JPP Journal of Pharmacy and Pharmacology

JPP 2001, 53: 1477–1487

Received May 2, 2001
Accepted July 18, 2001
ISSN 0022-3573

Effects of cholesterol and model transmembrane proteins on drug partitioning into lipid bilayers as analysed by immobilized-liposome chromatography

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Acknowledgements and Funding: We thank Karl Edman, Ingo Gottschalk and Christine Lagerquist Hägglund for discussions and advice, David Eaker, Karin Elenbring and Marie Sundqvist for amino acid analyses, D. E. also for linguistic advice, and AstraZeneca and SmithKline-Beecham for providing certain drugs. The work was supported by the Swedish Research Council for Engineering Sciences (Grant No. 1999–304), the O. E. and Edla Johansson Science Foundation and AstraZeneca, Södertälje, Sweden.

Abstract

We have analysed how cholesterol and transmembrane proteins in phospholipid bilayers modulate drug partitioning into the bilayers. For this purpose we determined the chromatographic retention of drugs on liposomes or proteoliposomes entrapped in gel beads. The drug retention per phospholipid amount (the capacity factor K_s) reflects the drug partitioning. Cholesterol in the bilayers decreased the K_s value and hence the partitioning into the membrane in proportion to the cholesterol fraction. On average this cholesterol effect decreased with increasing temperature. Model transmembrane proteins, the glucose transporter GLUT1 and bacteriorhodopsin, interacted electrostatically with charged drugs to increase or decrease the drug partitioning into the bilayers. Bacteriorhodopsin proteoliposomes containing cholesterol combined the effects of the protein and the cholesterol and approached the partitioning properties of red blood cell membranes. For positively charged drugs the correlation between calculated intestinal permeability and log K_s was fair for both liposomes and bacteriorhodopsin–cholesterol proteoliposomes. Detailed modeling of solute partitioning into biological membranes may require an extensive knowledge of their structures.

Introduction

The partitioning of drugs and other compounds into lipid bilayers and biological membranes can be used as one among several predictors to estimate drug uptake by diffusion across the bilayers of membranes in the body in the context of pharmacokinetics. Such partitioning has been studied by use of liposomes and membranes in suspension (Korten et al 1980; Betageri & Rogers 1988; Wright et al 1990; Ma et al 1991), by liposome capillary electrophoresis (Zhang et al 1995a), by immobilized-liposome chromatography (ILC) (Beigi et al 1995, 1998; Lundahl & Beigi 1997; Österberg et al 2001) and by chromatography on immobilized red blood cell membrane vesicles and red blood cells (Lundahl & Beigi 1997; Beigi et al 1998; Beigi & Lundahl 1999). ILC has also been used for partition studies of oligopeptides (Zhang et al 1995b; Hjorth Alifrangis et al 2000). Related methods include chromatography on immobilized artificial membranes (Yang et al 1996), pH-titration in the presence of liposomes (Avdeef et al 1998; Balon et al 1999a, b), micellar liquid chromatography (Molero-Monfort et al 2000) and surface plasmon resonance analysis of interactions with liposomes (Danelian et al 2000).

Biological membranes contain a variety of components which affect drug partitioning into, and diffusion across, the membranes. This article addresses the

questions how, and to what extent, cholesterol and transmembrane proteins modulate the partitioning of drugs into bilayers. Inclusion of cholesterol in bilayers has been reported to decrease the partitioning of thiopental (Korten et al 1980), teniposide (Wright et al 1990), propranolol and diclofenac (Balon et al 1999b) and the permeation of *p*-toluic acids (Xiang et al 1998) and *p*-methylhippuric acids (Xiang et al 2000). The permeability of glucose was decreased, whereas the permeability of the hydrophobic ionophore nigericin (LaBelle & Racker 1977) and the partitioning of n-alkyl *p*-aminobenzoates was increased (Ma et al 1991). A non-monotonic effect of increasing sterol concentration has been reported (Wang et al 1998). A few articles suggest protein effects on bilayer properties. For example, thiopental showed lower partition coefficient in biological membranes than in egg lecithin–cholesterol bilayers (Korten et al 1980); bacterioopsin (retinal-free rhodopsin) at high concentration decreased the hydration of the hydrophobic bilayer region (Dumas et al 1999); and non-channel, head-to-tail dimers of gramicidin enhanced the permeation of α-carbamoyl-*p*-methylhippuric acid but did not affect *p*-methylhippuric acid (Xiang & Anderson 2000; Xiang et al 2000).

