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Drug partition chromatography on immobilized porcine intestinal brush border membranes

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Abstract

We immobilized porcine intestinal brush border membrane vesicles (BBMVs) for chromatographic analyses of drug partitioning into the membranes determined as K_s, the drug retention per phospholipid amount. For positive and neutral drugs K_s decreased day by day, whereas K_s for negative drugs increased marginally. Similar results on vesicle–lipid liposomes indicated a gradual loss of negative charge from the columns. The K_s values for positive drugs were higher than those for negative drugs with the same octanol/water partitioning or the same K_s on egg yolk phospholipid bilayers. Electrostatic interactions seem to be important for the partitioning of charged drugs into brush border membranes.

Keywords: Liposomes; Drug partitioning; Immobilization; Phospholipid bilayers; Drugs

1. Introduction

Orally administered drugs have to pass the single epithelial cell layer in the small intestine on their way to their target organs in the body. The epithelial cells are lined by two different plasma membranes, the apical brush border membrane facing the interior of the intestine and the basolateral membrane pointing towards the adjacent intestinal cells and the blood vessels. The uptake of drugs and nutrients takes place at the brush border membrane in the small intestine. However, brush border membrane vesicles (BBMVs) have been used only scantily as an in vitro model for drug absorption because of undesirable properties, such as decreased protein activities between experiments and after storage [1]. The uptake and partition and distribution coefficients of a few drugs have been determined with BBMVs by use of centrifugation [2,3] and by filtration techniques in, for example [1,4–8].

The present work is part of a series of chromatographic analyses of drug partitioning into natural and artificial cell membranes for the purpose of characterizing drug–membrane interactions. We prepared and immobilized porcine intestinal BBMVs and BBMV–lipid liposomes in or on gel beads and analyzed the partitioning of positive, negative and neutral drugs into the bilayers. We compared our partition data with partitioning into octanol (P_{oct}) (data from [9]) and liposomes (data from [9,10]).

Drug partition chromatographic analyses have previously been performed on immobilized unilamellar and multilamellar liposomes, as reported, for example, in [9–12], and on proteoliposomes [12], red blood cell vesicles and red blood cells/ghosts [11]. Immobilized artificial membrane chromatography with monolayers of phospholipid analogues has been used for similar types of analyses [13]. Previous results showed that drug partitioning into immobilized red blood cell vesicles or red blood cells/ghosts was similar to that for the corresponding membrane–lipid liposomes, although the range of the partition coefficients was decreased due to the presence of membrane proteins [11]. Cholesterol in lipid bilayers decreased the partitioning of drugs and model transmembrane proteins affected the drug partitioning mainly by electrostatic interactions [12]. Drug partition data obtained on natural and artificial membranes correlate with the fraction of drug absorbed in the body [9–11]. The partition data obtained by chromatography on lipid bilayers reveals the drug distribution between aqueous compartments and membranes in the human body and can help to estimate the membrane permeability to various drugs. However, the concentration gradient that can be

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obtained across the membrane during drug transport depends essentially on the drug partitioning into the outskirts of the membrane, which may explain why no perfect correlation between drug absorption and drug partitioning into membranes has been obtained so far.

2. Experimental

2.1. Materials and solutions

We purchased glass columns (HR 5/2), Sephadex G-50 medium and Superdex 200 prep grade from Amersham Biosciences (Uppsala, Sweden), cholic acid (>99%) from Fluka (Buchs, Switzerland), and ATP (>99%), ethylene glycol-bis(β-amino ethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA, >97%), *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES, >99.5%), glucose kit 510-A containing glucose oxidase, L-lactic dehydrogenase (EC 1.1.1.27, from rabbit muscle, L-2500), D-mannitol (>98%), NADH (98%, reduced form), phosphoenolpyruvate (97%), pyruvate kinase (EC 2.7.1.40, from rabbit muscle, P-1506), streptavidin-derivatized 4% agarose gel beads, alprenolol, atenolol, dexamethasone, metoprolol, oxprenolol, prednisolone, promethazine and propranolol from Sigma (St. Louis, MO, USA). Desmethyldiazepam, ibuprofen, oxazepam and theophylline were gifts from SmithKline-Beecham (King of Prussia, PA, USA) and diclofenac, diflunisal, indomethacin, ketoprofen, lidocaine, metolazone, tolfenamic acid and warfarin from AstraZeneca (Södertälje, Sweden). Small intestine from pig was obtained from Swedish Meats (Uppsala, Sweden). Chemicals not listed were of analytical grade.

