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Characterization of Rose Bengal binding to sinusoidal and bile canalicular plasma membrane from rat liver

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The binding of Rose bengal, a model organic anion, to sinusoidal and bile canalicular membrane fractions isolated from rat liver was compared. The fluorescence change of Rose bengal after being bound to liver plasma membranes was utilized for measuring the binding. The dissociation constants ($K_d = 0.1–0.12$ μM) and the binding capacities ($n = 11–15$ nmol/mg protein) for Rose bengal are comparable between the two membrane fractions, although the n value for sinusoidal membrane is somewhat larger than that for bile canalicular membrane. The Rose bengal binding to both membrane fractions was inhibited by various organic anions at relatively low concentrations, i.e., the half-inhibition concentrations (IC_{50}) for Indocyanine green, sulfobromophthalein, Bromophenol blue and 1-anilino-8-naphthalene sulfonate were 0.1, 100, 1.5–2.5 and 100 μM, respectively, while taurocholate did not inhibit the Rose bengal binding to either membrane fraction at these low concentration ranges. The type of inhibition of sulfobromophthalein and Indocyanine green for Rose bengal binding is different between the two membrane domains. That is, in sinusoidal and bile canalicular membrane fractions, these organic anions exhibit mixed-type and competitive-type inhibition, respectively. It was suggested that the fluorescence method using Rose bengal may provide a simple method for detecting the specific organic anion binding protein(s) in the liver plasma membrane.

Introduction

The hepatic uptake and biliary excretion of various organic anions such as bilirubin, ICG, BSP and RB have been characterized as saturable and selective carrier-mediated processes, using whole animals [1], isolated perfused liver [2,3], and isolated hepatocytes [4–6]. Several kinetic processes such as membrane transport, intracellular metabolism and binding to plasma albumin [7] and intracellular binding proteins [8,9] affect the overall transport process of these organic anions. Therefore, it is important to analyze each kinetic process independently to understand the mechanism of the selective transport system. The first step in the carrier-mediated transport process is the binding of the ligand to the carrier(s). The binding of organic anions to the liver plasma membranes (LPM) has been described [10–12], and the organic anion binding proteins have been isolated from detergent-solubilized LPM in an attempt to identify specific carrier proteins [13–15].

Cornelius et al. [10] first detected saturable binding of BSP to liver plasma membranes, although its binding was not inhibited by bilirubin. Subsequently, Tiribelli et al. [11] characterized a high-affinity BSP binding ($K_d = 5$ μM) with a capacity of approx. 50 nmol/mg of protein, in which the binding was competitively inhibited by bilirubin. Wolkoff and Chung [14] also detected the high-affinity BSP binding ($K_d = 2–4$ μM) both in the zonal-heavy fraction (probably sinusoidal-enriched membrane fraction) and in the zonal-light fraction (canalicular-enriched membrane fraction). BSP glutathione competitively inhibited the BSP binding to the both membrane fractions, while taurocholate did not [14]. The binding capacity ($n_1 = 6.3$ nmol/mg protein) in the zonal-heavy fraction was higher than that ($n_2 = 2.6$ nmol/mg protein) in the zonal-light fraction. Most re-

Abbreviations: BSP, sulfobromophthalein; ICG, Indocyanine green; RB, Rose bengal; ANS, 1-anilino-3-naphthalenesulfonate; CMV, canalicular membrane vesicles; SMV, sinusoidal membrane vesicles; LPM, liver plasma membrane.

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cently, Reichen et al. [12] demonstrated the saturable binding of BSP to the membrane fractions 1 and 2 with very high affinity ($K_d = 0.02$ μM) and low capacity ($n = 0.635$ nmol/mg protein) that was inhibited by bilirubin and ICG but not by bile acids. They also showed the similarity of the binding parameters calculated for fraction 1 (canalicular-enriched membrane fraction) and fraction 2 (sinusoidal-enriched membrane fraction) and considered that this similarity might probably be due to the incomplete separation of sinusoidal (SMV) and canalicular (CMV) membranes [12].

