acid uptake in isolated rat hepatocytes

Hepatocytes in suspension were incubated in the presence of PCMBS and $HgCl_2$ for 5 min and then uptake of either 11 nm [^{3}H]taurocholate/5 μM taurocholate or 1.25 μM [^{14}C]cholate/5 μM cholate was measured. For each bile acid the inhibition is expressed by two parameters, the initial velocity (V_i) and the Δ10 min value. For details see in Methods and Materials. The kind of inhibition was determined by washing the cells twice with Tyrode buffer after 5 min preincubation with the particular SH-modifying reagent. These data are listed at the end of each column. Each experiment was performed with n different cell preparations ($n = 3$–4). * Significantly different from control with $P \leqslant 0.05$, using the BMDP2V-program.

	Cholate (pmol/mg protein)				Taurocholate (pmol/mg protein)			
	V_i		Δ10 min		V_i		Δ10 min	
	$x \pm$ S.D.	% Inh.	$x \pm$ S.D.	% Inh.	$x \pm$ S.D.	% Inh.	$x \pm$ S.D.	% Inh.
$HgCl_2$ ($n = 3$)								
Control	163.7 (19.7)		979 (109.4)		539.7 (78.4)		1393 (179.4)	
10 μM	130.7 (21.8)	23	751 (85.4)	24	307 (32.3)	44	1078.4 (147)	23
20 μM	102.7 (15.6)	38	434 (47.4)	56	254.3 (28.4)	53	886.4 (97.4)	37
100 μM	42.7 (9.7)	74	234 (27.4)	77	37.4 (9.4)	94	225.5 (37.4)	84
10 μM washed	113.7 (17.1)	31	694 (104.9)	30	231.7 (41.4)	58	931.7 (87.4)	33
PCMBS								
Control	185.4 (18.8)		1062.6 (87.4)		418.7 (71.4)		1589.7 (189.4)	
10 μM	175.4 (19.3)	6	871.4 (25.6)	18	412.7 (43.4)	4	1474.3 (129.4)	8
50 μM	165.7 (14.7)	17	732.3 (47.4)	32	293.6 (42.8)	30	903.3 (96.40	43
100 μM	120.2 (21.5)	36	670.8 (101.4)	37	19.8 (3.7)	94	169.7 (27.4)	89
200 μM	46.8 (24.7)	71	324.4 (59.4)	70	13.7 (4.7)	97	136.7 (30.4)	93
100 μM washed	178.4 (21.4)	9	883.4 (107)	17	197.4 (4.7)	53	804.5 (107.4)	50

% Inhibition = $[(V_i - V_s) : V_i] \cdot 100$

V_i, initial velocity of the control; V_s, initial velocity of the sulfhydryl experiment.

The IC_{50} values are determined by linear regression of the appropriate inhibition data. In order to prove the significance of differences a two-factorial variance analysis was performed with a BMDP2V-program.

Results

Inhibition by PCMBS

The uptake of [^{3}H]taurocholate and [^{14}C]cholate into isolated rat hepatocytes was measured in the presence of several sulfhydryl modifying reagents (Tables I and II). The initial velocity (V_i) and the Δ10 min value were selected as parameters to demonstrate the degree of inhibition. Among the compounds listed the most effective inhibitor of either cholate as well as of taurocholate uptake was PCMBS. A dose-dependent inhibition of bile acid uptake was observed in the range of 10–200 μM PCMBS (Table I). In order to produce 50% inhibition of the initial velocity of taurocholate uptake, a concentration of 72.3 μM PCMBS was necessary, while the IC_{50} value was 139.2 μM for the V_i of cholate uptake.

Inhibition by NEM, DTNP and bromosuccinimide

Since PCMBS blocks SH-groups by noncovalent binding mechanisms [28,29], bile acid uptake might even be stronger inhibited by covalent reacting SH-reagents. Thus *N*-ethylmaleimide (NEM), 2,2′-dithio-bis(5-nitropyridine) (DTNP) and bromosuccinimide were used

TABLE II

The effects of covalently attaching SH-group reagents, not belonging to heavy metals, on bile acid uptake

Hepatocytes in suspension were incubated in the presence of NEM, bromosuccinimide and DTNP for 5 min and uptake of 11 nM [^{3}H]taurocholate/5 μM taurocholate or 1.25 μM [^{14}C]cholate/5 μM cholate was measured. Details are described in legend of Table I.

	Cholate (pmol/mg protein)				Taurocholate (pmol/mg protein)			
	V_i		Δ10 min		V_i		Δ10 min	
	$x \pm$S.D.	% Inh.	$x \pm$S.D.	% Inh.	$x \pm$S.D.	% Inh.	$x \pm$S.D.	% Inh.
NEM ($n = 3$)								
Control	141.4		911.4		358.4		1183.3	
	(17.4)		(109.4)		(56.3)		(188.4)	
50 μM	146.4	0	891.4	9	418.4	0	1345.4	0
	(13.2)		(93.4)		(75.4)		(87.4)	
100 μM	131	8	720.4	26	372.4	0	1267.4	0
	(10.4)		(79.4)		(44.2)		(101.5)	
200 μM	44.1	69	236.4	76	294.7	18	944.4	21
	(6.6)		(49.4)		(32.3)		(107.4)	
200 μM	31.3	77	(165.6)	85	115.7	68	276	67
washed	(5.6)		(29)		(17.1)		(49.40	
Bromosuccinimide ($n = 3$)								
Control	220.4		1197.6		374.5		1076.4	
	(32.9)		(176.4)		(46.8)		(176.4)	
200 μM	178.4	20	905.6	25	318.4	15	964.7	11
	(17.4)		(111.4)		(63.3)		(140.7)	
500 μM	83.4	63	430.8	65	94.6	76	249.6	77
	(9.4)		(79.4)		(19.7)		(31.4)	
200 μM	147.4	36	706	41	282.7	25	749.4	31
washed	(27.1)		(81.4)		(47.3)		(71.4)	
DTNB ($n = 3$)								
Control	220.4		1197.6		374.5		1076.4	
	(32.9)		(176.4)		(46.8)		(176.4)	
100 μM	167.4	25	834.6	31	393.4	0	875.6	19
	(14.7)		(139.4)		(51.7)		(144.7)	
200 μM	76.9	66	481.4	60	158.4	58	635.4	41
	(9.60		(161.4)		(13.8)		(87.4)	
500 μM	51.4	77	247.4	79	38.8	90	197.4	82
	(7.1)		(47.4)		(3.7)		(94.7)	
100 μM	127.3	43	658.3	45	281.4	26	693.7	36
washed	(28.9)		(82.3)		(38.7)		(78.4)	