Solutions (pH values at 21 ± 2 °C): solution A, 150 mM NaCl, 1 mM Na_2EDTA, 10 mM Tris/HCl, pH 7.4; solution B, 0.3 M D-mannitol, 12 mM HEPES/NaOH, 5 mM EGTA, pH 7.1; solution C, 0.15 M NaCl, 10 mM HEPES/NaOH, 1 mM Na_2EDTA, pH 7.4; and solution D, 33 mM NH_4Cl, 50 mM NaCl, 1.67 mM $MgCl_2$, 0.25 M imidazole, pH 7.3.

2.2. Preparation of BBMVs

We prepared BBMVs at 4 °C from porcine small intestines (300–600 g, fresh on ice) by a modification of the procedure described in [14]. We rinsed the intestine with 1–2 l of 0.15 M NaCl, sliced it open piece by piece, removed the mucus with paper, scraped off the cells with the sharp edge of a glass slab, homogenized the cells in 150 ml of solution B in a Waring blender for 3 min, diluted six-fold with water, homogenized for 3 min and stored an aliquot at −70 °C for analyses (see Section 2.4). We added $MgCl_2$ to a concentration of 10 mM under stirring to precipitate all membranes except the apical brush border membrane, stirred for an additional 5 min, centrifuged once or twice (12,000 × *g*, 5 min) and discarded the pellet(s), recentrifuged the supernatant containing the BBMVs (24,000 × *g*, 40 min), suspended the new pellet in 120 ml of solution C, centrifuged (48,000 × *g*, 15 min), resuspended the pelleted vesicles in approximately 10 ml of solution C to 20 μmol phospholipid per milliliter and stored the suspension at −70 °C.

2.3. Preparation of BBMV–lipid liposomes

We extracted the BBMV lipids by a modification of the method described in [15]. We diluted an aliquot of the suspended BBMVs three-fold with solution A, mixed vigorously with chloroform/methanol (2:1, v/v), collected the organic phase, reextracted the aqueous phase, evaporated the solvents from the combined organic phases and washed twice with ether. The lipid film formed could not be rehydrated with solution A only. We therefore dissolved it with 250 mM cholate in solution A at pH 8.0, removed the cholate on a 37 cm × 2 cm Sephadex G-50 medium gel bed in solution A at 0.5 ml/min at 21 ± 2 °C, freeze–thawed (−75/+25 °C) the obtained liposomes twice and concentrated them by centrifugation (186,000 × *g*, 4 °C, 30 min). The liposome suspension contained 10 μmol phospholipid per milliliter and less than 0.02 g protein per gram of phospholipid.

2.4. Phospholipid and protein amounts and marker enzyme activities

We determined the phospholipid amount by phosphorus analysis [12,16] of aliquots of homogenate and BBMV, liposome and gel suspensions and the protein amounts by quantitative amino acid analyses [17] at our department. We determined sucrase activity by use of glucose oxidase essentially as in [18] and ouabain-sensitive Na^+/K^+-ATPase activity by a procedure modified from [19]. For the latter analyses we supplemented 5–50 μl homogenate or vesicle suspension with reagents in solution D to obtain 312 μM ATP, 8.8 μM NADH, 125 μM phosphoenolpyruvate, 7.2 U L-lactic dehydrogenase and 9 U pyruvate kinase in 1.2 ml, measured the absorbance at 340 nm during 90 s at 21 ± 2 °C, added 75 μl of 24 mM ouabain, mixed, and measured the absorbance during another 90 s to determine the non-ouabain-sensitive ATPase activity.