Recently, several methods which separate SMV and CMV have been reported [16–19]. The aim of the present study is to compare the binding properties for the organic anions between SMV and CMV. In this study, RB was used as an organic anion, utilizing its fluorescence change after being bound to LPM.

Materials and Methods

Materials

Ouabain, L-alanine, and sodium taurocholate were purchased from Sigma (St. Louis, MO, USA). Disodium adenosine triphosphate and disodium glucose-6-phosphate were purchased from Boehringer (Mannheim, F.R.G.). Sodium 2,6-dichlorophenolindophenol, disodium *p*-nitrophenyl phosphate, sodium β-glycerophosphate, sodium 1-anilino-8-naphthalenesulfonate (ANS), and Bromophenol blue were purchased from Tokyo Chemical Industries (Tokyo, Japan). Phenazine methosulfate and L-leucyl-β-naphthylamide were purchased from Daiichi Chemical (Tokyo, Japan). Rose bengal (RB) and unconjugated bilirubin (over 95% pure) were purchased from Wako (Osaka, Japan). ICG, BSP and phenolsulfophthalein were purchased from Daiichi Pharmaceutical (Tokyo, Japan). L-[2,3-^{3}H]Alanine (59 Ci/mmol) was purchased from Amersham International (UK).

Preparation of isolated rat liver plasma membrane vesicles

SMV and CMV were prepared from male Sprague-Dawley rat (280–300 g) liver according to the method of Inoue et al. [16,19] and suspended in 10 mM Hepes-Tris buffer (pH 7.4) containing 0.25 M sucrose/0.2 mM $CaCl_2$/10 mM $MgCl_2$ (standard buffer) and stored in small portions (3–10 mg protein per ml). In order to check the purity of prepared vesicles, Na^+/K^+-ATPase and Mg^{2+}-ATPase activities were determined by the method of Schoner et al. [20]. Alkaline phosphatase activities were determined in the mixture containing 5 mM *p*-nitrophenyl phosphate/5 mM $MgCl_2$/50 mM 2-amino-2-methylpropanol-HCl (pH 10.0). After 20 or 30 min of incubation at 37°C, the reaction was terminated by addition of 10% trichloroacetic acid. The denatured protein was removed by centrifugation, and the clear supernatant was neutralized by addition of NaOH, and the amount of *p*-nitrophenol released was determined colorimetrically (A_{420}). Acid phosphatase activities were determined in the mixture containing 5 mM β-glycerophosphate and 50 mM acetate/NaOH (pH 5.0). After 30 min of incubation at 37°C, the reaction was terminated by addition of trichloroacetic acid. Following the removal of denatured protein by centrifugation, the amount of inorganic phosphate in the supernatant was measured by the method of Fiske and SubbaRow [21]. Leucine aminopeptidase activities were determined by the method of Goldbarg and Rutenburg [22]. Glucose-6-phosphatase activities were determined by the method of Nordle and Arion [23]. Succinate dehydrogenase activities were determined by the method of Gutman et al. [24]. Protein was determined by the method of Lowry et al. [25] with bovine serum albumin as standard. The plasma membrane vesicles were stored at −70°C and were used within 1 month after the isolation.

Electron microscopy

LPM preparations were obtained as a pellet by centrifugation at $65000 \times g$ for 40 min, fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) and subsequently in 0.5% OsO_4 in 0.1 M sodium phosphate buffer (pH 7.4), dehydrated in ethanol and embedded in epoxyresin. Sectioning was performed with Ultra-Microtom, Sorvall. The sectionings were stained in uranyl acetate and lead nitrate solutions. Electron micrographs were obtained with a Hitachi HU IIB electron microscope (Hitachi, Tokyo, Japan).