In this work we used ILC to investigate the effects of cholesterol and the transmembrane model proteins, the glucose transporter GLUT1 (Barrett et al 1999; Seatter & Gould 1999; Lagerquist Hägglund et al 2000) and bacteriorhodopsin (Booth 2000; Krebs & Isenbarger 2000), on the partitioning of drugs into phospholipid bilayers and compared ILC data with drug permeabilities (P_{eff}). Various drugs which are well absorbed from the intestine were chosen to avoid the limitation inherent in analysis of homologous molecules and to confine the study to drugs taken up by the transcellular route.

Materials and Methods

Materials and solutions

Carboxymethyl-(CM-)Sepharose, Sepharose CL-6B, Sephadex G-50 medium and Superdex 200 prep grade gels and HR glass columns (i.d. 5 mm) were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), L-α-phosphatidylcholine (PC, M_r 760, > 99%), L-α-phosphatidylethanolamine (PE, M_r 746, > 99%) (both from porcine brain) and cholesterol (> 98%) from Avanti Polar Lipids (Alabaster, AL), n-octyl β-D-glucopyranoside (octyl glucoside) from Dojindo Laboratories (Kumamoto, Japan), cholic acid (> 99%) from Fluka (Buchs, Switzerland) and bacteriorhodopsin (polypeptide M_r 25501, from *Halobacterium halobium*), cholesterol oxidase (*Brevibacterium sp.*), alprenolol, atenolol, chlorpromazine, corticosterone, hydrocortisone, metoprolol, oxprenolol, pindolol, promethazine, propranolol and warfarin from Sigma (St Louis, MO). Diazepam, ibuprofen, oxazepam and theophylline were gifts from SmithKline-Beecham (King of Prussia, PA). Other drugs (Figures 1–4) were gifts from AstraZeneca (Södertälje, Sweden). Egg-yolk phospholipids ($M_r \approx$ 1000) were prepared as described by Mascher & Lundahl (1988) and contain phosphatidylcholine (71%), phosphatidylethanolamine (21%), lysophospholipids and minor components (Yang & Lundahl 1994). GLUT1 (polypeptide M_r 54117) and membrane lipids were prepared from human red blood cell membranes as described by Brekkan et al (1996). Copper nitrate, D-glucose, potassium dichromate, and other salts and buffers were reagent grade.

The following solutions were used (pH values at 23°C): A, 150 mM NaCl, 1 mM Na_2EDTA, 10 mM Tris-HCl, pH 7.4; B, 150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.4; C, 600 mM NaCl, 1 mM Na_2EDTA, 10 mM Tris-HCl, pH 7.4; D, 150 mM NaCl, 10 mM sodium phosphate buffer, pH 5.7.

Immobilized-liposome chromatography (ILC)

In general, liposomes were prepared by rehydration of a lipid film, which produces multilamellar liposomes, or by detergent removal from a lipid or protein–lipid solution, whereby unilamellar (proteo)liposomes are formed. Dry gel beads were rehydrated with each of the (proteo)liposome suspensions, after concentration in the detergent removal case. Liposome fusion, enlargement and entrapment were induced by freezing and thawing, which produces multilamellar structures. The non-immobilized material was removed by centrifugal washings. These procedures (Brekkan et al 1997; Lundqvist et al 1998) are based on work by Lundahl et al (1993), Yang & Lundahl (1994) and Brekkan et al (1995, 1996). The entrapped (proteo)liposomes are thought to be suspended in the dextran-coated microcavities of the agarose gel beads. A column can be prepared from lipids and gel beads in one day. Solute interaction with membrane proteins in proteoliposomes or membrane vesicles entrapped in the above way can be analysed by frontal affinity chromatography as reviewed (Gottschalk et al 2001, Lundqvist & Lundahl 2001). GLUT1 immobilized in this way retained its affinity for the inhibitor cytochalasin B for months at room temperature as shown by repeated chromatographic analyses (see the cited reviews).

The present applications are described in detail below. PC–PE (89:11, molar ratio) and cholesterol (0–46 mol% of the total lipid) were dissolved in chloroform which was evaporated. The material was dissolved in diethyl ether. Liposome suspensions were prepared by rehydrating (solution A) the lipid films formed by rotary evaporation of the ether. The mixtures were chosen to mimic a typical membrane composition in a simple way, to obtain low transition temperatures and to give stable entrapment. Higher PE ratios were less suitable from the point of view of stability. The reproducibility was tested by preparation of two columns with 33 mol% cholesterol. They provided similar results.

