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Research Article

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Expression of the Hepatocellular Chloride-dependent Sulfobromophthalein Uptake System in *Xenopus laevis* Oocytes

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Abstract

The expression of the basolateral chloride-activated organic anion uptake system of rat hepatocytes has been studied in *Xenopus laevis* oocytes. Injection of oocytes with rat liver poly(A)$^+$RNA resulted in the functional expression of chloride-dependent sulfobromophthalein (BSP) uptake within 3–5 d. This expressed chloride-dependent BSP uptake system exhibited saturation kinetics (apparent $K_m \sim 6.2$ μM) and efficiently extracted BSP from its binding sites on BSA. Furthermore, the chloride-activated portion of BSP uptake was inhibited by bilirubin (10 μM; inhibition 53%), 4,4'-diisothiocyano-2,2-disulfonic acid stilbene (DIDS, 100 μM; 80%), taurocholate (100 μM; 80%), and cholate (200 μM; 95%). In contrast to results with total rat liver mRNA, injection of mRNA derived from the Na$^+$/bile acid cotransporter cDNA (Hagenbuch, B., B. Stieger, M. Foguet, H. Lübbert, and P. J. Meier. 1991. *Proc. Natl. Acad. Sci. USA.* In press.) had no effect on BSP uptake into oocytes. Size fractionation of total rat liver mRNA revealed that a 2.0- to 3.5-kb size-class mRNA was sufficient to express the hepatic chloride-dependent BSP uptake system. These data indicate that "expression cloning" in oocytes represents a promising approach to ultimately clone the cDNA coding for the hepatocyte high affinity, chloride-dependent organic anion uptake system. Furthermore, the results confirm that the Na$^+$/bile acid cotransport system does not mediate BSP uptake. (*J. Clin. Invest.* 1991. 88:2146–2149.) Key words: expression-cloning • hepatic organic anion uptake

Introduction

Hepatocellular uptake of the organic anions bilirubin and sulfobromophthalein (BSP)[1] is generally assumed to be a carrier-mediated membrane transport process (1, 2). However, despite numerous efforts in recent years, neither the exact driving force(s) nor the putative transport protein(s) have been well defined. For example, evidence has been provided for both electrogenic (2, 3) and electroneutral (4) BSP uptake into hepatocytes. Furthermore, intracellular (or intravesicular) acidic (2) as well as alkaline (4) pH gradients have been proposed to drive hepatocytic BSP uptake. And finally, several distinct proteins have been identified as candidate BSP transporters, including the so called BSP/bilirubin binding protein (~ 55 kD) (1), the organic anion binding protein (~ 55 kD) (5, 6), and the bilitranslocase (~ 37 kD) (2). Although all these proteins have been suggested as having a role in BSP uptake, none have been characterized fully on the molecular level nor has their structure/function relationship(s) been established.

As recently demonstrated, a characteristic feature of hepatocellular BSP uptake is its dependence on extracellular chloride (3, 4, 7). In cultured rat hepatocytes this chloride-dependent uptake system has an exceptionally high affinity for BSP ($K_m \sim 0.3$ μM), resulting in efficient extraction of BSP from its binding sites on albumin (4, 7). Because none of the putative BSP-transporting polypeptides (see above) has been identified as mediating chloride-dependent BSP uptake, we adopted a functional expression cloning strategy to isolate and characterize the full length cDNA encoding this important hepatocytic transport system. In this study we report the successful expression of chloride-dependent BSP uptake in *Xenopus laevis* oocytes injected with either total rat liver mRNA or a 2.0–3.5-kb size-class thereof. Furthermore, the results demonstrate that the recently cloned hepatocellular Na$^+$/bile acid cotransporter (8) does not mediate BSP uptake, thus confirming the involvement of different transport systems in the hepatocyte uptake of bile acids and of non–bile acid organic anions (1, 2). Hence, expression cloning represents a promising approach to ultimately characterize the gene of the basolateral chloride-dependent BSP uptake system of rat liver.

Methods

Materials. ^{35}S-labeled BSP was prepared at a specific activity of 4 Ci/mmol by sulfonation of phenoltetrabromophthalein with $H_2{}^{35}SO_4$ as described (9). [6-^{3}H]Taurocholic acid (2.1–6.6 Ci/mmol) and [2,4-^{3}H]cholic acid (25 Ci/mmol) were obtained from Du Pont-New England Nuclear (Boston, MA). Guanidinium isothiocyanate, phenol, and sucrose were from Bethesda Research Laboratories (Gaithersburg, MD). Oligo (dT)-cellulose (type 7) was obtained from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Ethyl m-aminobenzoate (MS-222) was purchased from Sandoz Ltd. (Basel, Switzerland).