Measurement of binding of RB by fluorescence method

The fluorescence measurements were performed in the standard buffer at 37°C with 10-nm slit widths for both excitation and emission channels. The excitation and emission wavelengths for RB were 520 and 570 nm, respectively. In every measurement 2 μM RB in EtOH was used as a standard. If necessary, the fluorescence intensities were corrected for the inner filter effect according to the method of Chignell [26].

The binding of RB to LPM was determined by the fluorescence titration method. The infinite fluorescence intensity when the added RB was completely bound to LPM was determined by titration of 0.188 μM RB with LPM. The infinite fluorescence intensity thus obtained in the presence of large excess amounts of LPM shows the apparent fluorescence quantum yield of the bound RB. The apparent quantum yields contain the true quantum yields, the absorption coefficient and instrumental parameters. In some preparations, the fluorescence intensities did not reach the plateau even after the addition of approx. 200 μg LPM protein per ml, and the infinite intensities were derived from the double reciprocal plots (1/fluorescence intensity vs. 1/LPM concentration) [27].

A reverse titration of LPM (approx. 40 μg protein per ml) with RB (final concn. 0.06–5 μM) was then carried out to determine the binding isotherms. The concentrations of bound RB (C_b) and unbound RB (C_f) were calculated as follows:

$$C_b = \frac{(F - q_f \cdot C_t)}{(q_b - q_f)} \quad (1)$$

$$C_f = C_t - C_b \quad (2)$$

where C_t is the total RB concentration, F is the observed fluorescence intensity, and q_b and q_f represent the apparent fluorescence quantum yield of bound and unbound RB, respectively.

Inhibition of RB binding by organic anions

Inhibition of the RB binding to LPM by organic anions was determined by following the decrease in the fluorescence of RB. To cuvettes containing 1.6 ml of mixtures of LPM (approx. 30–40 μg/ml) and RB (0.12 μM), was added a maximum of 60 μl of the inhibitor in 1–3 μl aliquots. The solution was mixed after each addition and the fluorescence was measured. The stock solutions of bilirubin (0.5 mM) and ICG (0.5 mM) were freshly prepared in 0.01 M NaOH and distilled water, respectively. In this way, bilirubin up to a final concentration of about 15 μM was solubilized in the buffer.

To determine the type of inhibition, experiments were performed as follows [28]. Titration of approx. 40 μg/ml LPM with RB was performed in the absence or presence of inhibitors. The concentration of each inhibitor was used so that the appropriate inhibition for RB binding was obtained. The apparent K_d value and the maximal binding capacities were calculated from the Scatchard plots by a non-linear least-squares method.

Results

LPM preparation

The enzymatic profile of SMV and CMV preparations are shown in Table I. The specific activity of Na^+/K^+-ATPase, a marker of the sinusoidal membrane, in SMV was increased 24-fold relative to that in the crude homogenate, whereas in CMV, a 3.3-fold increase was observed. The specific activities of leucine aminopeptidase, alkaline phosphatase and Mg^{2+}-ATPase, which are the markers of the bile canalicular membrane, in CMV were increased 44-, 50- and 30-fold, whereas in SMV 3.4-, 6.3- and 5.8-fold increases were observed, respectively. The relative enrichment of the specific activity of glucose-6-phosphatase (a marker of microsomes), acid phosphatase (a marker of lysosomes), and succinate dehydrogenase (a marker of mitochondria) were small, if any.

Transmission electron microscopy demonstrated that SMV and CMV preparations contained predominantly small closed membranes vesicles as shown in Fig. 1. Rare contamination by other structures was seen.

