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Research Article

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Hepatocellular Uptake of Sulfobromophthalein and Bilirubin Is Selectively Inhibited by an Antibody to the Liver Plasma Membrane Sulfobromophthalein/Bilirubin Binding Protein

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Abstract

To clarify sulfobromophthalein (BSP) and bilirubin uptake mechanisms, isolated rat hepatocytes were incubated with [^{35}S]BSP. The initial uptake velocity (V_0), determined from the first, linear portion of the cumulative uptake curve, was saturable (Michaelis constant [K_m] = 6.2±0.5 μM; V_{max} = 638±33 pmol × min^{-1} per 10^5 hepatocytes), maximal at 37°C and pH 7.4, and competitively inhibited by bilirubin, but not by taurocholate, cholate, or oleate. Preloading with unlabeled BSP led to transstimulation of V_0. Sodium substitution or pretreatment of hepatocytes with ouabain or metabolic inhibitors had no effect on V_0; trypsin reduced V_0 by 39% ($P < 0.001$). A rabbit antiserum to the rat liver plasma membrane (LPM)-BSP/bilirubin binding protein selectively reduced V_0 of 5 μM [^{35}S]BSP and [^{14}C]bilirubin by 41 and 42%, respectively ($P < 0.01$); uptakes of [^{3}H]oleate, [^{3}H]cholate and [^{3}H]taurocholate were not affected. Hence, the LPM-BSP/bilirubin binding protein plays a role in the carrier-mediated uptake of BSP and bilirubin by hepatocytes.

Introduction

Conjugated bile acids, free fatty acids, and nonbile acid cholephils including sulfobromophthalein (BSP)[1] and bilirubin represent three classes of organic anions efficiently extracted from blood by the liver (1) despite tight binding to albumin (2–4). Hepatocellular uptake processes for these three classes of organic anions share common features. Each exhibits a series of kinetic phenomena that have come to be called the albumin receptor effect (5–8); for each, a specific liver plasma membrane (LPM) binding protein (9–12) and a discrete cytosolic receptor protein (13–15) have been identified. The hepatocellular uptake kinetics of bile acids (16–17), free fatty acids (18), and BSP and bilirubin (19–20) are all compatible with carrier-mediated transport, and the bile acid transport system has been partially reconstituted in artificial liposomes by incorporation of the LPM bile acid binding protein into synthetic lipid bilayers (21). A physiologic role for the LPM fatty acid binding protein has recently been established in studies showing that antibodies to this protein selectively inhibit hepatocellular fatty acid uptake (18). An analogous transport function for the LPM-BSP/bilirubin binding protein has been postulated (9, 22) but not yet established.

In the present study we demonstrate that a rabbit antibody to the rat LPM-BSP/bilirubin binding protein selectively inhibits [^{35}S]BSP and [^{14}C]bilirubin uptake by isolated rat hepatocytes. When added to available kinetic evidence, this observation strongly supports the concept that BSP and bilirubin enter the hepatocyte via a membrane protein–associated carrier mechanism.

Methods

Materials. 9,10-[^{3}H]Oleic acid (3 Ci/mmol), [^{3}H]taurocholic acid (6.8 Ci/mmol), and 2,4-[^{3}H]cholic acid (25 Ci/mmol) were from New England Nuclear (Boston, MA); [^{35}S]sulfobromophthalein (disodium phenoltetrabromophthalein di-[^{35}S]sulfonate; [^{35}S]BSP, 24 mCi/mmol) and 1′,2,3′,4,5,6′,7′,8-[^{14}C]bilirubin (63 mCi/mmol) were from Amersham International (Buckinghamshire, England). The unconjugated [^{14}C]-bilirubin was repurified as previously described (9). Unlabeled BSP, bilirubin, oleate, taurocholate, cholate, and trypsin were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of hepatocytes. Hepatocytes of male Wistar rats (250–280 g) were isolated by a modification of the procedure of Berry and Friend (23) previously described in detail (18). The resulting homogenous single cell suspension was washed twice by addition of 0.15 M NaCl/0.02 M phosphate buffer (PBS) (pH 7.4) and centrifugation at 50 *g* for 1.5 min. After dilution to a concentration of 2 × 10^6 cells/ml with PBS, the hepatocyte suspension was stored on ice for up to 2 h until use. Viability tests were routinely done on every preparation before and after each experiment, and included trypan blue exclusion and determinations of lactate dehydrogenase leakage, intracellular K^+ concentration, and stimulation of respiration by succinate (18).