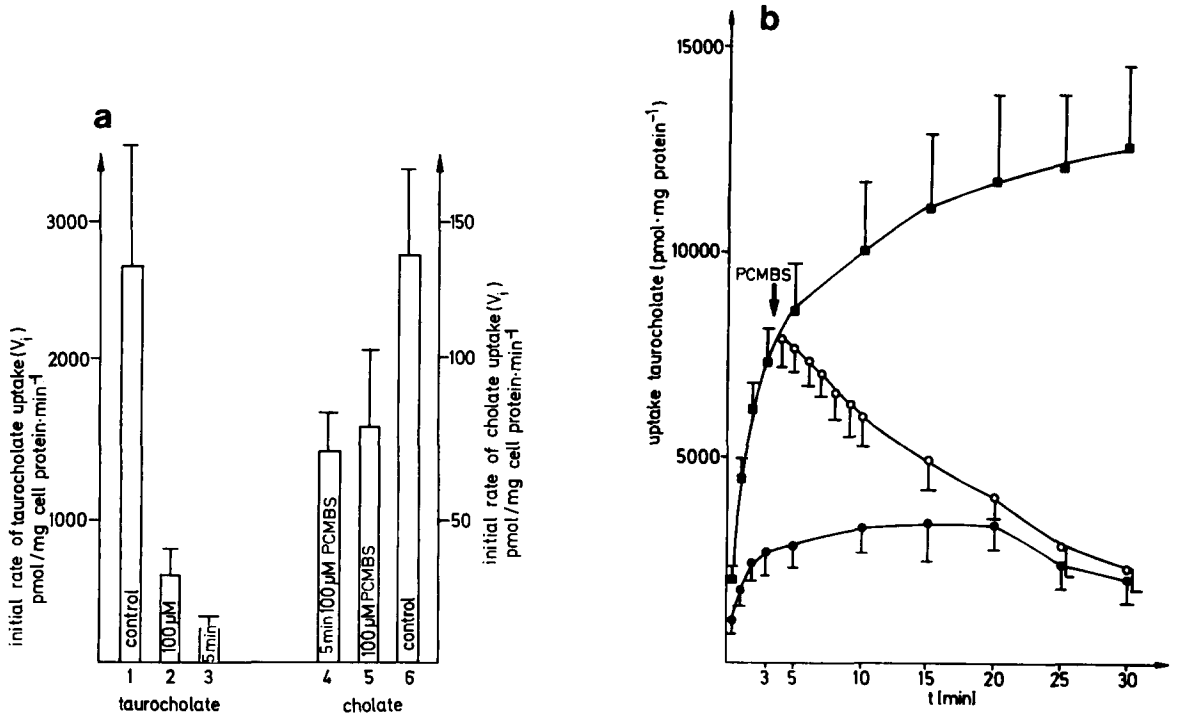


Fig. 1. Time course of inhibition by 100 μM PCMBS on bile acid uptake. (a) The initial velocity of taurocholate and cholate is calculated by linear regression of the 15, 45, 75 and 105 s values in the absence (1,6) and in the presence of 100 μM PCMBS for 5 min (3,4) and 15 s (2,5), respectively. Each V_i represents the mean value from experiments with three different cell preparations ($n = 3$). Bars indicate $\pm$S.D. (b) Time course of inhibition by PCMBS in an ongoing uptake experiment. Uptake of 11 nM [^{3}H]taurocholate/70 μM taurocholate is measured (■, control). These cells recieved 100 μM PCMBS after 3 min (○), while another cell sample recieved 100 μM PCMBS 5 min before bile acid uptake (●). Each point represents the mean value of four experiments with n cell preparations ($n = 4$). Bars indicate $\pm$S.D.

