Biochimica et Biophysica Acta, 1149 (1993) 278–284
© 1993 Elsevier Science Publishers B.V. All rights reserved 0005-2736/93/$06.00

BBAMEM 76020

Two distinct types of SH-groups are necessary for bumetanide and bile acid uptake into isolated rat hepatocytes

Matthias Blumrich and Ernst Petzinger

Institute of Pharmacology and Toxicology, Justus Liebig University Giessen, Giessen (Germany)

(Received 23 December 1992)
(Revised manuscript received 23 March 1993)

Key words: Organic anion transporter; Organic sulfhydryl reagent; Dithiobis(2-nitrobenzoic acid) derivative; Binding protein; Loop diuretic; (Liver)

Substances that block SH-groups were studied in respect to their effects on the uptake of the loop diuretic bumetanide and the bile acids cholate and taurocholate into isolated rat hepatocytes. SH-blockers, e.g., *p*-chloromercuribenzenesulfonate (PCMBS), *N*-ethylmaleimide (NEM), dithiobis-nitropyridine (DTNP) and dithiobis-2-nitrobenzoic acid (DTNB) reduced bumetanide transport in a concentration-dependent manner. Inhibition of the organic mercurial PCMBS was reversed by the addition of 500 μM dithiothreitol (DTT), indicating an interaction of this substance with free SH-groups. NEM irreversibly blocked SH-groups by covalent binding and was the most effective inhibitor of bumetanide and cholate uptake. In contrast, PCMBS was the most effective inhibitor of taurocholate uptake. Photoaffinity studies with [^{3}H]bumetanide and [^{3}H]7,7-azotaurocholate were performed with isolated rat hepatocytes in the presence of PCMBS and DTNP. Binding of the photolabels was not reduced by SH-group blockers. Newly synthesized sulfhydryl-modifying reagents such as dithio-sulfonate-ethyl-nitrobenzoic acid (DTSNB) and dithio-octyl-nitrobenzoic acid (DTONB), are derivatives of the alkylating agent DTNB. DTSNB is regarded as a selective blocker for SH-groups in a hydrophilic environment, while DTONB is more lipophilic and interacts with SH-groups in the transmembrane domain of transport proteins. The IC_{50}-values of these blockers for bumetanide uptake (DTSNB 250 μM, DTONB 141 μM) and for cholate uptake (DTSNB 250 μM, DTONB 115 μM) were almost identical. These findings support the concept of a common uptake mechanism for cholate and bumetanide and indicate that two distinct moieties of SH-groups are required for the uptake of both organic anions. One of these is probably located on the outer surface and the other within the membrane of hepatocytes.

Introduction

The functional importance of free SH-groups on the uptake of bile acids into isolated rat hepatocytes was investigated recently with SH-group blockers, and differences in the uptake of taurocholate versus cholate were verified by their specific effects [1]. It was assumed that two different classes of SH-groups are required to account for complete transport function. Recently, a sodium-dependent taurocholate transporter was cloned and the primary amino-acid sequence revealed seven cysteine-residues [2]. Two SH-groups are located on the outside of the membrane, while the others are placed in the transmembrane loops of the protein, supporting the findings with selective SH-blockers. The aim of the present investigation was to study the uptake of the loop diuretic drug bumetanide in the presence of SH-blocking substances. The transport of bumetanide into hepatocytes has been correlated with an uptake by bile acid transporters [3–6]. It was postulated that carrier proteins for bile acids translocate bumetanide and related loop diuretics across the basolateral membrane of hepatocytes.

The present results with standard and newly synthesized SH-reagents DTSNB and DTONB support this concept and indicate a common transport system for unconjugated bile acids and organic anions such as bumetanide, whereby this system is distinct from the taurocholate transporter.

Correspondence to: E. Petzinger, Institute of Pharmacology and Toxicology, Justus Liebig University Giessen, Frankfurter Strasse 107, D-6300 Giessen, Germany.

Abbreviations, PCMBS, *p*-chloromercuribenzenesulfonate; NEM, *N*-ethylmaleimide; DTNP, dithiobis-nitropyridine; DTNB, dithiobis-2-nitrobenzoic acid; DTSNB, dithio-sulfonyl-ethyl-nitrobenzoic acid; DTONB, dithio-octyl-nitrobenzoic acid; DTT, dithiothreitol.