Animals. Male Sprague-Dawley rats (SUT:SDT) weighing 200–250 g were obtained from the Süddeutsches Tierzuchtinstitut (Tuttlingen, Germany) and fed ad lib. until used. Mature *X. laevis* females were purchased from H. Kähler (Hamburg, Germany) and kept under standard conditions as described (10).

Isolation of rat liver mRNA. Total RNA from 10 g of rat liver was prepared and mRNA purified, size-fractionated, and precipitated as previously described (11). Isolated mRNA was resuspended in water and stored at −80°C. mRNA concentrations were estimated by measuring the absorption at 260 nm (12).

Expression of mRNA in Xenopus oocytes. Frogs were anesthetized by immersion for 15 min in a 0.1% solution of ethyl m-aminobenzoate (MS-222). Oocytes were removed and incubated at room temperature for 2 h in Ca^{++}-free OR-2 solution (10), supplemented with 2 mg/ml

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1. *Abbreviation used in this paper:* BSP, sulfobromophthalein.

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collagenase (type D; Boehringer Mannheim Biochemicals, Indianapolis, IN). They were then washed in a modified Barth's solution (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 15 mM Hepes-NaOH, pH 7.6, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 10 U/ml penicillin, 10 μg/ml streptomycin), supplemented with gentamycin (50 μg/ml) and soybean trypsin inhibitor (50 μg/ml). After an overnight incubation at 18°C in modified Barth's solution, healthy oocytes were injected either with 50 ng of rat liver mRNA (1 mg/ml) or with 0.5 ng of mRNA derived from the recently characterized "prLNaBA" cDNA clone encoding the hepatocyte Na^+/bile acid cotransport system (8). Control oocytes were either treated with corresponding volumes of H_2O or not injected at all. Subsequently, oocytes were cultured for 3–5 d at 18°C with a daily change of modified Barth's solution.

BSP uptake into oocytes. Cultured oocytes were washed twice at 25°C in chloride-containing or chloride-free uptake media consisting of either (in mM) 100 NaCl (or 100 choline chloride), 1.2 $MgCl_2$, 0.81 $MgSO_4$, 2.5 $CaCl_2$, 25 Hepes-Tris, pH 7.2, or 100 sodium gluconate (or 200 sucrose), 1.2 $Mg(gluconate)_2$, 0.81 $MgSO_4$, 2.5 $Ca(gluconate)_2$, 25 Hepes-Tris, pH 7.2, respectively. Between 7 and 10 oocytes were then incubated at 25°C in 100 μl of the corresponding uptake medium supplemented with 1–2 μCi of [^{35}S]BSP and 7.35×10^{-3} mM (0.05%) BSA. With the exception of the kinetic uptake studies, the concentrations of BSP in the uptake media were adjusted to 2 or 4 μM and the albumin/BSP ratios kept constant at 3.7:1 (7). After the indicated time interval, the uptake was stopped by the addition of 1 ml of ice-cold stop solution which was similar in composition to the NaCl uptake buffer except that 0.74 mM (5%) BSA was included in order to reduce nonspecific surface binding of tracer BSP. Furthermore, oocytes were additionally washed 3× with 8 ml of cold stop solution. Single oocytes were then dissolved in 500 μl of 10% (wt/wt) SDS. After addition of 5 ml of scintillation fluid (Opti-Fluor™; Packard Instrument International S.A., Zurich, Switzerland) the oocyte-associated radioactivity was determined in a Packard Tri-Carb™ 2200 CA liquid scintillation analyzer.

Taurocholate uptake into oocytes. The overall transport competence of mRNA-injected oocytes was routinely tested by parallel measurements of Na^+-dependent taurocholate uptake as previously described (11). Only mRNA samples expressing satisfactory Na^+-dependent taurocholate uptake were used for BSP uptake studies. In addition, oocytes injected with mRNA derived from the so-called prLNaBA cDNA clone (8) were used to examine a possible direct role of the hepatic Na^+/bile acid cotransporter in BSP uptake.

Statistical analysis of data. All results are expressed as means±SD. Statistical significance was calculated by the unpaired Student's *t* test.