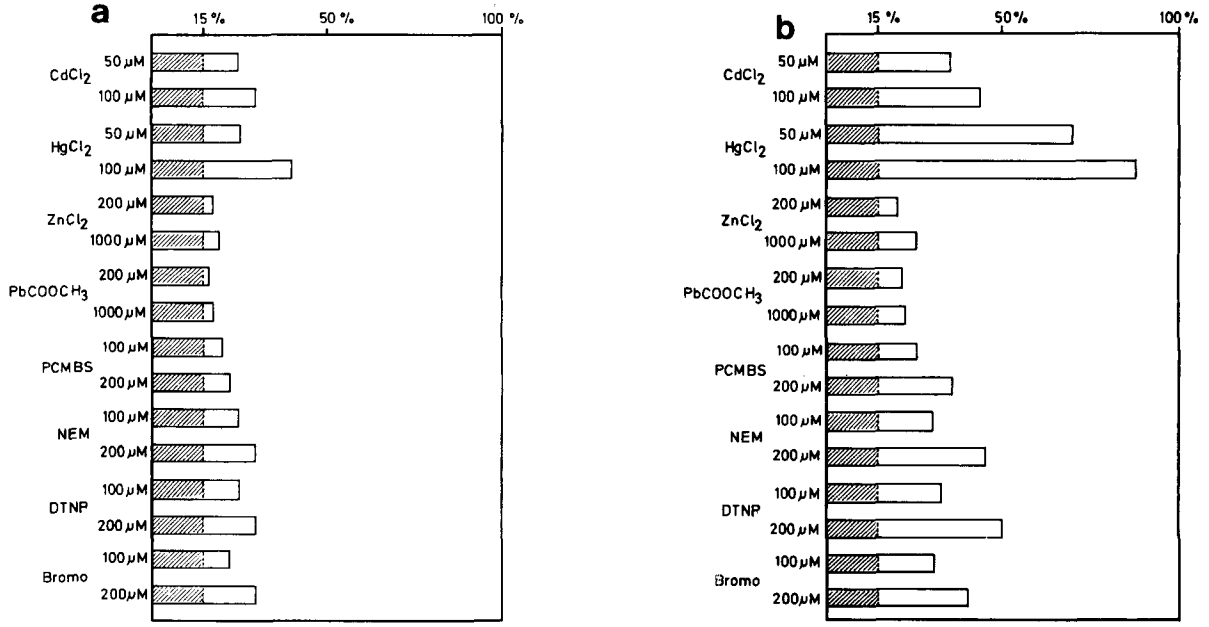
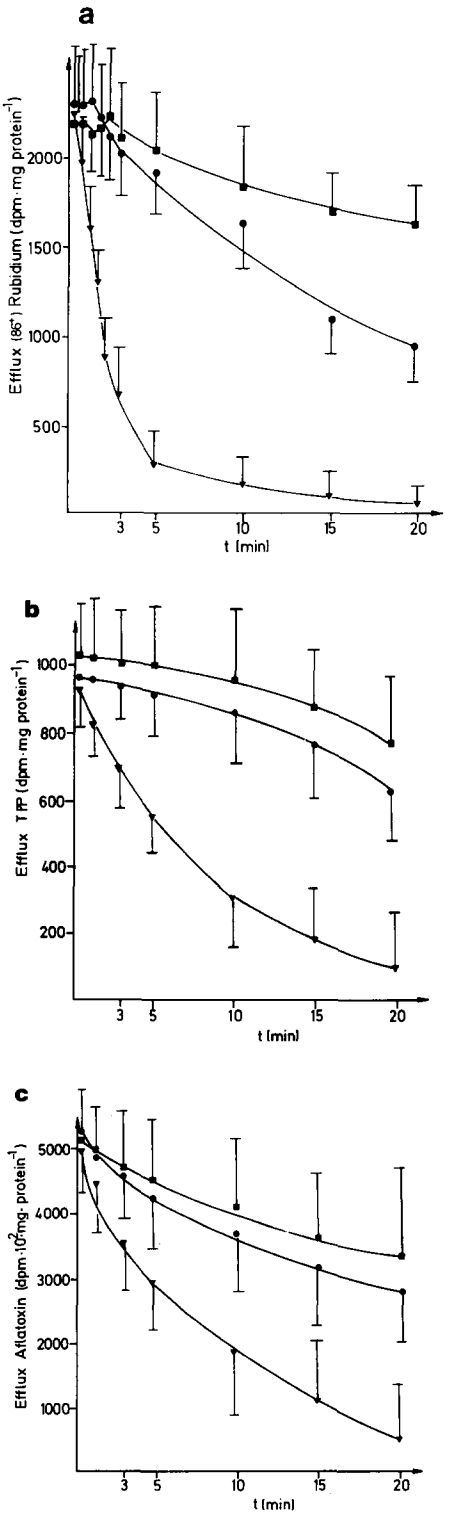


Fig. 2. (a and b) Trypan-blue index in hepatocyte suspensions treated with SH-modifying reagents. Isolated rat hepatocytes were incubated with the particular SH-blocker for 5 min (a) or 15 min (b). Cell viability was determined by the Trypan-blue exclusion test. The mean value for stained hepatocytes without inhibitor is 15%. Each value represents the mean value of duplicate experiments with at least three different cell preparations.

(Table II). They were all more effective inhibitors of the cholate uptake than of the taurocholate one. For example 200 μM NEM inhibited the initial velocity of the total cholate transport as well as the Δ10 min uptake to 70%, but only 20% of the taurocholate uptake was blocked at this particular concentration.



Inhibition by heavy metals

Besides the organic SH-reagents it was necessary to investigate the effects of SH-reactive heavy metals. The $HgCl_2$ inhibition was found to be dose dependent. At a concentration of 100 μM $HgCl_2$ the V_i of unconjugated and conjugated bile acids was blocked up to 74% and 94% while the Δ10 min values for unconjugated and conjugated bile acids were reduced to 77% and 84% (Table I). Other heavy metals viz. $CdCl_2$, $ZnCl_2$ and $Pb(CH_3COO)_2$ were also tested (not shown). $CdCl_2$ inhibited bile acid transport markedly at a concentration of 100 μM. In contrast $ZnCl_2$ showed moderate blocking potential only at high concentration of 1000 μM, while 1000 μM $Pb(CH_3COO)_2$ was without a significant effect on bile acid uptake. But at least part of their effects were accompanied by a cell destruction (Figs. 2a and b).

Kind of inhibition

To estimate the degree of reversibility of the inhibition, the cells were washed twice with Tyrode buffer after preincubation with the particular SH-reagents (Tables I and II). With the exception of PCMBS the inhibition of all reagents was irreversible. The inhibition of the V_i by 100 μM PCMBS on taurocholate was 94.3%, but was reduced up to 53.6% after the washing procedure (Table I). Similar results were obtained for the cholate uptake. 100 μM PCMBS blocked 36% of the initial velocity, while the inhibition was reduced up to 9% after washing the cells twice (Table I).

Onset of inhibition

Considering the type of inhibition, the PCMBS effect was studied in detail. The uptake of both bile acids was immediately blocked and accomplished within seconds (Fig. 1a). When the preincubation period was reduced from 5 min to 15 s, the bile acid transport was effected to the same extent. Similarly, when PCMBS was added in an ongoing taurocholate uptake trial, the transport was blocked immediately (Fig. 1b).