2.5. Chromatography on immobilized BBMVs and BBMV–lipid liposomes

We rehydrated dried Superdex 200 gel beads with a BBMV or liposome suspension and freeze–thawed in order to obtain vesicles or liposomes entrapped in the beads [12,20]. We packed the beads directly into HR columns of 5 mm i.d. and washed for 30–60 min, applied an analyte (20 μl, 0.1 mol^{-1}, <5% ethanol), eluted at 0.50 ml/min in solution A, and detected (Waters 484 or 486, Millipore, MA, USA) the analyte at 220 nm, all at 21 ± 2 °C. The columns were kept at 4 °C when not in use.

We expressed the drug partitioning into the bilayers as a K_s value (M^{-1}) [12]:

$$K_s = \frac{V_E - V_0 - V_G}{A} \quad (1)$$

where V_E is the elution volume of the drug; V_0 is the elution volume of $Cr_2O_7^{2-}$; V_G is the drug retention volume on an equally-sized column with empty Superdex 200 gel beads; and A is the amount of phospholipids in the gel bed determined after the series of runs. K_s can be converted to a partition coefficient (K_{LM}) by dividing with the molar volume of the immobilized phospholipids, for example, $0.755\,l\,mol^{-1}$ for egg yolk phosphatidylcholine [10,21].

3. Results

3.1. Preparation and immobilization of BBMVs and BBMV–lipid liposomes

BBMVs prepared from porcine intestine showed a yield of both phospholipid and protein of 1% of the homogenate (Table 1). The apical brush border membranes were enriched 18-fold, whereas the activity of ouabain-sensitive Na^+/K^+-ATPase, a marker for the basolateral membrane, stayed at the original level (Table 1).

Freeze–thawing of the BBMV/Superdex mixture immobilized 20% of the BBMV phospholipids to give 10 μmol phospholipid per milliliter gel bed. The yield for a BBMV–lipid liposome suspension was 66% and resulted in the same concentration of immobilized phospholipid as for the BBMVs.

When we alternatively biotinylated the BBMVs and adsorbed them onto streptavidin-derivatized agarose gel beads [17] only 6% of the phospholipids (0.9 μmol phospholipid per milliliter agarose gel bed) became immobilized, probably only on the surfaces of the beads. This resulted in smaller elution volumes and hence lower precision than by freeze–thaw entrapment.

Table 1
Yield and purity of porcine small intestine BBMVs as indicated by phospholipid and protein[a] amounts and the specific activity of sucrase (marker of the apical brush border membrane) and of ouabain-sensitive Na^+/K^+-ATPase (marker of the basolateral membrane) in the cell homogenate and the BBMV suspension

	Cell homogenate[b]	BBMV suspension[b]
Phospholipid (mmol)	19	0.22
Protein (g)	21	0.17
Sucrase (nmol min^{-1} mg^{-1})	3.0	56
ATPase (nmol min^{-1} mg^{-1})	4.7	4.6

[a] The protein amounts were determined by amino acid analyses [17]. A micro-Bradford membrane protein assay can also be used to analyze the protein amounts in membranes immobilized in gel beads [22].

[b] Values from a single preparation. Another preparation showed similar values.

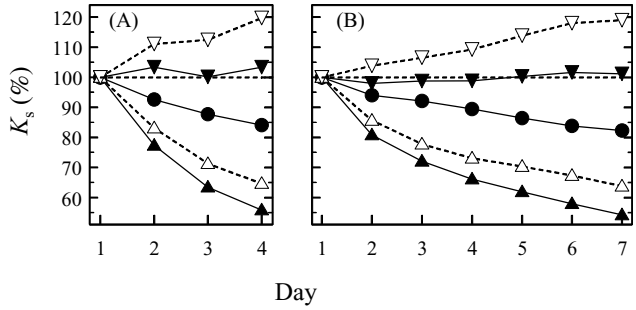

Fig. 1. Averages of K_s values (filled symbols, full lines), in percent, obtained on immobilized BBMVs (A) and BBMV–lipid liposomes (B) vs. the number of days, for positive (▲), negative (▼) and neutral (●) drugs. The decrease in K_s for the neutral drugs was presumably caused by loss of vesicles or liposomes, or free lipids and the average K_s values have therefore been corrected accordingly (open symbols, hatched lines). The drugs are those listed in Table 2. The averages in panel (A) are calculated from one BBMV column. Another BBMV column gave similar results (Section 3.2).