Uptake studies. After a 10-min preincubation period at 37°C, 250 μl of the hepatocyte suspension (2 × 10^6 cells/ml) was incubated with radiolabeled ligand in polypropylene tubes in a total volume of 1 ml PBS. At various time intervals 200-μl aliquots of the incubation mixture were pipetted onto the center of Whatman GF/C glass filters (24 mm, Whatman Chemical Separation, Inc., Clifton, NJ) and filtered under 50 mmHg vacuum pressure using a filtration apparatus (model 7 H, Hoefer Scientific, San Francisco, CA) (18). The filters were washed with 20 ml PBS. Washing with more PBS did not change the radioactivity remaining on the filters. The filters were placed in scintillation vials, 10 ml Aquasol (New England Nuclear) was added, and the radioactivity determined in a 1217 Rackbeta liquid scintillation counter (LKB Wallace, Turku, Finland). Quench correction was determined by a channels ratio method. Ligand absorption to the filters in the absence of cells was very reproducible for any given experimental circumstance and constituted 1–4%. The radioactivity absorbed to the filters without cells was routinely determined and subtracted from values measured with the cells. Studies of [^{35}S]BSP uptake included the following information: time course of

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1. *Abbreviations used in this paper:* BSP, sulfobromophthalein; LPM, liver plasma membrane.

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uptake, assessment of temperature dependency at 4°, 16°, and 37°C and of pH dependency by varying the pH of the incubation medium from 6.0 to 8.4 using the phosphate buffer system of Soerensen (24). The effect of various organic anions on [^{35}S]BSP uptake was analyzed by incubation of 250 μl of the hepatocyte suspension (2×10^6 cells/ml) with 5 μM [^{35}S]BSP either alone or in the presence of 10 or 40 μM unlabeled BSP, taurocholate, cholate, bilirubin, or oleate. Because of their limited solubilities the latter two compounds were originally dissolved in 0.1 N NaOH, and subjected to rapid neutralization and ultrasonication immediately before use.

The effect of trypsin pretreatment of hepatocytes on [^{35}S]BSP uptake was determined by preincubation of 2 ml of the hepatocyte suspension (2×10^6 cells/ml) with 1 ml trypsin solution (0.1 mg/ml) in 10 mM Tris/HCl, pH 8.0, for 15 min at 37°C. The cells were then washed three times with PBS, centrifuged and resuspended to 2×10^6 cells/ml. Controls were incubated with 10 mM Tris/HCl, pH 8, without trypsin and thereafter washed with PBS, centrifuged, and resuspended to 2×10^6 cells/ml. The viability of the cells after preincubation was >95%. 250 μl of the cell suspension was then incubated with 3 μM [^{35}S]BSP in 1 ml PBS at 37°C.

To evaluate the effect on [^{35}S]BSP uptake of preloading hepatocytes with unlabeled BSP, 4 ml of the hepatocyte suspension (2×10^6 cells/ml) was incubated with 0, 5, 10, 15, or 20 μM BSP in a total volume of 8 ml PBS at 37°C for 5 min. Cells were then washed three times with PBS, centrifuged and resuspended to 2×10^6 cells/ml (viability > 95%). Thereafter 250 μl of the pretreated cells were incubated with 10 μM [^{35}S]BSP in 1 ml PBS at 37°C.

Antibody inhibition studies. Uptake of ligands was examined in hepatocytes pretreated either with a monospecific rabbit antibody to the LPM-BSP/bilirubin binding protein (IgG concentration 10 mg/ml) newly prepared as previously described (9), or with the preimmune serum as control. 4 ml of the isolated hepatocytes (2×10^6/ml) were incubated for 1 h at 4°C with 400 μl of the appropriate serum, and subsequently centrifuged and washed three times in PBS. Antibody pretreated cell suspensions were then rediluted to a concentration of 2×10^6 cells/ml. 250 μl of the pretreated hepatocyte suspensions were incubated at 37°C and 4°C with increasing concentrations of [^{35}S]BSP as well as with other labeled organic anions such as bilirubin, oleate, taurocholate, or cholate at concentrations of 5 μM. Uptake was determined as described above.