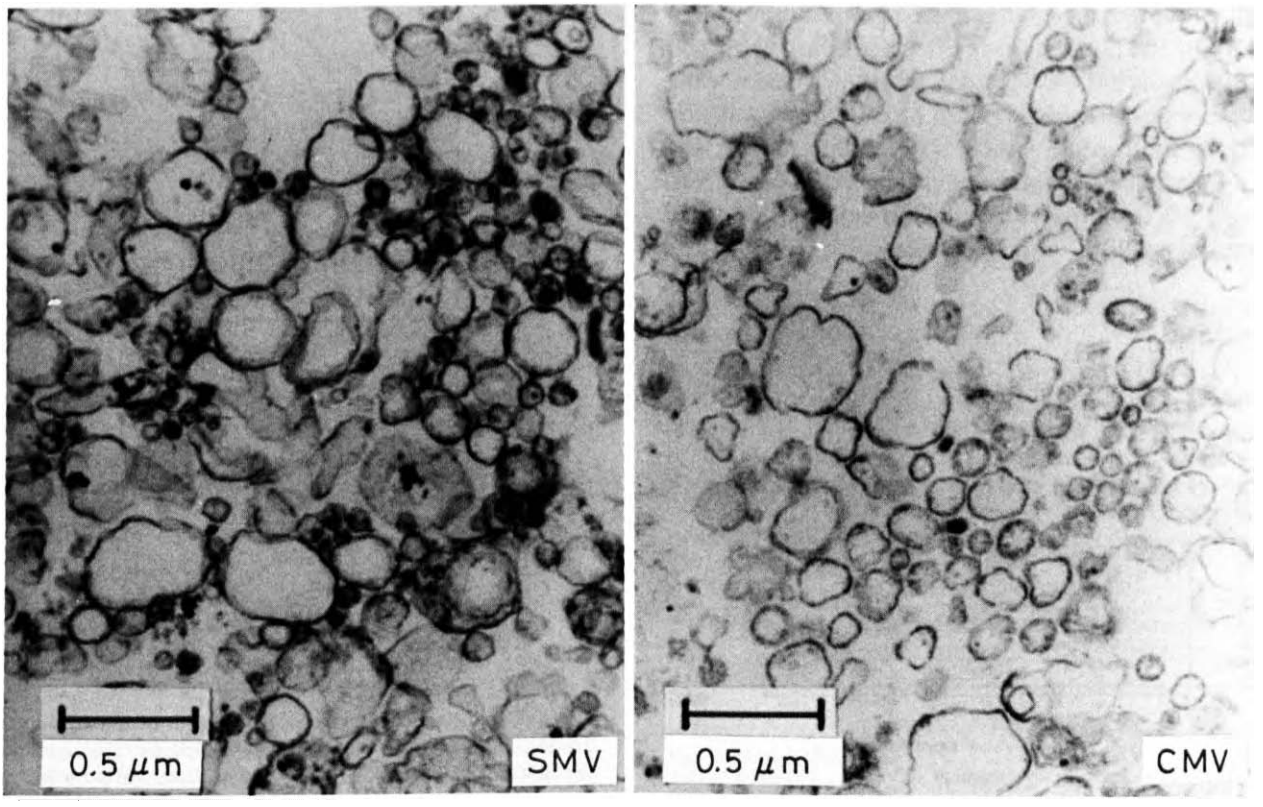

Fig. 1. Transmission electron micrograph of SMV and CMV. Each picture shows a uniform membrane profile of various shapes and sizes, free of nuclear debris or other cytoplasmic organelles. (Bar, 0.5 μm; magnification, ×30000).

TABLE I

Enzymatic characterization of SMV and CMV

Each value represents the mean ± S.E. from four independent preparations. Specific activities are expressed as μmol product formed/mg protein per h, except for succinate dehydrogenase, for which the corresponding units are μmol/mg protein per min. Enrichment is defined as the ratio of specific activity in the LPM to specific activity in the homogenate.