Fig. 3. Efflux studies on viable hepatocytes during treatment with SH-modifying reagents. (a) Isolated rat hepatocytes in suspension were preloaded with a concentration of 7.4 kBq/ml $^{86}Rb^+$. After a washing step the cells recieved either 100 μM PCMBS (●) or 100 μM $HgCl_2$ (▼). Control (■). Each point represents the mean value of four experiments ($n = 4$) with different cell preparations. Bars indicate $\pm$S.D. (b) Hepatocytes in suspension were incubated with a concentration of 18.4 kBq [^{3}H]TTP^+ per ml/10 μM TTP^+. Control (■), 100 μM PCMBS (●), 100 μM $HgCl_2$ (▼). The results of a typical experiment are shown ($n = 3$). (c) Hepatocytes in suspension recieved a concentration of 125 nM [^{3}H]aflatoxin. Control (■), 100 μM PCMBS (●), 100 μM $HgCl_2$ (▼). The results of a typical experiment are shown ($n = 3$).

Cell destruction by SH-group reagents

Since SH-group reagents might impair cell membrane integrity, the cell viability of hepatocytes was investigated with the Trypan-blue test (Figs. 2a and b). 100 μM PCMBS incubation for 5 and 15 min was elevating the Trypan-blue index only to 4.3% and 8.5%, respectively. The other SH-blockers increased significantly the ratio of stained hepatocytes, especially during a 15 min incubation period. For example 94.7% of the liver cells were stained blue within this time by 100 μM $HgCl_2$. 200 μM NEM increased the percentage of blue cells up to almost 30%.

In order to gain more information about the integrity of the cell membrane after interaction of SH-group blockers, the efflux of $^{86}Rb^+$ was measured. $^{86}Rb^+$ was allowed to become accumulated within the cells after a 30 min preincubation time [23,26]. 100 μM $HgCl_2$ caused an immediate and marked release of $^{86}Rb^+$, while 100 μM PCMBS slightly released the cation after a lag period of 10 min of incubation (Fig. 3a).

In a second approach the efflux of the lipophilic cation tetra[^{3}H]phenylphosphonium was investigated. This compound accumulates against a concentration gradient and has been used as a probe for noninvasive measurement of the membrane potential of isolated hepatocytes [25]. Again 100 μM $HgCl_2$ is producing a rapid and marked release of the cation, while 100 μM PCMBS is showing only an minimal efflux during the first 15 min (Fig. 3b).

Finally, the efflux of a bulky uncharged compound, aflatoxin B_1, was measured during exposure to both PCMBS or $HgCl_2$. This alkaloide permeates into liver cells by simple diffusion [30] and enhanced efflux might serve as a marker for nonspecific cell leakage of hepatocytes. 100 μM PCMBS was not able to evoke an increase of the aflatoxin B_1 efflux for the first 15 min (Fig. 3c).

Clearly 100 μM $HgCl_2$ (35%) but not 100 μM PCMBS reduced the content of ATP in isolated rat hepatocytes (Table III). The ATP level is similar with former investigation (947 ng/mg protein; Petzinger et al. (1988), Ref. 27).

TABLE III

The effect of PCMBS and $HgCl_2$ on the cellular ATP content of isolated rat hepatocytes

Hepatocytes in suspension were incubated in the presence of 100 μM $HgCl_2$ or 100 μM PCMBS for 15 min and ATP was measured as described in Methods. The reduction of the ATP content by the particular SH-group reagent is expressed in percent to the control values with data from four different cell preparations.

	ATP content (ng/mg protein)	
	$x \pm$S.D.	% reduction
Control	931 ± 215	
100 μM PCMBS	865 ± 254	7
100 μM $HgCl_2$	605 ± 157	35

TABLE IV

The effects of seven mercaptocompounds on the inhibition of the taurocholate uptake in isolated hepatocytes induced by 100 μM PCMBS

Hepatocytes in suspension were incubated in the presence of 100 μM PCMBS for 5 min. 11 nm [^{3}H]taurocholate/70 μM taurocholate was added and after 3 min the treated cells recieved 500 μM of the particular mercaptocompound. Each experiment was performed at least with n different cell preparations ($n = 3$). * Significantly different from control with $P \leq 0.05$, using the BMDP2V-program.

Mercapto-compound (500 μM)	Uptake of taurocholate after 20 min (pmol/mg protein)	reversibility (%)
L-cysteine	4669.8	39.5 *
L-glutathione	5513.4	46.3 *
2-Mercaptoethanol	6634.8	56.1 *
1,4-Dimercaptobutane	7142.6	60.4 *
1,2-Dimercaptoethane	7276.3	61.6 *
1,6-Dimercaptohexane	7437.4	63.5 *
1,4-Dithiothreitol	10321.4	87.4 *
Control	11808.3	
100 μM PCMBS	2840.7	

Kinetic analysis of PCMBS with taurocholate

Additional information about the kind of inactivation on the bile acid transport in hepatocytes was obtained by determing kinetic constants of taurocholate uptake before and after PCMBS preincubation. For taurocholate uptake without inhibitor a K_m value of 32 μM taurocholate and a V_{max} rate of 2350 pmol · min^{-1} · mg^{-1} cell protein were found, which are in good agreement with other investigations [31,32]. The $V_{max}$was reduced to 1282 pmol · min^{-1} · mg^{-1} cell protein, while the $K_m$was 38 μM in the presence of 80 μM PCMBS, indicating a type of non-competitive inhibition.

Effects of mercaptocompounds on the PCMBS induced inhibition

To define the specific interaction of PCMBS with SH-groups, bile acid uptake was blocked by PCMBS first. Subsequently the reactivation of the blocked uptake was achieved by adding a series of mercaptocompounds to the cells (Table IV). 500 μM 1,4-dithiothreitol (DTT) was the most effective one to antagonize the blockade, while 500 μM of six other mercaptocompounds viz. 2-mercaptoethanol, 1,2-dimercaptoethane, 1,4-dimercaptobutane, 1,6-dimercaptohexane, L-cysteine and L-glutathione reversed only partially the inhibition by 100 μM PCMBS. The time course of the DTT effect indicated a two-step process. The first step, which lasted approx. 7 min, had an immediate onset with a slow increase of bile acid uptake while during the second step bile acid uptake became accelerated steadily and reached nearly control values (100%).