GLUT1 proteoliposomes were prepared from GLUT1 and the membrane lipids co-purified with the protein (Brekkan et al 1996). For this purpose the detergent was removed at 6°C on a 2 × 38 cm Sephadex G-50 medium gel bed in solution A. These proteoliposomes were either entrapped or subjected to lipid extraction with chloroform–methanol (2:1) (Folch et al 1957). The lipids were separated from the denatured GLUT1 by filtration through filter paper and used to prepare entrapped liposomes. The lipid bilayers contained 0.1 weight% of denatured GLUT1. The reproducibility was tested by preparation of two similar columns, which provided similar results.

For the preparation of bacteriorhodopsin proteoliposomes, freeze-dried bacteriorhodopsin (1 or 3 mg, 0.7 mg mL^{-1}) was solubilized with 35–40 mM octyl glucoside in 20 mM sodium phosphate buffer, pH 6.9 supplemented with 4 μL of 200 mM egg-yolk phospholipids in 250 mM sodium cholate, pH 8.0. The mixture was bath-sonicated for 20 s to disperse the bacteriorhodopsin and was left in the dark at 23°C for 14–20 h. This is a modification of the procedure described by Dencher & Heyn (1982). More of the above egg-yolk phospholipid solution (43 μL) was added and the detergent was removed as above. The bacteriorhodopsin proteoliposomes were purple. Protein-free egg-yolk phospholipid liposomes were prepared similarly.

Bacteriorhodopsin–cholesterol proteoliposomes were prepared by solubilization of bacteriorhodopsin (5 mg) as above, addition of 650 μL of a cholesterol–cholate solution (see below) and 18 μL of the above egg-yolk phospholipid solution, followed by detergent removal as above. The cholesterol–cholate solution was prepared by dissolving 4.4 mg cholesterol in 0.8 mL of hot 96% ethanol and adding 0.8 mL of sodium cholate solution (750 mM, pH 8.4), whereby the cholesterol was precipitated, and then 1.0 mL of the cholate solution, after which the cholesterol was redissolved by heating. Protein-free egg-yolk phospholipid liposomes were prepared by rehydration of a lipid film. Proteoliposomes with 3.4, 6.4 and 21 weight% bacteriorhodopsin provided log K_s values in reasonable proportion to the bacteriorhodopsin percentage (0.031 and -0.016 Δ log K_s $(\%)^{-1}$ for positively and negatively charged drugs, respectively) after correction for the cholesterol amount in the proteoliposomes with 21% bacteriorhodopsin.

The (proteo)liposome suspensions prepared by detergent removal were concentrated by two-fold freeze-thawing (-75°C/$+25$ °C) and ultracentrifugation (230 000 *g*, 60–90 min). These concentrated suspensions or the suspensions prepared by rehydration were mixed with dry Superdex 200 prep grade (70–100 mg per mL suspension), degassed and then incubated for at least 15 min under nitrogen gas. The mixtures were five-fold frozen (-75°C) and thawed ($+25$°C) and washed in solution A (6 × 3 min at 150–350 *g*, 15–20°C). Gel beds (approximately 0.5 mL, 2–13 μmol phospholipid) were formed in HR 5/2 glass columns. Analytes (20 μL, 1–5 μg, < 5% ethanol) were applied and eluted in solution B (PC–PE–cholesterol liposomes) or in solution A (proteoliposomes). Solutions C or D were used when so stated. The different buffers in solutions A and B did not affect the drug retention in tests on bacteriorhodopsin–cholesterol proteoliposomes. The flow rate was 0.50 or 1.00 mL min^{-1}, the temperature was 23 ± 1°C unless otherwise stated, and the eluted drugs were detected at 220 nm (600 nm for Cu^{2+}) (Waters 484 or 486, Millipore, MA). The drug analyses were done in duplicates (PC–PE–cholesterol liposomes) or triplicates (proteoliposomes) on single columns, or duplicate columns (PC–PE–liposomes with 33 mol% cholesterol; GLUT1 proteoliposomes and corresponding liposomes without GLUT1). The average s.e.m. of log K_s values above 1.5 was ± 0.01. Lower log K_s values showed larger variation due to small retardation volumes.