Data analysis. For calculation of kinetic constants, values for the initial uptake rate (V_0) of [^{35}S]BSP at various BSP concentrations were fitted directly to the Michaelis-Menten equation or to its Lineweaver-Burk or Eadie-Hofstee linear transformations, employing version 24 of the simulation, analysis, and modeling (SAAM) program of Berman and Weiss (25) on the IBM mainframe computer system of the City University of New York. As previously described (25–27), the program uses an iterative nonlinear least-squares fitting procedure to determine the parameters of a mathematical function describing a data set, or the parameters of a compatible model. In the region of the least-squares solution, the program also calculates a variance-covariance matrix for these parameters, from which it estimates their uncertainties (standard deviations) and correlation coefficients. The significance of differences between two estimates of a given parameter may be determined from the calculated values and standard deviations of the two estimates using a Z distribution (28).

Results

Kinetics of [^{35}S]BSP uptake. Incubation of 10 μM [^{35}S]BSP with 10^6 hepatocytes in 2 ml PBS at 37°C demonstrated that the slope of the cumulative uptake curve was maximal and linear over the first 60 s (Fig. 1). The slope gradually decreased over the next minute and then remained constant between 2 and 7 min during which time the cells continued to accumulate isotope slowly. The initial uptake rate (V_0) was taken to be the slope of the linear portion of the cumulative uptake curve over the initial 60-s period. The reduced rate of net accumulation observed between 1 and 7 min reflects a balance between continued uptake and efflux of intracellular BSP, and not substrate depletion, as demonstrated for the hepatocellular uptake of other compounds (18). With increasing concentrations of [^{35}S]BSP (0.075–20 μM) incubated at 37°, V_0 increased nonlinearly (Fig. 2). In contrast, uptake at 4°C, considered to represent a nonspecific component of uptake and binding, was markedly slower and linear. When the total uptake curve (37°C) was corrected for nonspecific uptake (4°C), an apparent saturation curve was observed (Fig. 2). Analyzed according to the Michaelis-Menten equation, the estimated K_m was 6.2±0.5 μM and V_{max} 638±(SD) 33 pmol × min^{-1} per 10^5 hepatocytes. [^{35}S]BSP uptake was maximal at 37°C. Uptake was reduced by 54% at 16°C and by 81% at 4°C. The pH optimum of V_0 occurred at pH 7.4. Pretreatment of hepatocytes with trypsin yielded a 39% reduction of V_0 from 297±13 to 181±14 pmol × min^{-1} per 10^5 hepatocytes ($P < 0.001$).

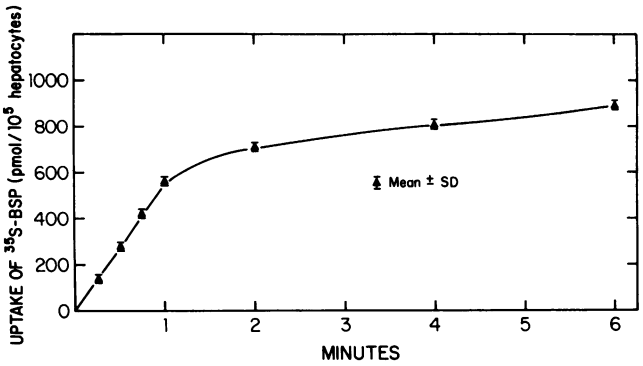

Figure 1. Time course of uptake of [^{35}S]BSP by isolated rat hepatocytes. See text for experimental details.

Preloading phenomenon. V_0 in control cells in these studies was 537±26 pmol × min^{-1} per 10^5 hepatocytes. In cells preloaded with 5, 10, and 15 μM BSP, V_0 increased nonlinearly to 613±19, 669±23, and 684±26 pmol × min^{-1} per 10^5 hepatocytes, respectively. All of these rates are significantly faster than in control cells (Student's t test, $n = 6$ per group, $P < 0.01$), and indicate that the BSP uptake mechanism is characterized by accelerative exchange diffusion. When cells were preloaded with BSP concentrations > 15 μM, no further increase in V_0 was observed.