Enzyme	Homogenate (specific activity)	SMV (enrichment)	CMV (enrichment)
Na^+/K^+-ATPase	1.47 ±0.67	24.4 ±7.9	3.3 ±2.0
Mg^{2+}-ATPase	6.40 ±1.07	5.8 ±0.9	29.4 ±3.8
Alkaline phosphatase	0.319±0.041	6.3 ±0.7	49.8 ±9.6
Leucine aminopeptidase	1.66 ±0.25	3.4 ±0.4	44.0 ±9.1
Glucose-6-phosphatase	5.26 ±0.59	1.3 ±0.1	1.0 ±0.5
Acid phosphatase	2.01 ±0.29	1.8 ±0.3	3.1 ±0.6
Succinate dehydrogenase	52.8 ±7.2	0.10±0.03	0.71±0.21

SMV preparations showed the Na^+-gradient-dependent L-alanine transport and the initial uptake rate was 6-times larger in the NaSCN gradient compared with the KSCN gradient, suggesting that prepared SMV fractions maintained the normal transport ability.

Binding of RB to LPM

As shown in Fig. 2, the fluorescence intensity of RB was increased by the interaction with either SMV or CMV. Also, a red shift of the fluorescence emission of RB after being bound to plasma membranes was observed, probably suggesting a change in environmental polarity of RB [29]. Fluorometric titrations at a fixed concentration of RB (0.188 μM) with each membrane fraction were performed in order to obtain the infinite fluorescence intensities, which correspond to the apparent fluorescence quantum yield of the bound RB. The mean (± S.E.) apparent fluorescence quantum yield of RB bound to SMV and CMV were 255.5 ± 9.7/μM RB ($n = 4$) and 172.6 ± 12.6/μM RB ($n = 6$), respectively, and the difference was statistically significant ($P < 0.01$). The apparent fluorescence quantum yield of the unbound RB was 38.9 ± 1.2/μM RB ($n = 10$). The difference in apparent fluorescence quantum yield between the membrane fractions may reflect the difference either in the polarity or in the viscosity of the binding sites [29].

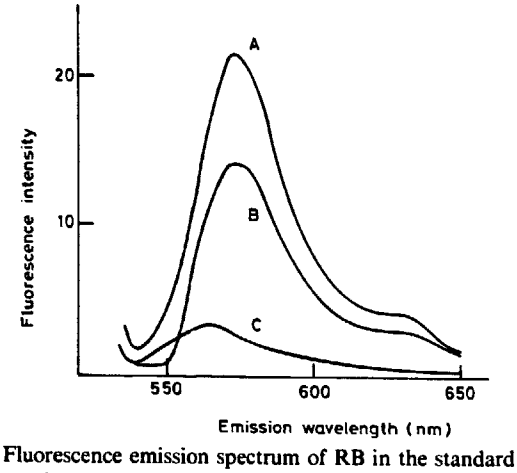

Fig. 2. Fluorescence emission spectrum of RB in the standard buffer. Spectrum of 0.188 μM RB in the presence of SMV (0.235 mg protein per ml) (A); CMV (0.185 mg protein per ml) (B); and in the absence of LPM (C) at 37 °C. The excitation wavelength was 520 nm.

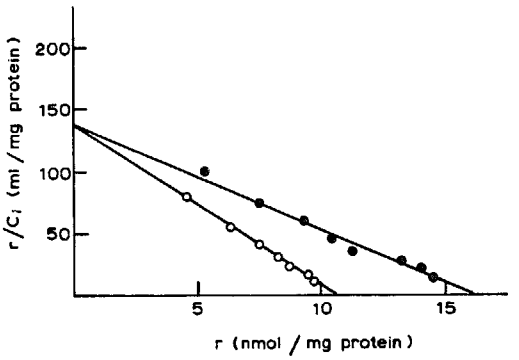

Fig. 3. Typical results of the Scatchard plot of the RB binding. The small aliquots of RB solution were titrated into the standard buffer containing SMV (●) or CMV (○) (about 40 μg protein per ml). The binding parameters were estimated by a non-linear least-squares method.