For control experiments 0.5-mL beds of CM-Sepharose and Sepharose CL-6B (mimicking the ion-exchanger matrix) were packed in HR 5/2 columns. The differences between the elution volumes of a drug on the two beds were determined for comparison with ILC data. Several drugs were tested.

Cholesterol, phospholipid and polypeptide amounts

The weight fractions of cholesterol in the mixtures used to prepare the (proteo)liposomes are given below, although the cholesterol fractions in the gels after the runs, determined by use of cholesterol oxidase essentially as described by Ahmed (1993), were on average 11% higher (Beigi 2000). The amounts of phospholipids and

polypeptide in the gels were determined after the runs by phosphorus analysis of gel samples (Bartlett 1959; Brekkan et al 1996, 1997) in solution A and by quantitative amino-acid analyses of hydrolyzed gel samples (Brekkan et al 1996, 1997) with minor corrections (Gottschalk et al 2000). The total phospholipid losses during the packing of the gel beds and the entire series of runs on each bed were 10–30%.

The capacity factor K_s

K_s (expressed as M^{-1}), which reflects the drug partitioning into the lipid bilayers, was defined by:

$$K_s = (V_R - V_0 - V_G \times h)/A \quad (1)$$

where V_R is the elution volume of the drug, V_0 is the elution volume for an analyte that presumably does not interact with the (proteo)liposomes, A is the amount of phospholipids, V_G is the drug retention volume on empty gel beads, per bed length, and h is the length of the (proteo)liposome gel bed used. As in Beigi et al (1998), V_G was neglected in the analyses on PC–PE–cholesterol liposomes. V_0 values determined by use of $Cr_2O_7^{2-}$, Cu^{2+} and D-glucose did not differ.

K_s for a given drug will depend slightly on the liposomal phospholipid composition because the structure and volume of the bilayer affects the partitioning. Effects of cholesterol or transmembrane proteins in bilayers of certain phospholipid compositions were therefore determined by use of cholesterol-free and protein-free bilayers of corresponding phospholipid compositions as references.

The difference, $\log K_{s,i} - \log K_{s,j}$, between the log K_s values for two drugs, i and j, on a column containing entrapped phospholipid liposomes equals $\log (V_{R,i} - V_0 - V_{G,i} \times h) - \log (V_{R,j} - V_0 - V_{G,j} \times h)$. If the $\log K_{s,i}$ value for a drug, i, is known, the $\log K_{s,j}$ value for any other drug, j, can be determined from the values of $V_{R,i}$, $V_{R,j}$, $V_{G,i}$ and $V_{G,j}$, without determination of the phospholipid amount.

Calculation of intestinal permeability values (P_{eff})

The chemical structures of the compounds were drawn in their neutral form using ChemDraw (Cambridge Soft Corporation). The SMILES string for the structures were created in ChemDraw, transferred to SYBYL (version 6.6, Molecular Modeling Software, Tripos Associates, St Louis, MO) and processed using CONCORD to yield 3D structures, which were minimized by use of the AM1 method (keywords PRECISE, XYZ and NOMM). The number of hydrogen-bond donors (HBD) was estimated by counting the number of hydrogens connected to N- and O-atoms. The polar surface area (PSA), defined as the surface area of the oxygens, nitrogens, sulfurs and hydrogens bonded to any of these atoms, was calculated using Savol (Pearlman, R. S., Savol3: Molecular Surface Areas, Volumes, and Atomic Contributions Thereto, software available from the author, College of Pharmacy, University of Texas, Austin TX 78712). A solvent probe of 0.0 Å radius was used in order to obtain PSA values comparable to those reported by Winiwarter et al (1998). Theoretical octanol/water partitioning values ($\log P_{oct}$ values) were calculated using the method of Ghose & Crippen (1986) with the help of a SYBYL SPL-script. Permeability values were calculated using:

$$\log P_{eff} = -3.128 - 0.0088\ PSA + 0.172 \log P_{oct} - 0.215\ HBD \quad (2)$$

which is a variation of an equation previously presented by Winiwarter et al (1998). The above equation was derived by Winiwarter, S., Ax, F., Lennernäs H., Hallberg, A., Pettersson, C. and Karlén, A. by use of data for 13 passively absorbed drugs with known human jejunal permeability values.