Materials and Methods

Cell preparation

Hepatocytes were prepared from male Wistar rats (body wt. 260–300 g) by using a modified protocol of Berry and Friend [7,1]. Isolated hepatocytes were incubated for 30 min in Tyrode buffer with (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). As judged by Trypan blue test, 85–90% of the isolated cells were viable. Protein concentration was determined using the Bio-Rad reagent according to the method of Bradford [8].

Transport experiments

Uptake studies were performed either in 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) or with sodium free buffer B (in mM: 137 choline chloride, 12 $MgCl_2$, 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.

Uptake of radioactive compounds was measured by a rapid centrifugation method [9] after addition of 60 nM [^{3}H]bumetanide or 11 nM [^{3}H]taurocholate or 1.25 μM [^{14}C]cholate with varying amounts of unlabeled substrate. Details of the experimental protocol were reported elsewhere [1]. Cell-associated radioactivity was measured in Lipoluma/Lumasolve/water mixture (100 : 10 : 2 (v/v/v), Baker, Phillipsburg, NJ, USA) with a Packard counter 2660.

Experiments with sulfhydryl-reacting reagents

p-Chloromercuribenzene sulfonate (PCMBS) was dissolved in Tyrode buffer, *N*-ethylmaleimide (NEM) was used in a 96%-ethanol solution; 2,2-dithiobis-5-nitropyridine (DTNP), 5,5-dithiobis-2-nitrobenzoic acid (DTNB) and the two DTNB derivatives DTSNB and DTONB were dissolved in DMSO. The solvent concentrations were 1–2% in the hepatocyte suspension and had no measurable effect on the uptake of the radiolabeled substrates. In experiments with PCMBS an equimolar amount of Na_2EDTA was added simultanously to chelate free mercury that might have been released from the organic mercurial [10]. Cell suspensions were incubated with these sulfhydryl reagents for 5 min and substrate uptake was performed in the presence of these reagents.

Estimation of the octanol–water partition coefficient

The octanol–water partition coefficient (log *p*) was estimated by computer calculation using the Quantum Chemistry Program Exchange (QCPE) 608 program. The structural information of the particular SH-reagent was transformed in the type CRT mode [11]. This program is based on the group contribution method and classifies carbon and nitrogen atoms in terms of their environment.

Experiments with dithiothreitol

4 ml hepatocyte suspension was incubated for 5 min with the respective sulfhydryl-blocking reagents. Radioactive bumetanide was added and residual uptake was measured for 3 min. Then the cell suspension was divided into two parts. One received dithiothreitol (DDT) to reverse the inhibition by the particular SH-reagent, the other served as a control.

Photoaffinity labeling

Freshly isolated hepatoyctes ($4 \cdot 10^6$ cells/ml) were incubated for 5 min in the absence or presence of 200 μM PCMBS, NEM or DTNP. 1 ml of the suspension was placed into a quartz pipette, at which time the cells received either 10 μCi [^{3}H]7.7-azotaurocholate or 100 μCi [^{3}H]bumetanide for one minute. Photoactivation was performed with a photoflash device as reported previously [4]. The hepatocytes were then immediately frozen in liquid nitrogen. After thawing, unbound radioactivity was removed by washing the hepatocytes two times with phosphate-buffered saline containing the following proteinase inhibitors: freshly prepared leupeptin (50 μg/ml), PMSF (1 mM), iodoacetamide (1 mM) and benzamidine (1 mM) as well as 1 mM Na_2EDTA. The radiolabeled membrane proteins were solubilized by 0.2% SDS for 5 min at 95°C and separated by SDS-PAGE [12]. Bound radioactivity was determined after slicing the gels into 2-mm slices and measuring the samples by liquid scintillation counting.

Materials

[*carboxyl*-^{14}C]Cholic acid (spec. act. 2.07 GBq/mmol) was obtained from Amersham (Braunschweig, Germany) and New England Nuclear (Dreieich, Germany) supplied [^{3}H]taurocholic acid (spec. act. 77.7 GBq/mmol). [^{3}H]Bumetanide (spec. act. 555 GBq/mmol) was synthesized as previously described [3]. [^{3}H]7,7-Azotaurocholate was a gift from Dr. Dr. W. Kramer, Hoechst (Frankfurt, Germany). Collagenase was purchased from Boehringer-Mannheim (Mannheim, Germany) and the Bio-Rad Protein Assay was from Bio-Rad Labaratories (München, Germany). Bumetanide was a gift from Dr. P.W. Feit, Leo Pharmaceuticals (Ballerup, Denmark). The DTNB-derivatives DTSNB and DTONB were synthesized by Prof. Dr. H. Faulstich (Heidelberg, Germany) [13]. All the other chemicals were used in highest purity available.