A single class high-affinity binding of RB was observed both in SMV and CMV. Fig. 3 shows the typical result of the RB binding expressed as the Scatchard plot. The dissociation constant (K_d) and the maximal binding capacity (n) of RB binding for SMV were

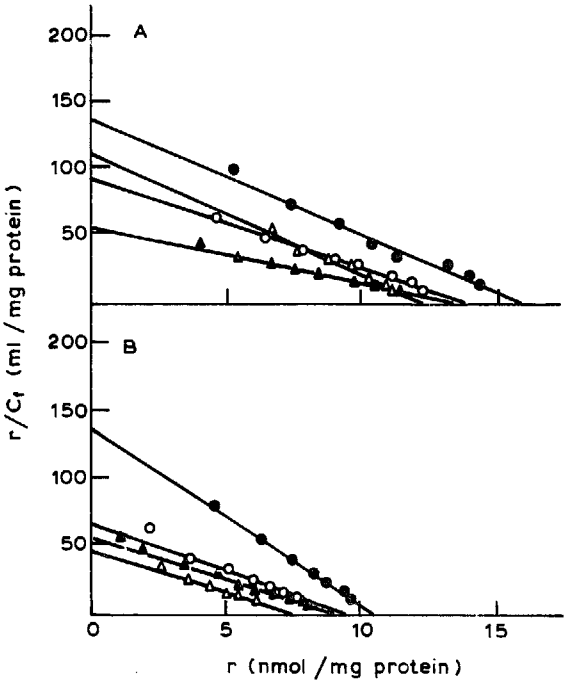

Fig. 4. Typical results of the Scatchard plot of the RB binding in the presence and absence of other organic anions. The small aliquots of the RB solution were titrated into the standard buffer containing SMV (A) or CMV (B) at 37 °C, in the absence (●) or presence of organic anions; 20 μM BSP (▲); 0.2 μM ICG (○); and 10 μM bilirubin (△).

TABLE II

Apparent K_d and n values of RB binding in the absence or presence of other organic anionic dyes

Each value represents the mean ± S.E. derived from three to five determinations. Binding parameters were estimated by a non-linear least-squares method. * $P < 0.05$ (compared to the control); ** $P < 0.01$ (compared to the control).

		Control	+20 μM BSP	+0.2 μM ICG	+10 μM bilirubin
SMV	K_d (μM)	0.12 ± 0.01	0.26 ± 0.04 *	0.32 ± 0.02 **	0.12 ± 0.02
	n (nmol/mg protein)	15.1 ± 0.5	12.3 ± 0.4 *	7.3 ± 0.3 **	12.0 ± 0.2 *
CMV	K_d (μM)	0.10 ± 0.01	0.18 ± 0.02 **	0.24 ± 0.07 *	0.18 ± 0.05
	n (nmol/mg protein)	10.9 ± 2.1	12.4 ± 1.2	7.7 ± 1.0	9.5 ± 1.0

0.12 ± 0.01 μM and 15.3 ± 0.4 nmol/mg protein ($n = 4$), and those for CMV were 0.10 ± 0.01 μM and 10.9 ± 2.1 nmol/mg protein ($n = 6$), respectively. The K_d values were thus comparable in both membranes, while the binding capacity for CMV was somewhat smaller than that for SMV, although the difference was not statistically significant.

Inhibition of RB binding by other organic anions

Fig. 4 shows the typical Scatchard plots of RB binding to SMV (A) and CMV (B) in the presence and absence of various organic anions. The apparent K_d values thus obtained from a number of experiments are summarized in Table II. In the binding to SMV, 20 μM BSP and 0.2 μM ICG increased the apparent K_d value for RB by a few times, whereas 10 μM bilirubin did not affect it. ICG also decreased the binding capacity to half of the control, but the decreases in the binding capacity by BSP and bilirubin were relatively small (approx. 20%). In the binding to CMV, all the organic anions tested increased the apparent K_d values by approx. 2-times, although the changes in the binding capacity were small and not statistically significant. These results tentatively indicate that BSP and ICG competitively inhibit the RB binding to CMV, while they show a mixed-type of inhibition in SMV.