Results

Effects of cholesterol and temperature on drug partitioning into bilayers

The log K_s values decreased rectilinearly with the cholesterol content in the PC–PE bilayers (Figure 1), indicating that the cholesterol lowered the partitioning of the drugs into the bilayers. However, the effect differed between different drugs. Firstly, the slopes became on average steeper the higher the log K_s values were, and, secondly, for drugs of similar log K_s value the slopes also differed. The rectilinear correlation line for the log $K_{s,46\%}$ values for the drugs on bilayers with 46 mol% cholesterol versus the log K_s values on cholesterol-free bilayers, $\log K_{s,0\%}$, had the equation:

$$\log K_{s,46\%} = 0.79 \log K_{s,0\%} + 0.08\ (R^2 = 0.97) \quad (3)$$

The individual $\log K_{s,46\%}$ values fell between 0.74 log $K_{s,0\%}$ and 0.93 $\log K_{s,0\%}$. This variation far exceeds the s.e.m. (s.e.m for log K_s values above 1.5 was on average ± 0.01; lower log K_s values showed larger variation)

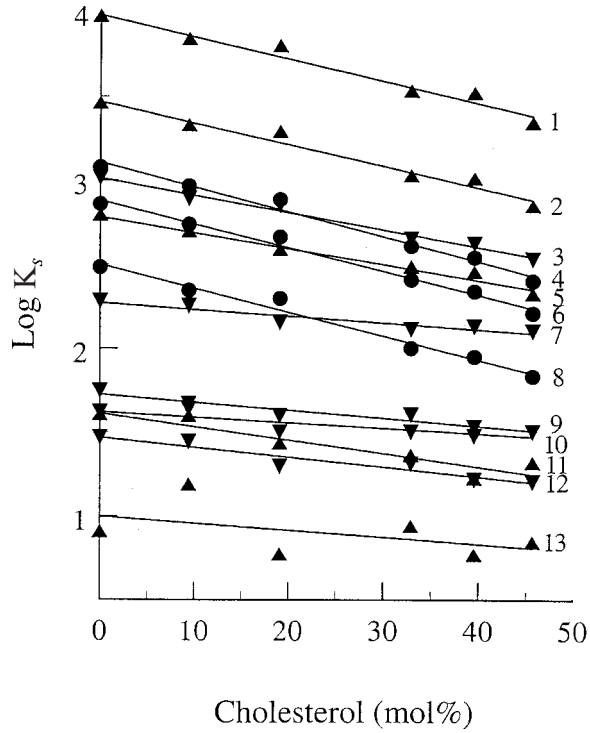

Figure 1 Effect of cholesterol on drug partitioning into bilayers: log K_s values at 23°C, pH 7.4, on PC–PE–cholesterol liposomes vs the fraction of cholesterol in the bilayers. 1, chlorpromazine; 2, promethazine; 3, indometacin; 4, oxazepam; 5, propranolol; 6, diazepam; 7, flurbiprofen; 8, corticosterone; 9, naproxen; 10, sulindac; 11, oxprenolol; 12, ketoprofen; 13, metoprolol. Positively charged (▲), negatively charged (▼) and neutral drugs (●) are denoted separately. The average s.e.m for log K_s values above 1.5 was ±0.01; lower log K_s values showed larger variation.