Results and Discussion

In order to exclude mere activation of an endogenous transport system, it was first important to exclude the presence of chloride-dependent BSP uptake in untreated oocytes. As demonstrated in Fig. 1, uninjected oocytes exhibited only low BSP uptake. Similar low BSP uptake was also found in water-injected oocytes (data not shown). Furthermore, no increased BSP uptake was observed in oocytes injected with total rat liver mRNA if only sucrose was present in the extracellular medium. However, in the presence of outside NaCl (100 mM) the mRNA-injected oocytes exhibited three- to sixfold higher BSP uptake as compared to results in the presence of outside sucrose or uninjected oocytes (Fig. 1). In addition, extraction of BSP from albumin was highest in the presence of chloride in the medium and was significantly reduced with gluconate substitution (Fig. 2). These BSP uptake characteristics are identical to the ones previously described in the isolated perfused rat liver (7), in cultured rat hepatocytes (4, 7), and in isolated rat liver sinusoidal vesicles (3). These results indicate de novo expression of this hepatocyte chloride-dependent organic anion transport system in 5-d cultured oocytes.

We next characterized the kinetics of the expressed BSP

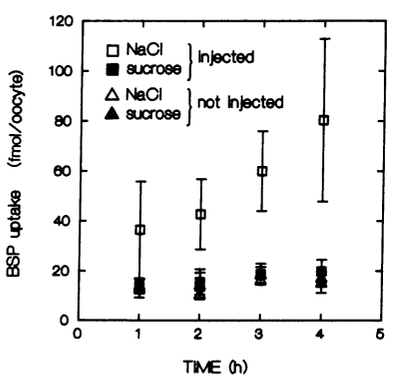

Figure 1. Expression of BSP uptake in oocytes injected with total rat liver mRNA. Oocytes were either untreated (not injected) or injected with 50 ng of liver mRNA (injection volume: 50 nl). After 5 d of culturing at 18°C, the oocytes were washed twice in NaCl or sucrose medium and uptake of [^{35}S]BSP (2 μM) was determined at 25°C in the presence (□, △) or absence (■, ▲) of extracellular NaCl, respectively. The illustrated uptake values represent the mean±SD of 10–20 measurements in two separate oocyte preparations.

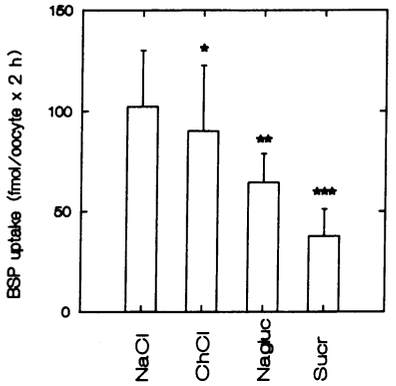

Figure 2. Effects of extracellular ion substitutions on BSP uptake into mRNA injected oocytes. Oocytes were injected with 50 ng of total rat liver mRNA (injection volume: 50 nl). After 5 d of culturing at 18°C, uptake of [^{35}S]BSP (4 μM) was determined at 25°C for 2 h in the presence of outside NaCl, choline chloride, sodium gluconate (each at a concentration of 100 mM) or sucrose (200 mM). The illustrated uptake values represent the mean±SD of 17–19 determinations in two separate oocyte preparations. *Not significant vs NaCl; **, significantly different from NaCl ($P < 0.001$) and choline chloride ($P < 0.01$); ***, significantly different from NaCl ($P < 0.001$), choline chloride ($P < 0.001$), and sodium gluconate ($P < 0.01$).

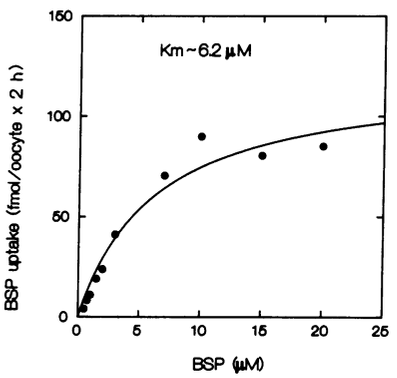

Figure 3. Kinetics of BSP uptake in mRNA injected oocytes. Oocytes were injected with 50 ng of rat liver mRNA and cultured for 3 d at 18°C. 2-h BSP uptake measurements (25°C) were then performed in the presence of increasing substrate concentrations (0.5–20 μM) in the presence of 100 mM NaCl or 200 mM sucrose in the incubation medium. The ratio of albumin/BSP was kept constant at 18.4:1 to minimize changes in the fraction of unbound ligand (7). The individual data points represent the difference between uptake values obtained in the presence and absence of outside NaCl. Data represent the means of 10–20 determinations in two separate oocyte preparations. The curve was fitted by a computer-based nonlinear regression analysis.