Effect of other organic anions on [^{35}S]BSP uptake. 5 μM [^{35}S]BSP was incubated either alone or in the presence of 10 and 40 μM unlabeled BSP, bilirubin, oleate, taurocholate, or cholate. V_0 of [^{35}S]BSP (404±23 pmol × min^{-1} per 10^5 hepatocytes) was reduced both in the presence of cold BSP (10 μM: 227±34, $P < 0.005$; 40 μM: 107±15, $P < 0.001$) and in the presence of cold bilirubin (10 μM: 207±13, $P < 0.001$; 40 μM: 118±20, $P < 0.001$). Uptake in the presence of excess unlabeled oleate, taurocholate, or cholate remained unaltered. In incubations conducted in the presence of 5 μM bilirubin, V_{max} (698±49 pmol × min^{-1} per 10^5 hepatocytes) was identical to that in the control incubation, whereas K_m (13.9±0.4 μM vs. 7.0±0.7 μM) was significantly increased ($P < 0.0001$), indicating purely competitive inhibition of BSP uptake by bilirubin. These results are compatible with the hypothesis that BSP and bilirubin share a common uptake mechanism, which is distinct from the uptake mechanism of the other classes of organic anions tested.

Effects of ion substitution and inhibitors. Substitution of 110 mM KCl/25 mM NaCl, 143 mM LiCl, or 246 mM sucrose for 143 mM NaCl in the incubation medium had no effect on V_0

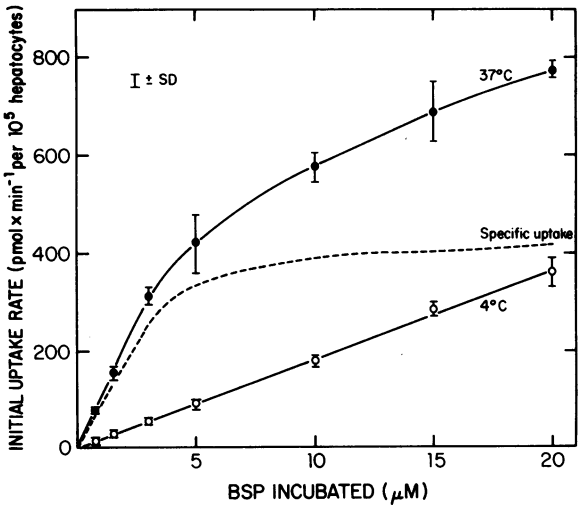

Figure 2. Uptake of [^{35}S]BSP by isolated rat hepatocytes at 37°C and 4°C, as a function of ligand concentration. Values at 4°C are taken to represent nonspecific uptake. Specific uptake (dashed line) is depicted as the difference between two curves observed at 37°C and 4°C.

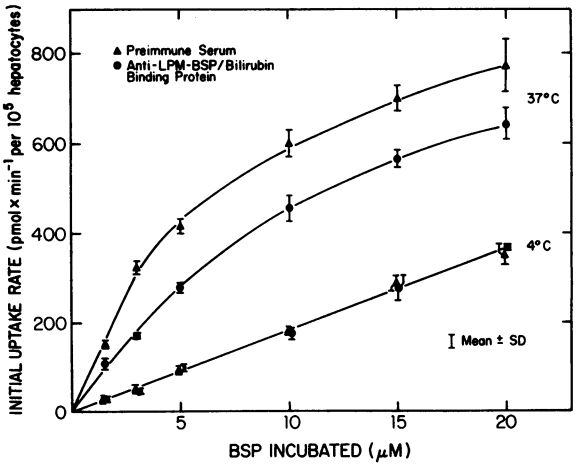

Figure 3. Effects on [^{35}S]BSP uptake velocity of an antibody to the LPM-BSP/bilirubin binding protein. The antibody significantly inhibited total [^{35}S]BSP uptake at 37°C at all BSP concentrations studied, but had no effect on the nonspecific uptake component measured at 4°C. Consequently, specific uptake, representing the difference between the curves measured at 37°C and 4°C was also significantly inhibited.

of [^{35}S]BSP uptake. Preincubation of hepatocytes with 200 μM phloretin, 1 mM ouabain, 100 μM carbonylcyanide-m-chlorophenyl-hydrazone, 4 mM 2,4-dinitrophenol (2,4-DNP), 1 mM KCN, 0.8 μg/μl antimycin, or with dithiothreitol (DTT) or *N*-ethylmaleimide according to previously described protocols (18), also had no effect on V_0 of [^{35}S]BSP.