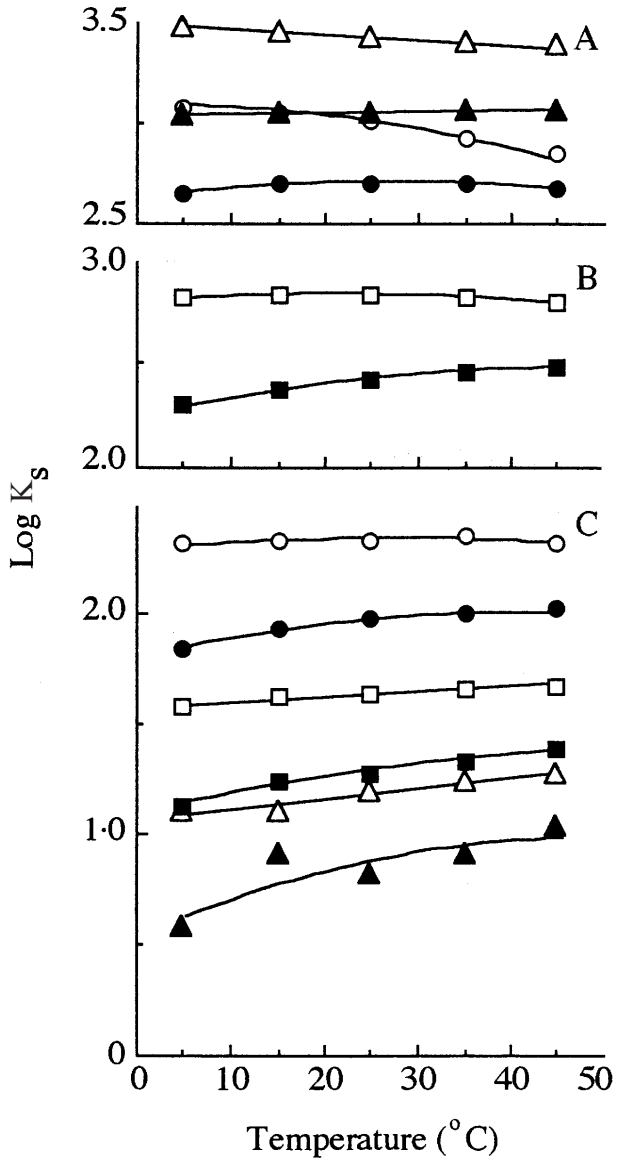

Figure 2 Temperature-dependence of cholesterol effects on drug partitioning: log K_s values on PC–PE liposomes (open symbols) and on PC–PE–cholesterol (33 mol%) liposomes (filled symbols) vs temperature. A. promethazine (△, ▲), indometacin (○, ●). B. diazepam (□, ■). C. alprenolol (○, ●), oxprenolol (□, ■), metoprolol (△, ▲). The temperature was controlled as in Lundqvist & Lundahl (1997).

and indicates different responses among the drugs to the cholesterol content of the bilayer.

Inclusion of 33 mol% cholesterol into PC–PE bilayers lowered the log K_s values over the temperature range 5–45°C for six drugs chosen to cover the log K_s interval 1–3.5 (Figure 2). The bilayers, with or without cholesterol, assumed the liquid-crystalline state throughout the temperature range, since the difference between the log K_s values obtained without and with cholesterol decreased gradually with increasing temperature to become, on average, 60±10% at 45°C of the difference at 5°C.

Effects of transmembrane proteins on drug partitioning into bilayers

The transmembrane proteins in the bilayers of immobilized proteoliposomes influenced the drug partitioning (Table 1). GLUT1 gave considerable decreases in log K_s for the positively charged drugs, which agrees with the positive net charge of the protein, +6 per monomer at pH 7.4 (Lu et al 1993; Henriksson et al 1995). However, decreases also for the neutral drugs indicated a non-electrostatic contribution to the effect. For the negatively charged drugs GLUT1 caused net decreases in the log K_s values. The non-electrostatic

Table 1 Effects of transmembrane proteins on drug partitioning into lipid bilayers as judged by ILC.

	GLUT1 proteoliposomes[a]		Bacteriorhodopsin proteoliposomes[b]		Bacteriorhodopsin–cholesterol proteoliposomes[c]	
	Log K_s[d]	Δ log K_s	Log K_s[d]	Δ log K_s	Log K_s[d]	Δ log K_s
Theophylline[e]	1.00	−0.07	0.932	0.12	0.61	0.37
Metoprolol[e]	1.63	−0.20	1.33	0.21	1.17	0.51
Alprenolol[e]	2.76	−0.25	2.42	0.23	2.42	0.40
Propranolol[e]	3.22	−0.26	2.84	0.24	2.84	0.43
Loperamide[e]	3.68	−0.34	3.37	0.25	3.39	0.19
Promethazine[e]	3.83	−0.27	3.47	0.24	3.50	0.28
Chlorpromazine[e]	4.33	−0.25	4.00	0.28	4.00	0.23
Corticosterone[f]	2.25	−0.15	2.37	0.00	2.42	−0.26
Phenytoin[f]	2.56	−0.20	2.71	−0.02	2.76	−0.31
Warfarin[g]	1.30	−0.11	1.48	−0.05	1.46	−0.29
Indometacin[g]	2.51	−0.06	2.86	−0.13	2.97	−0.59
Diflunisal[g]	2.73	−0.04	3.16	−0.19	3.23	−0.61