3.2. Drug partitioning into BBMVs and BBMV–lipid liposomes

The average of the K_s values for positive drugs decreased appreciably with time on BBMVs and BBMV–lipid liposomes (Fig. 1A and B, respectively, filled symbols). The average K_s for neutral drugs decreased less than for positive drugs and that for negative drugs increased marginally. We assumed that the decrease for the neutral drugs only represented a gradual loss of membranes or liposomes from the columns, or a loss of lipids from these materials, and corrected the averages of the K_s values accordingly for all drugs (Fig. 1, open symbols, hatched lines). The resulting large decreases in K_s for the positive drugs and small increase for the negative drugs indicated that the charged drugs interacted electrostatically with the bilayers. Another BBMV column showed similar results with an average decrease in K_s of 25% for positive drugs and 15% for neutral drugs after 4 days of partition analyses. Streptavidin–biotin-immobilized vesicles gave similar results as the entrapped vesicles (data not shown).

The individual $\log K_s$ values obtained on BBMVs correlated very well with the values shown by BBMV–lipid liposomes ($y = 0.94x + 0.17$, $r^2 = 0.99$, with BBMV values on the y-axis) (Table 2).

For the BBMV and BBMV–lipid liposome columns the range of initial elution volumes (V_E–V_0) were 0.02–25 ml for both materials (data not shown).

3.3. Drug partitioning into BBMVs, octanol and liposomes

For the entire set of drugs, partitioning into BBMVs versus partitioning into octanol ($\log P_{oct}$ values) (Fig. 2A) or egg yolk phospholipid (EPL) bilayers (Fig. 2B) showed scattered plots ($r^2 = 0.43$ and 0.77, respectively). However, $\log K_s$ on BBMVs correlated fairly well with $\log P_{oct}$ for the

Table 2
Drug partitioning into BBMVs and BBMV–lipid liposomes. Log K_s values for positive, negative and neutral drugs

Drug	Charge at pH 7.4	Log K_s	
		BBMVs[a]	Liposomes[b]
Alprenolol	+	2.85 ± 0.17	2.83
Atenolol	+	1.18 ± 0.19	1.14
Lidocaine	+	1.56 ± 0.09	1.44
Metoprolol	+	1.70 ± 0.12	1.66
Oxprenolol	+	2.07 ± 0.17	2.06
Promethazine	+	3.77 ± 0.13	3.86
Propranolol	+	3.35 ± 0.16	3.36
Theophylline[c]	+	1.14 ± 0.17	0.96
Diclofenac	−	2.34 ± 0.08	2.26
Diflunisal	−	2.56 ± 0.13	2.48
Ibuprofen	−	1.53 ± 0.15	1.38
Indomethacin	−	2.25 ± 0.04	2.17
Ketoprofen	−	1.27 ± 0.16	1.24
Tolfenamic acid	−	3.00 ± 0.06	2.92
Warfarin	−	1.62 ± 0.17	1.53
Desmethyldiazepam	0	2.72 ± 0.06	2.81
Dexamethasone	0	1.94 ± 0.08	2.03
Metolazone	0	2.11 ± 0.19	2.13
Oxazepam	0	2.64 ± 0.05	2.68
Prednisolone	0	1.90 ± 0.04	1.77

[a] Average log K_s value ± S.E.M. of four BBMV columns from two BBMV preparations. The used initial partition data from the two columns analyzed for several days have been corrected to lipid loss as in Fig. 1A.
[b] Data from one column. The initial partition data have been corrected to lipid loss as in Fig. 1B.
[c] Average BBMV log K_s value ± S.E.M. of three BBMV columns.

negative drugs alone ($r^2 = 0.94$) and at the same log P_{oct} value the log K_s values for positive drugs were much higher than those for negative drugs (Fig. 2A). For positive drugs log K_s on BBMVs showed a very good correlation with log K_s on EPL bilayers ($r^2 = 0.99$, Fig. 2B) and also here, the positive drugs partitioned more strongly into the BBMVs than did the negative drugs at the same log K_s value on EPL liposomes (Fig. 2B).