Table I. Effects of Various Organic Anions on Chloride-dependent BSP Uptake in mRNA-injected Oocytes

	BSP uptake	
	fmol/oocyte × 2 h	%
Controls	64.5±28.3	100
Probenecide (1 mM)	41.8±11.1 (NS)	65
Bilirubin (0.01 mM)	30.5±8.6 ($P < 0.01$)	47
DIDS (0.1 mM)	19.0±28.1 ($P < 0.01$)	29
Indocyanine green (0.1 mM)	12.6±6.4 ($P < 0.001$)	20
Taurocholate (0.1 mM)	12.6±6.4 ($P < 0.001$)	20
Cholate (0.2 mM)	3.5±3.7 ($P < 0.001$)	5

Oocytes were injected with 50 ng of total rat liver mRNA. After 3 d in culture (18°C), chloride-dependent portions of BSP (4 μM) uptake were determined at 25°C for 2 h in the absence (controls) and presence of the indicated concentrations of the various compounds. The absolute values are given as means±SD of 7–10 uptake measurements in one representative oocyte preparation. Levels of significance are given in parentheses. NS, not significant.

uptake system. As demonstrated in Fig. 3, if BSP uptake values (2 h) in the absence of outside chloride (isoosmotic substitution of NaCl with sucrose) were subtracted from uptake values in the presence of extracellular chloride (100 mM NaCl), the chloride-dependent BSP uptake portion exhibited clearcut saturability with increasing substrate concentration. Furthermore, at all substrate concentrations, BSP was efficiently extracted from albumin, because the BSP/albumin concentration ratio (1:18.4) was kept constant throughout the experiment. These results are compatible with carrier-mediated uptake of BSP into oocytes and further support the conclusion of successful expression of the hepatocytic chloride-dependent organic anion uptake system.

However, as also indicated in Fig. 3, the analysis of the saturation kinetics of the expressed chloride-dependent BSP uptake system revealed an apparent K_m value of 6.2 μM. While this value is consistent with various "albumin-free" BSP uptake studies in isolated rat hepatocytes (range of reported K_m values: 6.2–9.0 μM) (13–15) and in cultured Hep G2 cells (K_m ~ 12 μM) (16), it is ~ 20–30-fold higher than the value found for the chloride-dependent BSP uptake system in short-term cultured rat hepatocytes (K_m ~ 0.3 μM) (4, 7). The reason for this considerably lower BSP affinity of the chloride-dependent BSP uptake system in oocytes as compared to cultured hepatocytes is unknown at present, but there are several potential explanations. These include differences in the physicochemical properties between rat and Xenopus oocyte plasma membranes, and/or the inability to determine BSP uptake at substrate concentrations below 0.5 μM because of low signal/noise ratios (< 1 μM). This situation could not be improved by increasing the incubation temperature from 25°C to 37°C, because, in our hands, the oocytes did not tolerate such a high temperature for longer than 60 min (data not shown). Hence, as the expressed BSP uptake system was still able to efficiently extract BSP from albumin (K_a of BSA for BSP ~ 0.33 μM^{-1}; reference 4), the apparently lower BSP affinity in mRNA-injected oocytes does not invalidate the conclusion of successful expression of the hepatocyte chloride-dependent BSP uptake system. More accurate kinetic transport studies will become possible once a specific cDNA clone has been isolated. This should permit BSP uptake to be determined at lower substrate concentrations because of considerably higher transport rates (8).

The current paradigm holds that BSP and bilirubin are taken up into hepatocytes by the same membrane transport system (1, 2, 7, 17, 18). Therefore, bilirubin should strongly inhibit BSP uptake via the expressed transport system. In fact, as indicated in Table I, 10 μM bilirubin exerted a *cis*-inhibitory effect of 53%, which is in close agreement with the reported K_i value of 6.7 μM in cultured hepatocytes (7). Furthermore, even stronger *cis*-inhibitions were found with the anion transport inhibitor DIDS (4,4′-diisothiocyano-2,2′stilbene disulfonate), indocyanine green, and also with relatively high concentrations of the bile acids taurocholate and cholate (Table I). Although some previous studies have suggested competition between hepatocellular uptake of bile acids and BSP (15, 19), most studies agree that physiologic concentrations of bile acids and nonbile acid organic anions such as BSP are taken up into hepatocytes by different transport systems (for reviews see 1, 2, 18). Therefore, we also investigated whether the recently cloned Na^+/bile acid cotransporter (8) could indeed mediate BSP uptake. As demonstrated in Table II, while Na^+-dependent uptake of tau-