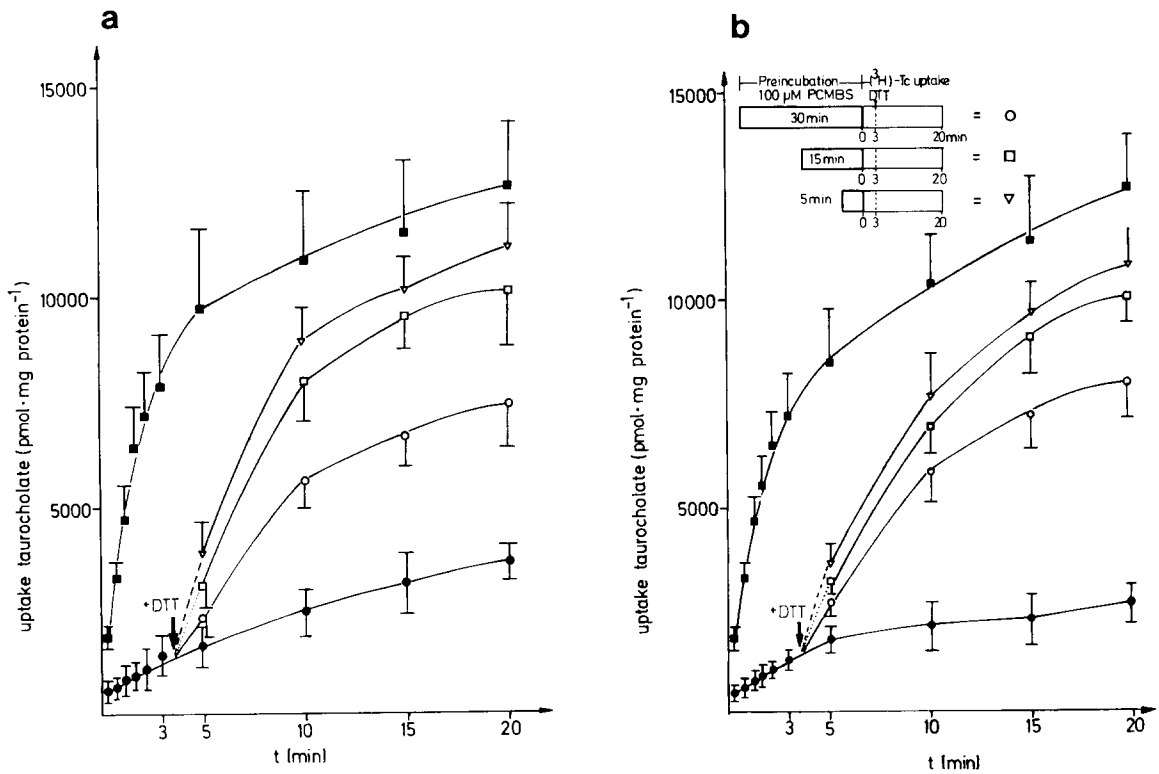


Fig. 4. (a) Isolated rat hepatocytes were incubated in the presence of 100 μM PCMBS for 5 min. Uptake of 11 nM [^{3}H]taurocholate/70 μM taurocholate was measured either in untreated (■) and PCMBS-labeled cells (●). After 3 min the treated cells recieved 25 μM (○), 250 μM (□), 1000 μM (▽) dithiothreitol (DTT). (b) Isolated rat hepatocytes were incubated in the presence of 100 μM PCMBS for 5 min (▽), 15 min (□) and 30 min (○). The same taurocholate mixture is used as in Fig. 4a. PCMBS-treated cells recieved 250 μM DTT after the addition of the radioactive bile acids. Values are means ± S.D. of three experiments with n cell preparations (n = 3).