Partition coefficients (log K_{LM}) obtained on liposomes composed of cholesterol, phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine to mimic the small intestine cell membrane [10] correlated fairly well with the log K_s values we obtained on BBMVs ($y = 0.74x + 0.73$, $r^2 = 0.93$) and on BBMV–lipid liposomes ($y = 0.77x + 0.61$, $r^2 = 0.92$) for the eight drugs in common to the two studies.

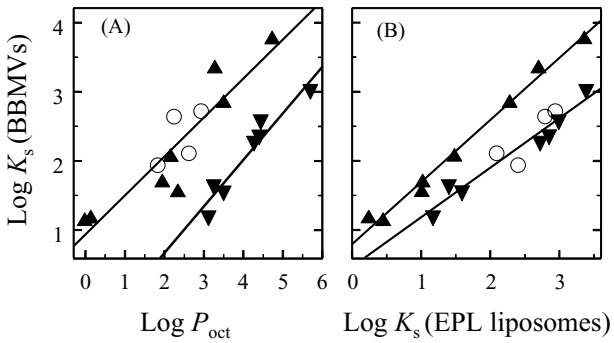

Fig. 2. Log K_s values for positive (▲), negative (▼) and neutral (○) drugs on BBMVs vs. (A) log P_{oct} and (B) log K_s on EPL liposomes. The log P_{oct} and log K_s values on EPL liposomes were taken from [9]. The drugs are those listed in Table 2 except prednisolone, for which no values were given in [9].

4. Discussion

The retention volumes and hence the K_s values of the positive drugs on BBMVs and on BBMV–lipid liposomes decreased with time (Fig. 1). A less dramatic decrease occurred for the neutral drugs, which we assume was largely related to a loss of vesicles, liposomes or lipids. Because the effects on positive and negative drugs were opposite we propose that changes in the amounts of charged groups in the bilayers were involved. Furthermore, because the effects were the same with the BBMVs and the protein-free BBMV–lipid liposomes, probably lipid charges were lost and not protein charges. The small amount of residual protein in the liposomes might nevertheless be involved in the process. The large loss in retention volume for positive drugs compared to negative drugs thus indicates that the columns lost negative charges of lipids, which is emphasized in Fig. 1 (open symbols, hatched lines) where the negative drugs show increased average K_s values, whereas positive drugs show decreased average K_s values. BBMVs from porcine small intestine contain 65% (w/w) protein and 35% lipid (6% free fatty acids, 12% cholesterol, 31% sphingoglycolipids, 17% phosphatidylcholine, 17% phosphatidylethanolamine, 6% phosphatidylserine, 5% phosphatidylinositol and 5% sphingomyelin) [23]. The negative lipids in the BBMVs are phosphatidylserine, which was retained in the membranes as showed by amino acid analysis; fatty acids, which would be released from the membrane at a higher rate than the phospholipids; and the slightly charged phosphatidylethanolamine. A gradual loss of fatty acids may thus be responsible for the changes in the partitioning of charged drugs. However, drug partitioning into EPL liposomes containing 50 mol% arachidic acid showed constant K_s values for four positive, one neutral and two negative drugs over 4 days of analyses [24], whereas extraction of fatty acids from BBMVs with bovine serum albumin decreased the partitioning of the neutral solutes nitrobenzene and toluene into the vesicles [2]. Two possible causes of the effect we observed are that a large proportion of the fatty acids in the BBMVs may have particularly short alkyl chains, and leave the membrane quickly, or that modifications of some lipid(s) occur such that negative charges are lost. The stability of the BBMVs and BBMV–lipid liposomes might be enhanced if the experiments were performed at other temperatures and the stability of the elution volumes might increase if, e.g. fatty acids were supplemented into the running buffer.