Calculation and statistics

The initial rates of substrate uptake were determined by linear regression from the 15, 45, 75 and 105-s uptake values and expressed as pmol/min per mg cell protein. All data are presented as mean ±S.D. of at least three experiments with n different cell preparations.

The IC_{50} values were determined by logarithmic regression of the appropriate inhibition data. In order to prove the significance of differences, a two factorial variance-analysis was performed using the BMDP2V-program [14].

Results

Inhibition of bumetanide uptake by standard SH-blockers

The uptake of [^{3}H]bumetanide into isolated rat hepatocytes was measured in the presence of several sulfhydryl-blocking substances (Table I). Dose-dependent inhibition of the transport process was observed with 50–200 μM of each of the tested SH-blockers. Within these concentrations cell viability of freshly isolated hepatocytes was not altered as judged by the Trypan blue test, ATP-level or rubidium efflux from these cells [1]. Considering the cell-damaging potential of these SH-reagents, the IC_{50}-value for a particular SH-reacting substance was calculated only from such inhibition data where cell viability was not altered.

The IC_{50}-value for bumetanide uptake was 196 μM in the case of the organic mercurial PCMBS and 132 μM in the case of NEM. NEM is a cyclic maleimide derivative with a *cis*-double bond, which interacts with free SH-groups by a nucleophilic covalent addition reaction [15]. Therefore, the transport inhibition by NEM was irreversible.

Two other organic SH-reacting substances showed similar IC_{50}-values, 181 μM for DTNB and 194 μM for DTNP. The latter compound is commonly used when studies are performed at physiological pH [16]. Both compounds are characterized by a disulfide-group that has a higher standard oxidation-reduction potential than SH-groups along an aliphatic hydrocarbogen chain. The interaction with SH-groups is by a disulfide–SH-group exchange reaction. The DTNB derivatives DTSNB and DTONB react in the same way [13].

Effects of DTT on the PCMBS-induced transport inhibition

1,4-dithiothreitol (DTT) is a highly-effective mercaptocompound, which in isolated rat hepatocytes antagonized the PCMBS-induced blockage of the uptake of conjugated and unconjugated bile acids [1]. 500 μM DTT also reversed the inhibition effect of 100 μM PCMBS on bumetanide uptake (Fig. 1), providing evidence of the specific interaction of PCMBS with free SH-groups. However, DTT was unable to antagonize the blockage by NEM, DTNP, or DTNB-derivatives of bumetanide uptake (data not shown).

TABLE I

The effects of standard SH-blockers on bumetanide uptake in isolated rat hepatocytes

Hepatocytes in suspension were incubated in the presence of different concentrations of NEM, PCMBS, DTNP and DTNB for 5 min, then uptake of 60 nM [^{3}H]bumetanide/7 μM bumetanide was measured. The inhibition is expressed by comparing the initial velocities (V_i). Each experiment was performed with n different cell preparations ($n = 3$–4). * Significantly different from control with $P < 0.05$, using the BMDP2V-program.

	Bumetanide (pmol/mg protein)	
	V_i mean ± S.D.	% Inhibition
NEM ($n = 3$)		
Control	124.4 (14.4)	
50 μM	92.6 (13.2)	26.6 *
100 μM	76.1 (10.4)	38.9 *
200 μM	40.1 (6.6)	67.8 *
DTNP ($n = 3$)		
Control	106.4 (12.9)	
50 μM	90.4 (12.4)	15.5
100 μM	76.4 (9.4)	28.2 *
200 μM	50.7 (12.1)	52.4 *
PCMBS ($n = 4$)		
Control	96.4 (12.3)	
50 μM	86.4 (11.4)	11.4
100 μM	72.4 (14.2)	25.1 *
200 μM	45.7 (8.3)	52.6 *
DTNB ($n = 3$)		
Control	107.5 (16.8)	
50 μM	87.4 (13.3)	18.7
100 μM	68.6 (15.7)	36.2 *
200 μM	48.1 (9.3)	55.2 *

Inhibition by NEM and PCMBS of bumetanide versus bile acid uptake

Fig. 2 describes the inhibition of NEM and PCMBS on bumetanide uptake versus bile acid uptake. NEM at

a concentration of 200 μM inhibited the initial velocity of bumetanide by 68%. NEM in same concentration reduced cholate uptake to almost the same percentage (69%), indicating a strong correlation between these two transport processes. In contrast, 100 μM PCMBS blocked the taurocholate uptake almost completely (94% inhibition), but reduced bumetanide transport only by 25% and cholate uptake by 36%. Obviously, the SH-dependence of bumetanide and cholate uptake to NEM and PCMBS is equivalent. The uptake process for conjugated bile acids, however, showed a different inhibition pattern in this respect.