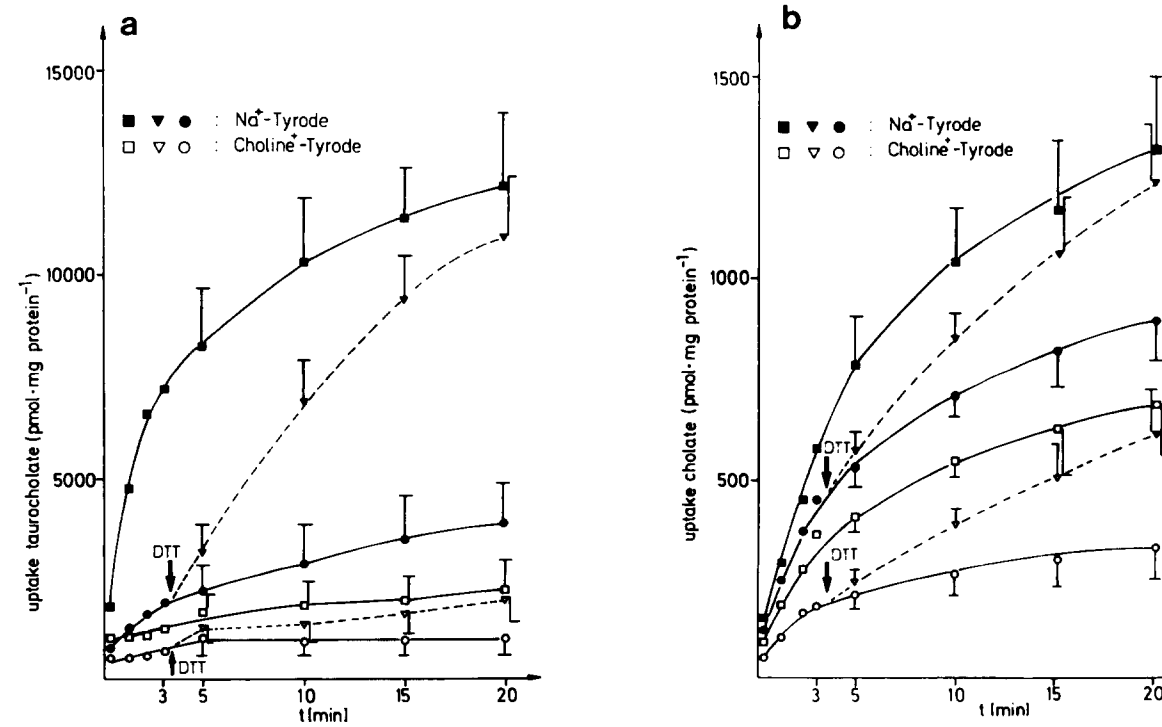


Fig. 5. Inhibition of sodium-dependent and sodium-independent uptake of bile acids into hepatocytes by PCMBS and reversal by DTT. (a) Isolated rat hepatocytes were incubated in Na^+-Tyrode and recieved 100 μM PCMBS for 5 min (●). Bile acid uptake was measured with 11 nM [^{3}H]taurocholate/70 μM taurocholate (■, control). After 3 min the PCMBS-treated cells recieved 250 μM DTT (▼). Identical experiments were performed in choline$^+$-Tyrode (□, ○, ▽). (b) Comparable experiments as in Fig. 5a were performed with 1.25 μM [^{14}C]cholate/5 μM cholate. Values are means ± S.D. of three experiments with n cell preparations (n = 3).

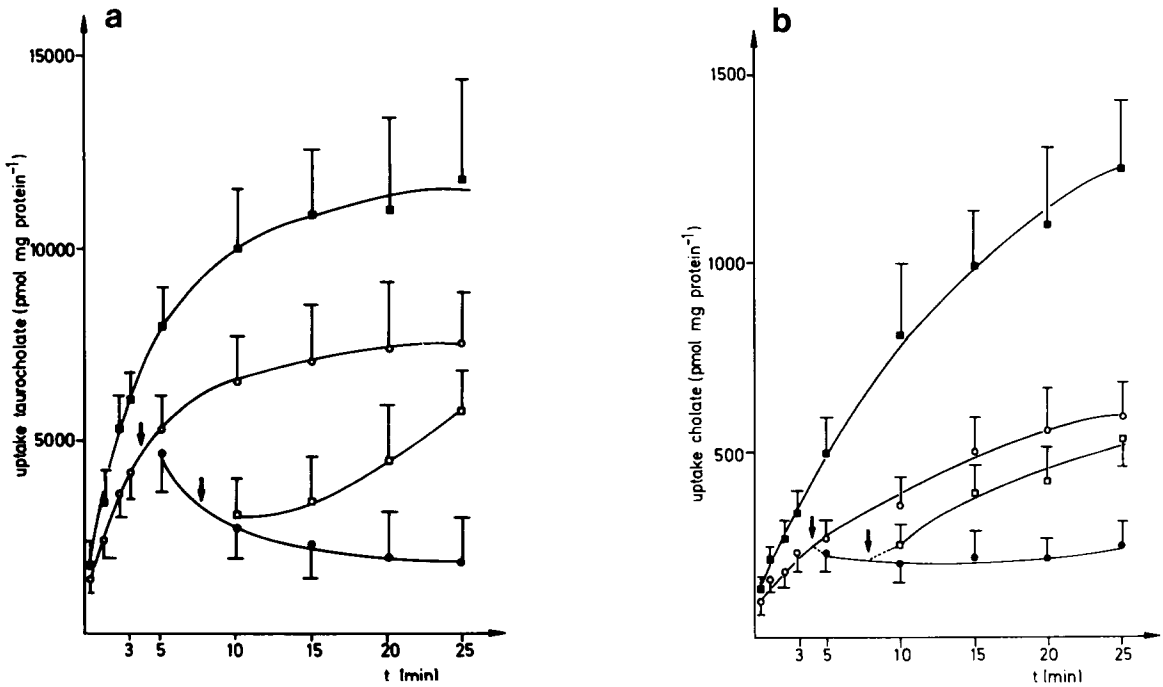


Fig. 6. Additive inhibition of bile acid uptake by treatment of hepatocytes with DTNP and PCMBS in series. (a) Hepatocytes in suspension were incubated in the absence (■) or presence of 100 μM DTNP (○) for 5 min. Uptake was initiated with 11 nM [^{3}H]taurocholate/70 μM taurocholate. An aliquot of the DTNP-treated cells recieved after 3 min (first arrow) PCMBS at a final concentration of 100 μM (●). In the last step (second arrow) one portion of the DTNP and PCMBS-treated cells recieved DTT at a final concentration of 500 μM (□). (b) Comparable experiments as in Fig. 6a with 1.25 μM [^{14}C]cholate/5 μM cholate. Values are means ± S.D. of three experiments with different cell preparations ($n = 3$).