Effect of antibodies to the LPM-BSP/bilirubin binding protein. Initial uptake of all [^{35}S]BSP concentrations tested (1.5–20 μM) was significantly reduced at 37°C in cells pretreated with anti-LPM-BSP/bilirubin binding protein, when compared to controls pretreated with preimmune serum (Fig. 3). In contrast the linear, nonspecific uptake component at 4°C was not affected by the antibody. Consequently, the effect of the antibody was limited to the specific uptake component, represented by the difference between rates measured at 37°C and 4°C. Computer analysis indicated that V_{max} was reduced by 42% in antibody pretreated cells, from 655±34 to 383±24 pmol × min^{-1} per 10^5 hepatocytes ($P < 0.0001$), while K_m was altered by only 5% (6.1±0.5 μM vs. 5.8±0.6 μM, $P = 0.38$). The anti-LPM BSP/bilirubin binding protein thus appears to act as a noncompetitive inhibitor of [^{35}S]BSP uptake. When 5-μM concentrations of various radiolabeled organic anions were incubated with 250 μl of the hepatocyte suspensions (2×10^6 cells/ml) pretreated with the anti-LPM-BSP/bilirubin binding protein, uptakes of [^{35}S]BSP and [^{14}C]bilirubin were 59±6% and 58±10%, respectively, of that in control cells treated with preimmune serum ($P < 0.01$). By contrast, uptakes of [^{3}H]oleate, [^{3}H]taurocholate, and [^{3}H]cholate (95±11%, 106±18%, and 97±15% of control, respectively) were not significantly reduced. Trypan blue exclusion remained >90% at the end of these studies, and no increased leakage of LDH into the medium was observed, indicating lack of hepatocellular toxicity from the antibody employed.

Discussion

The hepatocellular uptake of conjugated bile acids, free fatty acids and non-bile acid cholephils such as BSP and bilirubin, representing the three major physiologic classes of albumin-bound organic anions in plasma, now appears to occur by distinct but highly analogous class-specific, membrane-associated transport processes (1). Historically, for each class, the kinetics of hepatocellular uptake were shown to be compatible with carrier-mediated transport (16–20); high affinity class-specific binding sites were demonstrated on isolated liver plasma membranes (11, 29–31); and a specific membrane binding protein or proteins was isolated and characterized (9–12). Transport functions for the LPM-bile acid and fatty acid binding proteins were recently established by liposome reconstitution (21) and antibody inhibition studies (18), respectively. In the present report, the parallelism is again restored, as a monospecific antibody to the LPM-BSP/bilirubin binding protein is shown to effectively inhibit the hepatocellular uptake of BSP and bilirubin, but not of representative bile acids or nonesterified fatty acids. Lack of effect on bile acid or fatty acid uptake, and lack of evidence of cytotoxicity as measured by trypan blue exclusion or LDH release, are important indicators of the specificity of the antibody effect for BSP and bilirubin transport.

In fact, three groups have reported the existence of LPM-BSP/bilirubin binding proteins. That reported by Wolkoff and Chung (10), designated organic anion binding protein (OABP), appears closely related to the protein initially reported from our laboratory (9, 22), on the basis of similarities of subunit molecular weight, sialic acid content, ligand specificity (9), and at least partial immunologic crossreactivity (32). The relation if any, of these proteins to the "bilitranslocase" reported by Tiribelli and associates (33) remains to be established. The most recently reported subunit structure and molecular weight estimates (34) suggest, that they are in fact unrelated. The reported inhibition of BSP transport in the isolated perfused rat liver by an antibody to bilitranslocase (35) lacked evidence of specificity and any investigation of antibody toxicity, which we have found to be a significant problem with some antibody preparations in studies of this type.

One technical issue requires comment. The current studies are done in the absence of albumin, which ordinarily serves as the physiologic carrier for the anions studied. This decision was

made in order to avoid obscuring the fundamental transport kinetics of the cellular uptake mechanism with the complexities of ligand-albumin binding and of the so-called albumin receptor effect (5–8, 36–38). Recent analyses suggest that binding phenomena may so greatly modify uptake kinetics as to render interpretation of such studies problematical (39). The absence of albumin may have modified some of the quantitative transport variables measured in our study. Nevertheless, this should in no way alter the straightforward interpretation of the role of anti-LPM-BSP/bilirubin binding protein.

A complete understanding of a transport process requires not only recognition of the molecular machinery involved, but also of the driving forces. In this regard, knowledge of BSP and bilirubin uptake lags behind that of bile acids and free fatty acids, both of which are sodium-linked, energy-dependent processes (16–18). BSP uptake does not appear to be sodium coupled or to require directly cellular energy in the form of ATP (40). Recent preliminary studies in vesicles (41) and hepatocytes (42) suggest some relationship of BSP uptake to that of chloride by an as yet unexplained mechanism. Determination of the driving forces for BSP and bilirubin uptake appears to be the next major challenge in the thorough understanding of this transport process.

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