Photoaffinity labeling of isolated rat hepatocytes with [^{3}H]7,7-azotaurocholate and [^{3}H]bumetanide in the presence of PCMBS and DTNP

Two fractions of membrane proteins, in the range of 48–49 kDa and 52–54 kDa, become labeled with photoreactive bile acid analogs [17–19]. These proteins are believed to be involved in the hepatocellular transport process of bile acids (for review see Ref. 20). In addition, a 52–54 kDa integral protein in basolateral membranes from rat liver was labeled most specifically by [^{3}H]bumetanide [4,12]. However, the binding of [^{3}H]bumetanide or [^{3}H]7,7-azotaurocholate to the membrane proteins of isolated hepatocytes was not altered in the presence of 200 μM PCMBS, viz., 200 μM DTNP (Fig. 3). This demonstrates that covalent binding of the photoreactive labels to their particular binding sites does not require SH-groups.

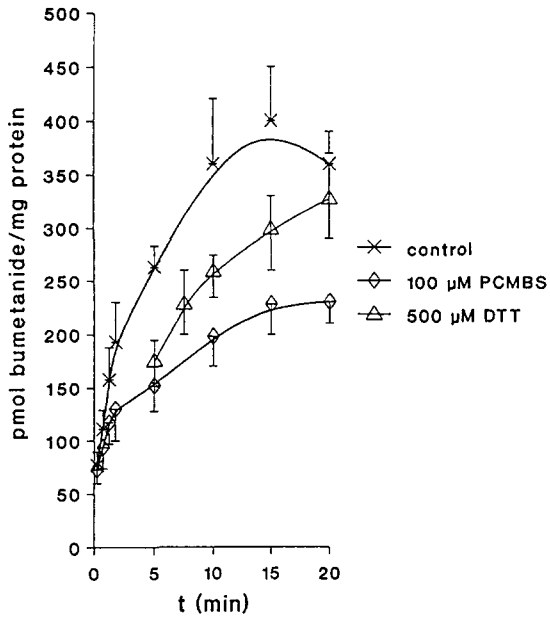

Fig. 1. Effect of DTT on the PCMBS induced inhibition. Hepatocytes in suspension were incubated in the absence (control) and in the presence of 100 μM PCMBS. Uptake was initiated with 60 nM [^{3}H]bumetanide/7 μM bumetanide. After 3 min the treated cells received 500 μM DTT. Values are means ±S.D. of three experiments with n cell preparations (n = 3).

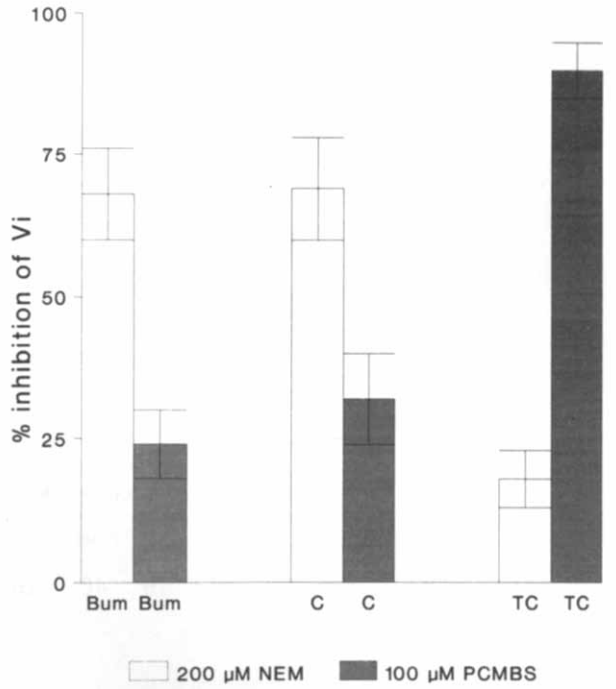

Fig. 2. Effect of NEM and PCMBS on bumetanide and bile acid uptake. Isolated rat hepatocytes were incubated in the presence of either 200 μM NEM or 100 μM PCMBS for 5 min. Uptake of bumetanide, cholate, and taurocholate was measured as described in Materials and Methods. Values are means ±S.D. of three experiments with n cell preparations (n = 3).