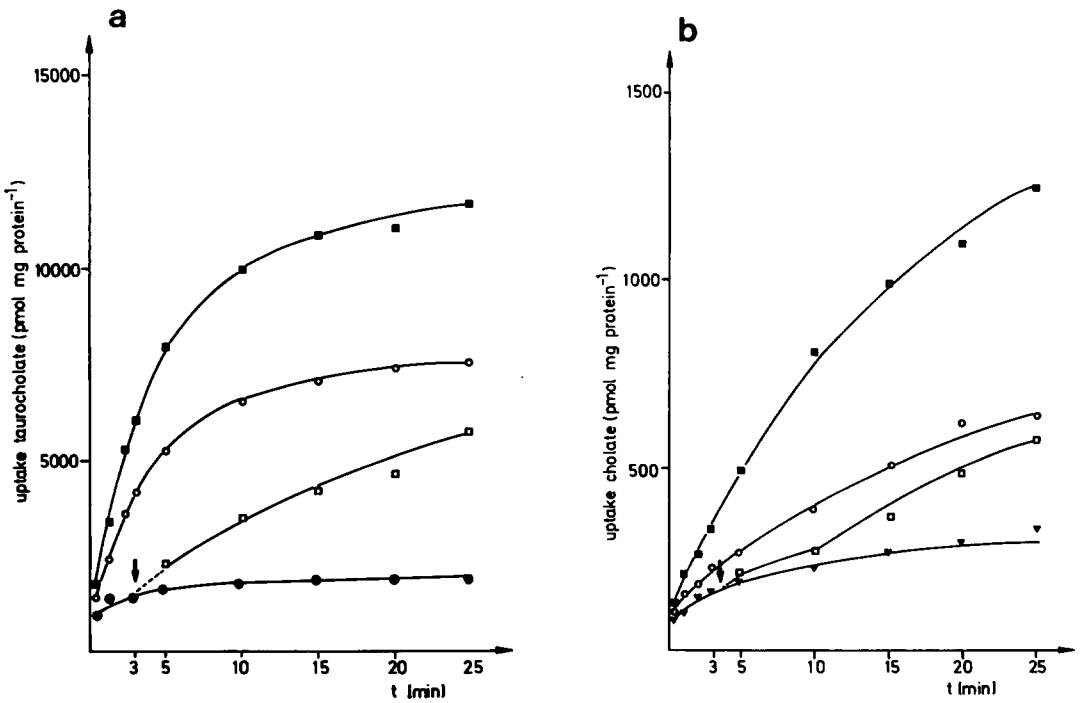


Fig. 7. Synergistic inhibition of bile acid uptake by two SH-group blockers. (a) Hepatocytes in suspension were incubated in the absence (■) or in the presence of 100 μM DTNP (○) or in the presence of 100 μM DTNP and 100 μM PCMBS (●). The uptake was initiated with 11 nM [^{3}H]taurocholate/70 μM taurocholate. After 3 min the 100 μM PCMBS and 100 μM DTNP-treated cells recieved 500 μM DTT (□). (b) Comparable experiments as in Fig. 7a with 1.25 μM [^{14}C]cholate/5 μM cholate. Values are means ± S.D. of three experiments with different cell preparations ($n = 3$).

The antagonizing effect of DTT was documented even at a concentration of 25 μM. Here DTT was still able to reverse the inhibition of 100 μM PCMBS up to 50% (Fig. 4a). However, DTT was unable to antagonize the NEM, DTNP and bromosuccinimide induced inhibition (data are not shown). The effect of DTT was dependent on the exposure time of the hepatocytes to PCMBS. The best results of DTT were found, if the thiol was given shortly after the inhibitor. But so far, 500 μM DTT was able to reverse the inhibition by 100 μM PCMBS up to 50% even after a preincubation period of 30 min (Fig. 4b).

Inhibition of bile acid uptake by PCMBS in choline buffer

It has been reported that PCMBS is able to increase the permeability of intestinal brush border plasma membrane vesicles to small inorganic cations, especially sodium ions, thereby lowering the driving forces for all substrates transported via a sodium-coupled transport system [33]. If such an indirect mechanism is expected, the inhibition of the uptake of the bile acids might be exclusively restricted to their sodium-dependent part of the total uptake. However, 100 μM PCMBS had a moderate blocking potential for the sodium-independent uptake of cholate and taurocholate. These experiments were carried out in a sodium free choline-Tyrode buffer. Under these conditions the sodium-dependent uptake is eliminated and therefore the control uptake (without PCMBS) for cholate and taurocholate is reduced up to 50% and 85%, respectively [6]. The remaining part of the bile acid uptake was blocked by the organic mercurial compound. Again 500 μM DTT were able to reverse the PCMBS blockade on sodium-independent bile acid uptake (Figs. 5 a and b).

Two different moities of SH-groups are involved in the uptake of bile acids

It was an interesting question whether the covalent, non penetrating SH-reagent, DTNP, and the slow penetrating organic mercurial, PCMBS [28,29,34], were interacting with the same SH-groups at the bile acid carrier proteins. Therefore liver cells were exposed to 100μM DTNP for 5 min and then one aliquot of these cells recieved 100 μM PCMBS in addition. There was an additive effect and the uptake of the bile acid was blocked almost totally. If a concentration of 500 μM DTT was applied the uptake returned back to values of DTNP induced inhibition (Figs. 6 a and b). If the sulfhydryl reagents DTNP and PCMBS were administered simultanously, similar results were obtained for either cholate or taurocholate. Again the values of the bile acid uptake could be reversed to the level of the DTNP induced inhibition, if DTT was added to the hepatocyte suspension (Figs. 7 a and b).

Discussion

Several transport activities in rat hepatocytes have shown to be sensitive to sulfhydryl modification [11–13], but so far no investigation has provided details concerning the relation between free SH-groups and the uptake of conjugated and unconjugated bile acids in isolated rat hepatocytes.

The present report describes the inhibition of [^{3}H]taurocholate and [^{14}C]cholate uptake into freshly prepared hepatocytes by several anorganic and organic SH-modifying reagents. It could be demonstrated that heavy metals were potential inhibitors of the bile acid uptake. But at least part of their effects were accompanied by a cell destruction, making these heavy metals inappropriate as a tool on living hepatocytes.

Organic SH-reagents, viz. DTNP, NEM and bromosuccinimide also exhibit blocking activities. But it was the water-soluble organic mercurial PCMBS, which was the most effective one among this group of chemicals. This particular agent is regarded to have a high specific tendency to react with free sulfhydryl groups exposed on the cell surface [28,29,38]. It was documented in another report that PCMBS is penetrating cells very slowly [34]. Therefore it was concluded that during a short incubation time the main target of PCMBS is the outer part of the cell membrane.