Table II. Effects of mRNA Derived from the Na^+/Bile Acid Cotransporter cDNA (prLNaBA) on Taurocholate, Cholate, and BSP Uptake in X. laevis Oocytes

Oocyte treatment	Medium	Taurocholate (17 μM)	Cholate (0.5 μM)	BSP (2 μM)
		fmol/oocyte × 1 h		*fmol/oocyte × 2 h*
Not injected	NaCl	90±13	24±1	12±4
	Choline chloride	104±24	13±2	ND
	Sucrose	ND	ND	11±2
Total mRNA	NaCl	300±80	ND	55±20
(25–50 ng)	Choline chloride	146±57	ND	ND
	Sucrose	ND	ND	12±3
prLNaBA-mRNA	NaCl	3044±1155*	223±47‡	15±5*
(0.5–5 ng)	Choline chloride	140±52*	16±1‡	ND
	Sucrose	ND	ND	10±2*

Oocytes were either not treated or injected with total rat liver mRNA or prLNaBA-cDNA–derived mRNA. *0.5 ng; ‡5 ng. Oocytes were cultured for 3 d and uptake of the various compounds determined as described in Methods. The values represent the means±SD of 10–20 determinations. ND, not determined.

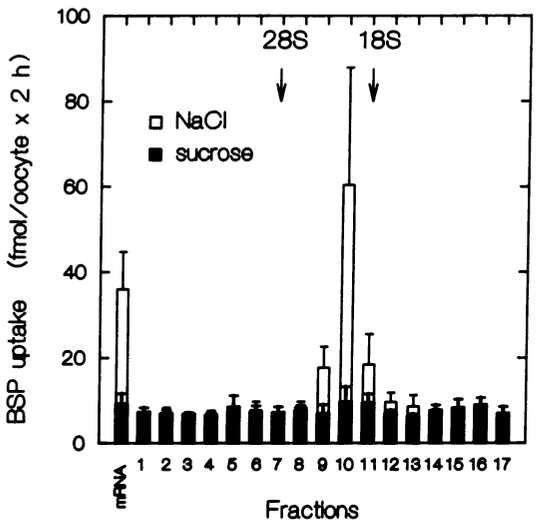

Figure 4. Fractionation of the mRNA on a linear sucrose density gradient. 150 μg of total rat liver mRNA was size-fractionated on a linear (6–20% [wt/wt]) sucrose gradient. After centrifugation 17 × 1-ml fractions were collected and the mRNA from each fraction was precipitated, washed, and resuspended in H_2O. 25-ng RNA samples (50 nl) were injected into oocytes. After 3 d in culture the oocytes were used for BSP (2 μM) uptake measurements (2 h values) in the presence of either 100 mM NaCl (□) or 200 mM sucrose (■) in the outside medium. The uptake values represent the mean±SD of 7–10 determinations in two separate oocyte preparations.

rocholate and cholate were increased 22- and 14-fold, respectively, injection of mRNA derived from the Na^+/bile acid cotransporter cDNA had no effect on BSP uptake into oocytes. In contrast, chloride-dependent BSP uptake was stimulated approximately fivefold after injection of total rat liver mRNA into oocytes. These data first demonstrate that, similar to intact rat hepatocytes (20) and isolated basolateral rat liver plasma membrane vesicles (21), the cloned bile acid uptake system (8) also mediates Na^+-dependent uptake of the unconjugated bile acid cholate in addition to its taurine-conjugated analogue taurocholate. Furthermore, the results confirm the concept that chloride-dependent BSP uptake occurs independent of Na^+-dependent bile acid uptake in rat hepatocytes. However, these studies do not exclude the possibility that Na^+-independent portion of hepatocellular bile acid uptake (22) might, at least in part, also occur via the chloride-dependent BSP uptake pathway.

Finally, total rat liver mRNA was size-fractionated on a linear sucrose gradient in order to enrich the mRNA species encoding the chloride-dependent BSP uptake system. As shown in Fig. 4, the most active mRNA was found within one subfraction, which, based on additional agarose gel electrophoresis (data not shown), corresponded to a mRNA size-class between 2 and 3.5 kb. This active mRNA subfraction is now being used to construct and screen a cDNA library in order to isolate and characterize a full-length cDNA encoding the hepatocellular chloride-dependent BSP uptake system. Hence, "expression cloning" in oocytes should ultimately provide a definite answer about the molecular structure and function of the chloride-activated hepatocellular nonbile acid organic anion uptake system.

Acknowledgments

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