Similarly, the effects of varying the concentrations of seven organic anions on the fluorescence of RB with SMV and CMV were determined, and the results are shown in Fig. 5. The half-inhibition concentrations (IC_{50}) are summarized in Table III. For either SMV or CMV, ICG and Bromophenol blue are strong inhibitors of RB binding, while bilirubin, BSP, ANS and taurocholate are less potent. Comparing the two membrane fractions SMV and CMV, the degrees of inhibition of RB binding by most of the organic anions tested were about the same.

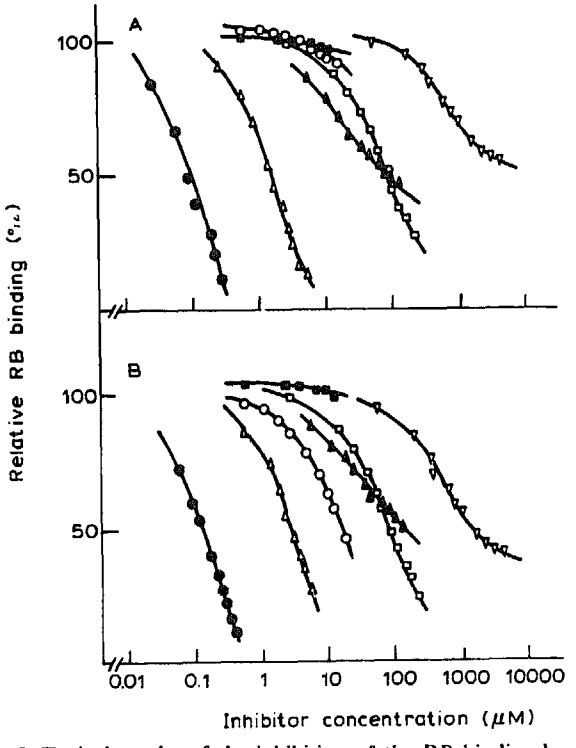

Fig. 5. Typical results of the inhibition of the RB binding by other organic anions. Aliquots of inhibitors were titrated into 0.12 μM RB in the standard buffer containing SMV (A) or CMV (B) (30–40 μg protein per ml) at 37 °C. ICG (●); bilirubin (○); BSP, (▲); Bromophenol blue (△); taurocholic acid (▽), phenolsulfophthalein (■); and ANS (□).

TABLE III

Half-inhibition concentration (IC_{50}) of various compounds for the RB binding to liver plasma membranes

Since the competitive inhibition was demonstrated only for some compounds (Fig. 5), IC_{50} values are shown instead of the inhibition constant. The mean value determined using two separate preparations is shown, except for cases marked *, where the means ± S.E. from three separate experiments are shown; **, inhibited by 7% at 15 μM of bilirubin (a higher concentration cannot be used due to the inner-filter effect caused by bilirubin); ***, inhibited by 43% at 4000 μM of taurocholate.

	Half-inhibition concentration (μM)	
	SMV	CMV
BSP	94 ± 25 *	110
ICG	0.08	0.12
Bromophenol blue	1.4 ± 0.1 *	2.5
ANS	105 ± 18 *	92
Bilirubin	> 15 **	16
Taurocholate	> 4000 ***	1500

Discussion

Novel methods have become available recently to selectively separate canalicular and sinusoidal (basolateral) rat liver plasma membrane fractions [16–19]. It has been shown that the presence of Na^+/K^+-ATPase activity in CMV is a sensitive criterion to test for the eventual cross-contamination with the sinusoidal membrane fragments, and that the activities of leucine aminopeptidase and alkaline phosphatase are those to test for the cross-contamination with the canalicular membrane fragments [16–19]. Table I indicates that each membrane preparation is maximally contaminated with the other membrane fraction to an extent of approx. 10–20%. The analysis of RB-binding isotherms to these membrane fractions revealed that both the affinity ($K_d = 0.1–0.12$ μM) and the binding capacity ($n =$ 11–15 nmol/mg protein) are comparable between the two membrane fractions (Table II). In addition the IC_{50} values of various organic anions for both membrane fractions are also comparable, except for bilirubin (Table III). The binding capacity detected by us is somewhat larger than that reported by Wolkoff et al. [14], and it is smaller than that reported by Tiribelli et al. [11]. The similar binding characteristics of RB between the two membrane fractions we detected in the present studies is consistent with the recent finding by Stremmel et al. [13] that the organic anion binding protein exists on all surface domains of rat hepatocytes. This was revealed by immunohistochemical studies using a rabbit antibody raised against the BSP-binding protein purified from rat liver plasma membrane fractions.