When PCMBS was tested for half-maximal inhibition values (IC_{50}), differences in the uptake of conjugated and unconjugated bile acids were observed. The IC_{50} value for the initial uptake of taurocholate was 72.3 μM, but the IC_{50} was 139 μM PCMBS for cholate uptake. Kinetic analysis revealed a remarkable decrease of V_{max}, which was reduced to almost 50% in the presence of 80 μM PCMBS for conjugated bile acids. The K_m was only slightly reduced, indicating a noncompetitive type of inhibition. While PCMBS was more effective in blocking the taurocholate transport, NEM inhibited the cholate uptake preferentially. This difference in the effect of these SH-modifying substances as well as the divergent IC_{50} data point out, that the moieties of SH-groups might not be homogenous in the 48–49 and 52–54 kDa bile acid proteins.

The inactivation of the bile acid uptake by 100 μM PCMBS is correlated directly to a blockade of free SH-groups, since it was reversed by seven mercapto-compounds. Dithiothreitol was the most effective antagonist for noncovalent bound PCMBS. When DTT was used in a high concentration (500 μM DTT versus 100 μM PCMBS), the bile acid uptake reached almost control values, while even 25 μM DTT were effective to reverse 50% inhibition. The reverse reaction by DTT on the complex of PCMBS/SH-group is chemically defined as a double disulfide exchange reaction [39,40]. By the same chemical reaction a high concentration of DTT is able to reduce cysteine-cysteine bridges [41]. It

was obvious, that there was no change of bile acid uptake due to the opening of cystine S-S groups since DTT alone had no effect on bile acid uptake. Thus only the re-exposure of originally free SH-groups rather than the addition of new reduced sulfhydryl groups reactivated bile acid transport.

The other thiocompounds, viz. mercaptoethanol, dimercaptoethane, dimercaptobutane, dimercaptohexane, cysteine and glutathione, antagonized the PCMBS blockade only partially. These compounds differ from DTT not only in the number but also in the position of SH-groups along an aliphatic carbon chain. As it was pointed out by Lenzen et al. [42] in an investigation on the effects of mercaptocompounds on alloxane induced inhibition of the liver glucokinase activity, thiols with only one SH-group (cysteine, 2-mercaptoethanol) as well as thiols with vicinal SH-groups (1,2-dimercaptoethane) are less effective than those bearing two end SH-residues. Generally these observations were confirmed by the present data. The high degree of the reversibility by DTT can be explained with its two terminal SH-groups, since it is well known that grouping of at least two sulfhydryl groups into one molecule is necessary for protecting mammalian cells against certain toxic effects of SH-blockers [36,37]. However, it was unexpected that 1,4-dimercaptobutane, a thiol with two end SH-groups in the same position as in DTT, was only able to reverse 50% of inhibition in contrast to the almost 100% by DTT. But 1,4-dimercaptobutane is much more lipophilic than DTT, because the two hydroxyl groups of DTT are missing. Under the assumption of an essential SH-group in a hydrophilic area within the transport proteins the lipophilic thiols like 1,4-dimercaptobutane may not reach the PCMBS-sensitive sites at the outer cell surface in a sufficient concentration.

A crucial point in the present study was the cell viability and membrane integrity. Whereas $HgCl_2$ rapidly permeabilized hepatocytes, PCMBS did not alter the cell viability. In contrast to the other SH-modifing reagents, PCMBS is elevating the Trypan-blue index of the isolated hepatocytes only to a minimal extent. The ATP content of isolated rat hepatocytes treated with 100 μM $HgCl_2$ was reduced to 35%. Similar data for ATP reduction in skate hepatocytes by $HgCL_2$ were obtained by another group [43]. PCMBS was almost without any effect on the cellular ATP level.Its high degree of blocking activity cannot be explained with unspecific cell death. This was confirmed by experiments on the efflux of an anorganic and an organic cation, viz. $^{86}Rb^+$ and TTP^+, as well as of an uncharged bulky compound, aflatoxin B_1. Clearly $HgCl_2$ but not PCMBS severely permeabilized the cell membrane of hepatocytes. The conditions were stable for at least 10 min during an incubation with 100 μM PCMBS. Within that time period PCMBS inhibition could be reversed almost completely by DTT. However 'late effects' of PCMBS occurred. Preincubation of hepatocytes for 30 min with PCMBS resulted in an inhibition, which was now only partially antagonized by DTT. In contrast to these 'late effects' the inhibition on the bile acid uptake takes place within seconds.

Will and Hopfer [33] have reported that PCMBS is inhibiting sodium-dependent uptake of valine and glucose into intestinal brush border vesicles by dissipating the electrochemical gradient of sodium ions. In order to prove this aspect, sodium independent-taurocholate and cholate uptake was investigated by substitution experiments (exchange of sodium versus choline) with liver cells, which were exposed shortly to PCMBS. Not only the sodium-dependent but also the sodium-independent uptake was blocked. This ability to reduce the sodium-independent bile acid uptake underscorses the specific interaction with essential SH-groups in the bile acid carrier proteins by PCMBS and argues against an unspecific effect on the sodium ion gradient.

It seems to be that on living hepatocytes two different moieties of SH-groups might be important for bile acid uptake. One group is susceptible to DTNP and NEM, whereas PCMBS is able to block further SH-groups in addition. This was indicated by an additive inhibition effect when both compounds (PCMBS/DTNP) were given subsequently. For another anion transport system on hepatocytes Passamonti and Sottocasa [13] argued for two independent classes of SH-groups in the uptake of sulfobromophthalein. Also titration of SH-groups in the human erythrocyte anion exchange protein indicated that NEM-unreactive sulfhydryl residues are still accessible to other mercurials [44]. Further investigations are necessary to distinguish these SH-moieties in the bile acid uptake, since in an abstract report it was claimed that also NEM titrable SH-groups are involved in the sodium-dependent uptake of taurocholate into sinusoidal plasma membrane vesicles [35].

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