Two distinct types of SH-groups are involved in the uptake of bumetanide

Bile acid uptake is dependent on two different moieties of SH-groups [1]. This was concluded from experiments which showed that PCMBS and DTNP inhibit bile acid uptake processes to different degrees. The newly synthesized sulfhydryl-modifying reagents, dithio-sulfonate-ethyl-nitrobenzoic acid (DTSNB) and dithio-octyl-nitrobenzoic acid (DTONB) are derivatives of the alkylating agent dithiobis-nitrobenzoic acid (DTNB). Their structures are shown in Fig. 4. DTSNB is thought to be a selective blocker for SH-groups in hydrophilic regions, while DTONB is more lipophilic and therefore interacts easily with SH-groups in transmembrane domains of proteins. An estimation of their octanol–water coefficient (log p) was determined using the QCPE 608 program. The program provided log p values of 3.9 for DTNB, 0.46 for DTSNB and 6.6 for DTONB. Both derivatives inhibited bumetanide transport in the same dose-dependent manner as DTNB (Fig. 5). The IC_{50}-value for DTONB was 141 μM but 250 μM for DTSNB (Table II). The IC_{50} values for cholate uptake (DTONB 115 μM, DTSNB 250 μM) were almost identical. It appears that 'hydrophobic' SH-groups are more important than hydrophilic groups for bumetanide/cholate uptake. In contrast, the IC_{50} values for taurocholate were 117 μM for DTSNB and 164 μM for DTONB, which demonstrates that free