It has been shown that bilirubin, ICG, BSP, RB and other organic anions share a common carrier and mutually compete for hepatic uptake [1,30,31]. Sugiyama et al. [31] has shown that the uptake of ANS by isolated rat hepatocytes is competitively inhibited by BSP and by other organic anions such as ICG, RB and Bromophenol blue, but not by phenolsulfophthalein, taurocholate and oleate. When the Ic_{50} values determined in the present study (Table III) are arranged in the increasing order, the relative affinities for the different compounds are RB ≡ ICG > Bromophenol blue > ANS > BSP > bilirubin > phenolsulfophthalein > taurocholate. This is approximately consistent with the order of inhibitory effects for ANS uptake by hepatocytes [31]; RB > ICG ≡ Bromophenol blue > BSP > phenolsulfophthalein ≡ taurocholate. These findings may suggest that the RB binding we determined in the present study represents the binding to the transport carrier(s) in the membranes.

However, the inhibition observed by the decrease in RB fluorescence intensity when organic anions are added could originate from an indirect effect on the environment of the binding site, rather than from a competition for the same binding site. If the fluorescence change is due to an indirect effect, the interpretation of the experimental results should be cautiously made. In fact, the type of inhibition of BSP and ICG is different between SMV and CMV (Fig. 4, Table II); i.e., in SMV, BSP and ICG showed an increment in K_d and a reduction in n value (mixed inhibition), while in CMV, a reduction only in the apparent K_d was observed (competitive inhibition). This finding may indicate the different interaction in different domains between RB and other organic anions (BSP, ICG) in SMV. While in CMV, these organic anions may share the common binding sites. Bilirubin, on the contrary, decreases only the n value (non-competitive inhibition) in SMV.

We previously identified two classes (high and low affinities) of binding sites for bile acid both on SMV and CMV [33]. The low-affinity binding capacities for bile acids (10–20 nmol/mg protein) are comparable with that for RB. 10 μM RB showed no inhibition for chenodeoxycholate binding to SMV [32], and the IC_{50} value of taurocholate for RB binding SMV was extremely high (Table III). These results may support the prevailing concept that bile acids and organic anions are taken up into the hepatocytes by different carriers [30].

We used RB in the present study as a model organic anion instead of BSP, which has been often used. The use of RB made it possible to determine the binding to the liver plasma membranes by use of the fluorescence change. By this method, the binding isotherms over a wide range of RB concentrations (0.06–5 μM) could be obtained with small amounts of the liver plasma membranes (approx. 40 μg). However, one has to pay attention to the limitations of the fluorescence method in the binding studies. First, the analysis of the binding isotherms assumes a constant fluorescence quantum yield and absorption coefficient for all the binding sites, namely the assumption of independent and identical binding sites. Second, higher-affinity binding sites may be revealed if we use a lower concentration range of RB (< 0.06 μM).

Furthermore, the binding of RB is of special interest in view of the specificity of photooxidation with this dye [32]. RB may be able to photooxidize the active site on the membranes and may thus be used as a photoaffinity probe for the membrane carrier in future.

In conclusion, Rose bengal showed comparable binding to SMV and CMV, and the fluorescence method using Rose bengal could be a simple and novel method to detect the organic anion binding sites in liver plasma membranes.

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