The results with BBMVs were independent of the immobilization method. The streptavidin–biotin immobilization gave only small amounts of immobilized phospholipid and hence small elution volumes. A higher biotin–avidin immobilization yield may be obtained with superporous agarose beads [25] owing to the larger surface area available to the vesicles. BBMVs have been immobilized by adsorption to Triton X-100-substituted Sepharose 4B gel beads [26].

The plot of $\log K_s$ values determined on BBMVs versus $\log P_{oct}$ values gave a scattered plot (Fig. 2A) resembling the plot in Fig. 1A in [9]. The $\log P_{oct}$ did thus not well represent the partitioning into the BBMVs, although fair correlations were obtained when negative drugs and other drugs were treated separately. The discrepancy between $\log K_s$ on BBMVs and $\log P_{oct}$ may largely be due to the fact that $\log P_{oct}$ is determined at pH values that provide neutral drugs. Negative charges of the BBMVs seemed to enhance the partitioning of positive drugs into the brush border membranes compared to the EPL bilayers, whereas negative and neutral drugs partitioned more weakly into the BBMV membranes than into the EPL liposomes (Fig. 2B).

The partition coefficients obtained by Liu et al. on unilamellar liposomes composed to mimic intestinal cell membranes [10] correlated fairly well with the partition data on both BBMVs and BBMV–lipid liposomes (Section 3.3). The partition data obtained on BBMVs versus data obtained on BBMV–lipid liposomes correlated very well (Section 3.2, Table 2). The membrane proteins in the BBMVs do thus not seem to affect the drug partitioning remarkably or different membrane proteins have opposite effects, leading to no net effect. Previous results showed that electrostatic interactions are important when drugs partition into a lipid bilayer containing transmembrane proteins [12]. The authentic BBMV lipids provided anyhow liposomes that resembled the drug partitioning into BBMVs more closely than do the liposomes employed in [10].

Calculated permeability values ($\log P_{eff}$) versus $\log K_s$ obtained on BBMVs indicated differences among neutral, positive and negative drugs (data not shown) similarly as in [12], where $\log P_{eff}$ was compared with $\log K_s$ obtained on EPL liposomes. At low $\log K_s$ values the negative drugs have much higher permeability than the positive drugs, probably because they interact differently with the zwitterionic headgroups of phospholipid molecules, as illustrated in Fig. 3. In principle, electrostatic effects hinder the entrance of positive drugs and promote the entrance of negative drugs into the hydrophobic region of the membrane.

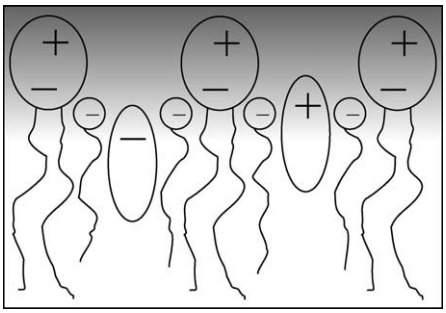

Fig. 3. Examples of electrostatic interactions affecting the partitioning of charged drugs across a bilayer of common zwitterionic phospholipid molecules, for example, phosphatidylcholine, supplemented with charged lipids such as fatty acids. The permittivity is indicated by the shading. Low permittivity enhances electrostatic interaction. In this example the diffusion of the negative drugs across the membrane from the position shown will be favored compared to the diffusion of the positive drug.

5. Conclusions

Immobilized BBMVs allow chromatographic drug partition analyses only during a short time for each column due to an apparent gradual loss of negative charge or lipid from the membranes entrapped in or on the gel beads. Electrostatic interactions seem to be of importance for the partitioning of charged drugs into the brush border membranes.

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