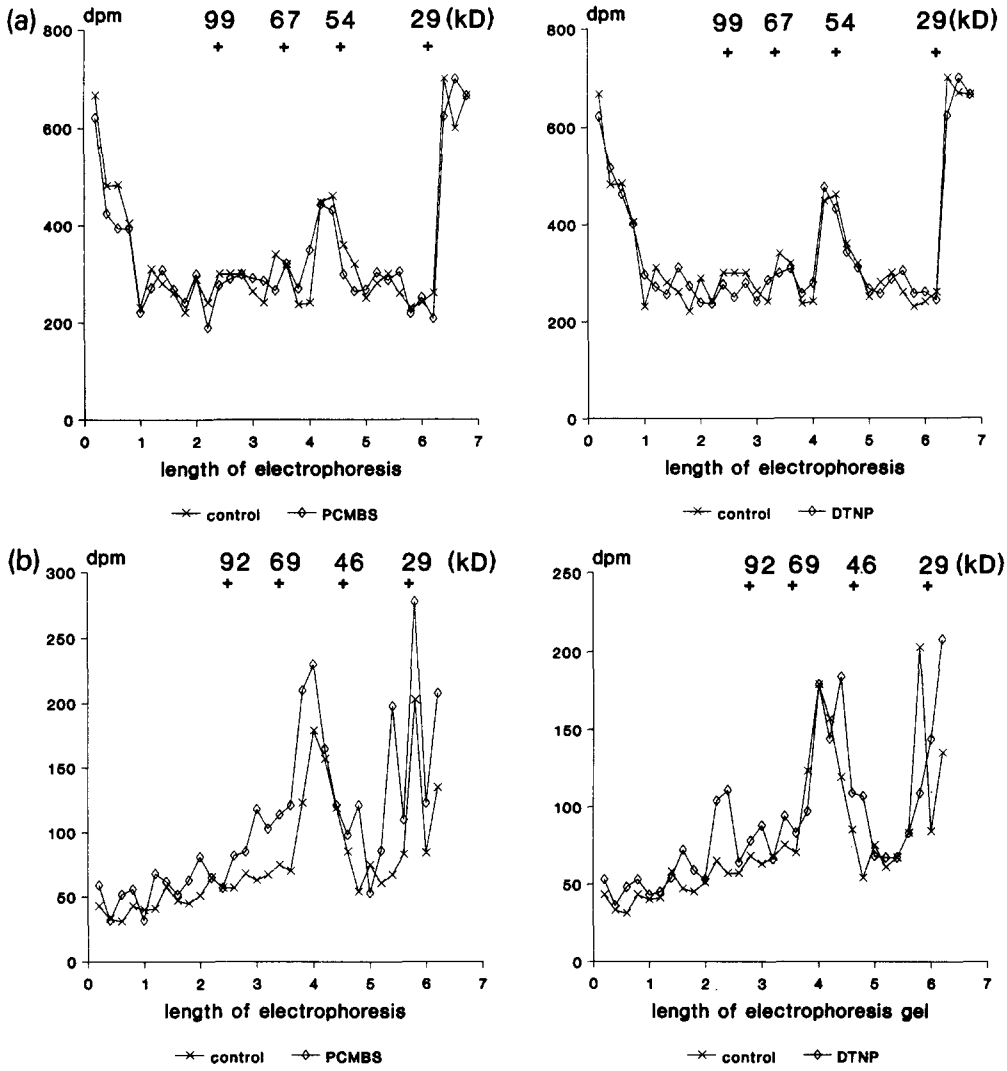

Fig. 3. Photoaffinity labeling of hepatocytes with [^{3}H]bumetanide or with [^{3}H]7,7-azotaurocholate in the presence of PCMBS or DTNP. (a) 1 ml cell suspension ($4 \cdot 10^6$ hepatocytes/ml) was incubated with either 200 μM PCMBS or with 200 μM DTNP for 5 min. The cells received 100 μCi [^{3}H]bumetanide for 1 min and the membrane proteins were labeled using a photoflash device as described. The distribution of the radioactivity was measured after protein separation in SDS-polyacrylamide gels (see Materials and Methods for details). (b) Photoaffinity labeling was performed with 10 μCi [^{3}H]7,7-azotaurocholate as described in Fig. 3a.

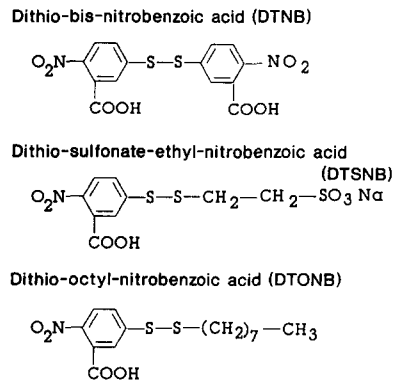

Fig. 4. The structures of DTNB-derivatives DTSNB and DTONB.

SH-groups in a hydrophilic area in this transport protein are important.

Inhibition by DTONB and DTSNB on the Na^+-independent uptake of bumetanide and bile acids

Bumetanide, as well as bile acid uptake, occurs by both Na^+-independent and Na^+-dependent transport [3,21]. In the abscence of sodium ions the IC_{50} values for DTONB were almost identical for all three substrates; 58 μM for bumetanide, 60 μM for cholate and 68 μM for taurocholate, providing evidence for the importance of SH-groups in the transmembrane area

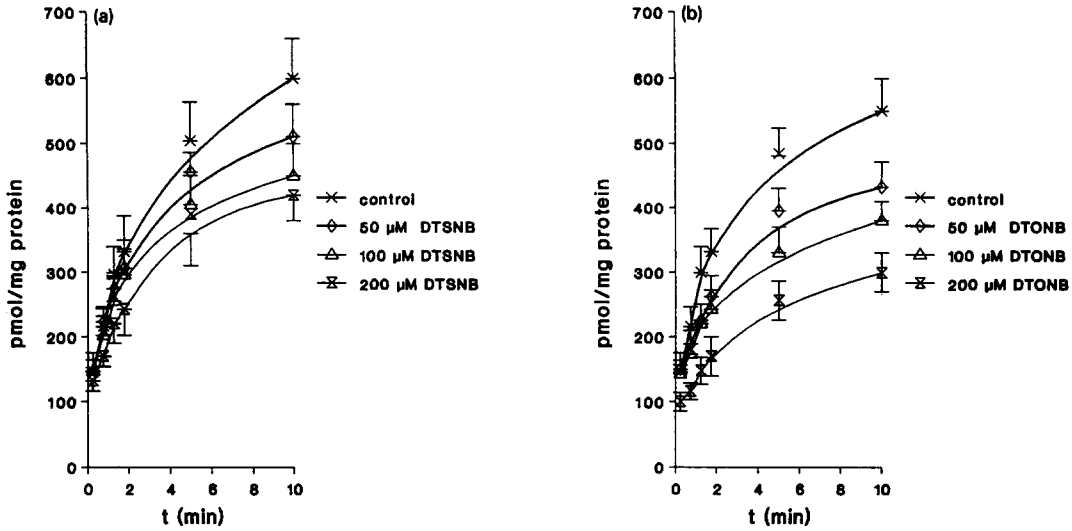

Fig. 5. Inhibition of bumetanide uptake by newly synthesized SH-modifying reagents. (a) Hepatocytes in suspension were incubated in the presence of 50, 100 and 200 μM DTSNB for 5 min. The uptake was initiated with 60 nM [^{3}H]bumetanide/7 μM bumetanide. (b) Comparable experiments as in Fig. 4a with 50, 100 and 200 μM DTONB. Values are means ±S.D. of three experiments with different cell preparations ($n = 3$).

TABLE II

Inhibition data of DTSNB and DTONB on bumetanide and bile acid uptake in presence and absence of Na^+-ions

Hepatocytes in suspension were incubated with 50, 100 or 200 μM of the particular DTNB-derivative for 5 min. Uptake was initiated as described in the legend of Fig. 2. The initial velocity of uptake was determined by linear regression from the 15, 45, 75 and 105-s values. The IC_{50}-values were determined graphically in a semilogarithmic plot. Values are means ±S.D. of four experiments with different cell preparations ($n = 4$).

	Sodium-Tyrode buffer	Choline-Tyrode buffer
IC_{50} value for DTSNB		
Bum	250±47	451±43
C	250±30	505±67
TC	164±20	554±86
IC_{50}-value for DTONB		
Bum	141±25	58±12
C	115±22	60± 8
TC	117±19	68±10

(Table II). In contrast, hydrophilic SH-groups might not be involved in the Na^+-independent transport of these substrates, because the apparent IC_{50}-values for DTSNB under these condition were in the range of 500 μM (Table II).

Discussion

In the present study we used four standard SH-modifying reagents, PCMBS, NEM, DTNP and DTNB and two newly synthesized DTNB-derivatives DTSNB and DTONB as inhibitors of bumetanide uptake into isolated rat hepatocytes. So far, all tested SH-blocking substances inhibited the transport process in a concentration-dependent manner. The specificity of the PCMBS inhibition was investigated in the presence of dithiothreitol. This thiol-compound reversed the blockage in a time-dependent manner by a disulfide exchange. We concluded that bumetanide uptake is dependent upon free SH-groups within the transport protein.

These SH-groups belong to two different classes. The presence of at least two different moieties of SH-groups has been also documented for complete uptake of bile acids [1] and other organic anions such as BSP [22,23]. In the case of bile acid transport synergistic inhibition by PCMBS and NEM or PCMBS and DTNP was observed [1]. The newly synthesized DTNB-derivatives have a common reaction type but are either selective inhibitors for SH-groups at the outer surface of the cell membrane, or for SH-groups in the transmembrane domains of transport proteins. The lipophilic compound DTONB inhibited the uptake of all three substrates bumetanide, cholate, and taurocholate in a concentration-dependent manner with almost identical IC_{50}-values. On the other hand, the hydrophilic blocker DTSNB preferentially blocked taurocholate uptake. The primary amino-acid sequence of the Na^+-dependent taurocholate transporter revealed the presence of seven SH-groups [2]. Two SH-groups are located on the outer surface of the membrane, whereas five other cysteine residues are located in the transmembrane domains. In this context it was recently published that Na^+-dependent taurocholate transport into membrane vesicles from terminal ileum depends on vicinal SH-groups [24]. The precise mechanism of the transloction of taurocholate and organic

anions is still unknown. If SH-groups are involved, rapid conformational changes might occur by means of a disulfide/sulfhydryl exchange process within the transport protein as has been reported for organic cation transport in renal basolateral membrane vesicles [25].

This renal cation transporter contains SH-groups distal from the substrate binding site [25]. From our results it appears that with respect to organic anion uptake into liver cells, SH-groups are not present in the substrate-binding region of the proteins. A 52–54 kDa integral protein in the basolateral membranes from rat liver was labeled specifically by [^{3}H]bumetanide. The binding of bumetanide to this protein was not altered by PCMBS and DTNP. In addition, when bile acid binding was investigated with the photoreactive bile acid analog [^{3}H]7,7-azotaurocholate, the binding was not reduced in the presence of PCMBS and DTNP. It was also not possible to protect the inactivation by NEM in the presence of excessive concentrations of the substrates as has been reported for *N*-methyl-nicotinamide uptake into renal brush border membrane vesicles [26].

The substrate-dependent protection from the inactivation by SH-blockers was, however, observed in amino acid transport of hepatoma cells but not of non-transformed hepatocytes [10]. Such diverging properties of functional SH-groups indicate that the inactivation of transport activities by their blockers can indeed be accomplished by means other than direct chemical modification of substrate recognition sites. For instance, if the protein is a dimer or multimer, rapid conformation changes may be required to allow specific substrates to pass the cell membrane. A multimeric composition of the hepatic bile acid transport systems was suggested [27,28]. It is also likely that those SH-groups which are embedded in the transmembrane spanning regions are reponsible to maintain the tridimensional structure of such multimeric transport protein complexes [15].

One type of SH-groups, the hydrophilic type, seems not to be essential for sodium-independent bumetanide and bile acid transport. DTSNB did not significantly reduce the uptake of all three substrates in the sodium-free incubation buffer. In contrast, the IC_{50}-values for the lipophilic SH-blocker, DTONB were rather low, approx. 60 μM. Therefore, the sodium-independent transport systems are likely without hydrophilic SH-groups and may be related to each other.

Acknowledgements

We gratefully acknowledge the generous gift of the DTNB-derivatives DTSNB and DTONB by Prof. Dr. H. Faulstich, Max-Planck Institut für Medizinische Forschung, Heidelberg. We thank also Dr. B. Boschek for critically reading the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 249).

References

1 Blumrich, M. and Petzinger, E. (1990) Biochim. Biophys. Acta 1029, 1–12.
2 Hagenbuch, B., Stieger, B., Foguet, M., Lübbert, H. and Meier, P.J. (1991) Proc. Natl. Acad. Sci. USA 88, 10629–10635.
3 Petzinger, E., Müller, N., Föllmann, W., Deutscher, J. and Kinne, R.K.H. (1989) Am. J. Physiol. 256, G78–G86.
4 Petzinger, E., Honscha, W., Schenk, A., Föllmann, W., Deutscher, J., Zierhold, K. and Kinne, R.K.H. (1991) Eur. J. Pharmacol. Mol. Sect. 208, 53–65.
5 Petzinger, E., Föllmann, W., Blumrich, M., Schulz, S., Hahnen, J. and Feit, P.W. (1993) Am. J. Physiol. 260, in press.
6 Zimmerli, B., Valantinas, J. and Meier, J.P. (1989) J. Pharm. Exp. Ther. 250, 301–308.
7 Berry, M.N. and Friend, D.S. (1969) J. Cell Biol. 43, 506–520.
8 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
9 Klingenberg, M. and Pfaff, E. (1967) Methods Enzymol. 10, 680–684.
10 Chiles, T.C., Dudeck-Collart, K.L. and Kilberg, M.S. (1988) Am. J. Physiol. 255, C340–C345.
11 Suzuki, T. (1991) J.-CAMD 5, 149–166.
12 Honscha, W., Ottallah, M., Schenk, A., Schuh, K. and Petzinger, E. (1992) Eur. J. Pharmacol. Mol. Sect. 226, 53–65.
13 Faulstich, H., Tews, P. and Heintz, D. (1993) Anal. Biochem. 208, 357–362.
14 Dixon, W.J. (1987) BMDP statistical software. University of California Press, Berkeley.
15 Russo, A. and Bump, E.A. (1988) Methods Biochem. Anal. 33, 165–241.
16 Grassetti, D.R. and Murray, J.F. (1967) Arch. Biochem. Biophys. 119, 41–49.
17 Kramer, W., Bickel, W, Buscher, H.P., Gerok, W. and Kurz, G. (1982) Eur. J. Biochem. 129, 13–24.
18 Von Dippe, P., Drain, P. and Levy, D. (1983) J. Biol. Chem. 258, 8890–8895.
19 Ziegler, K., Frimmer, M. and Fasold, H. (1984) Biochim. Biophys. Acta 769, 117–129.
20 Frimmer, M. and Ziegler, K. (1988) Biochim. Biophys. Acta 947, 75–99.
21 Anwer, M.S., Kroker, R. and Hegner, D. (1978) Hoppe-Seyler's Z. Physiol. Chem. 359, 181–192.
22 Passamonti, S. and Sottocasa, G.L. (1989) Biochim. Biophys. Acta 979, 294–298.
23 Passamonti, S. and Sottocasa, G.L. (1990) Biochim. Biophys. Acta 1021, 9–12.
24 Kramer, W., Nicol, S.B., Girbig, F., Gutjahr, U., Kowalewski, S. and Fasold, H. (1992) Biochim. Biophys. Acta 1111, 93–102.
25 Zimmermann, W.B., Byun, E., McKinney, T.D. and Sokol, P.P. (1991) J. Biol. Chem. 266, 5459–5463.
26 Sokol, P.P., Holoham, P.D. and Ross, C.R. (1986) J. Biol. Chem 261, 3282–3287.
27 Elsner, R. and Ziegler, K. (1989) Biochim. Biophys. Acta 983, 113–117.
28 Elsner, R. and Ziegler, K. (1992) J. Biol. Chem. 267, 9788–9783.