The Δ log K_s values represent the differences between the normalized drug retention on proteoliposomes and on the corresponding liposomes. For the latter the log K_s values are given. [a]14.3 weight% protein; [b]6.4 weight% protein; [c]21 weight% protein, 40 mol% cholesterol; [d]Log K_s values for phospholipid liposomes. The GLUT1 proteoliposomes and the corresponding liposomes were prepared with endogenous co-purified red blood cell membrane lipids. Different egg-yolk phospholipid batches have been used for the bacteriorhodopsin proteoliposomes and the corresponding liposomes on one hand and the bacteriorhodopsin–cholesterol proteoliposomes and the corresponding liposomes on the other. [e]Positively charged drugs; [f]neutral drugs; [g]negatively charged drugs.

effect was thus larger than the electrostatic effect. In contrast, bacteriorhodopsin showed electrostatic effects only. The positively charged, neutral and negatively charged drugs had increased, similar and decreased log K_s values, respectively, on bacteriorhodopsin proteoliposomes compared with protein-free liposomes, consistent with the net electric charge of bacteriorhodopsin, –3 per monomer, at pH 7.4. The orientation of the proteins in the bilayers was presumably random and did not contribute to a transmembrane potential. The fact that a non-electrostatic protein effect was observed with GLUT1 but not with bacteriorhodopsin may suggest differences between the hydrophobic protein surfaces and their interactions with the bilayer lipids.

The chromatographic retention volumes for charged drugs on the cation-exchanger, CM-Sepharose, in solution A, were small compared with the retention volume changes caused by bacteriorhodopsin in proteoliposomes (6.4% protein), even though the ionic capacity of the ion-exchanger was about 1000-fold that of the net protein charge in the proteoliposomal gel bed. For example, at the ionic strength, I, of 0.16, propranolol was retarded by 3 mL by the bacteriorhodopsin alone (0.35 mg protein, 0.04 μmol net bacteriorhodopsin charge) and by 0.83 mL on a CM-Sepharose column (ionic capacity 55 μmol), in agreement with the known fact that a single-site electrostatic interaction in an aqueous phase is weak at physiological ionic strength. As a control, a Sepharose CL-6B gel bed of the same volume as the CM-Sepharose bed gave 0.10 mL retardation of propranolol.

The electrostatic interaction between the drugs and the membrane protein took place, at least partly, in a region in or adjacent to the aqueous phases, as shown by the fact that the ionic binding between the positively charged drugs alprenolol, propranolol, loperamide, promethazine and chlorpromazine and negative charges on bacteriorhodopsin was weakened by an increase in I from 0.16 (solution A, containing 150 mM NaCl) to 0.6 (solution C, containing 600 mM NaCl). The difference between the log K_s values on the bacteriorhodopsin proteoliposomes (6.4%) and on protein-free liposomes decreased by 37 ± 8%, from 0.25 ± 0.02 at I = 0.16 to 0.16 ± 0.02 at I = 0.6. For neutral drugs the increase in ionic strength had no effect. For the negatively charged drugs indometacin, diflunisal and warfarin, the numerical value of the difference decreased from 0.12 ± 0.05 to 0.00 ± 0.04. At a pH value equal to the isoelectric point 5.7 of bacteriorhodopsin (solution D), the effect on bacteriorhodopsin proteoliposomes was essentially the

same as at pH 7.4, indicating that the pK_a values of the majority of the amino-acid side chains involved are < 5.7 or > 7.4, as for the pK_a values of the drugs.

A combined effect of bacteriorhodopsin and cholesterol on the drug partitioning into bilayers

On the entrapped bacteriorhodopsin–cholesterol proteoliposomes (21 weight% bacteriorhodopsin, 40 mol% cholesterol; Table 1, Figure 3) positively and negatively charged drugs showed increased and decreased log K_s values, respectively, compared with the values on corresponding liposomes, similarly as for bacteriorhodopsin proteoliposomes (Table 1). All log K_s values were lowered by the cholesterol. Therefore the values for the neutral drugs were also decreased. The effects of bacteriorhodopsin and cholesterol were approximately additive. The inclusion of cholesterol together with bacteriorhodopsin into the bilayers made the partitioning for the positively charged drugs theophylline, atenolol, metoprolol, pindolol, oxprenolol, alprenolol, propranolol, loperamide, promethazine and chlorpromazine fairly similar to the partitioning into cytoskeleton-depleted human red blood cell membrane vesicles (Beigi et al 1998) (not shown). The straight-line equation for the bacteriorhodopsin–cholesterol values vs the vesicle values was $y = 1.20x - 0.45$, $R^2 = 0.96$. With red blood cell membranes instead of vesicles the slope became steeper ($y = 1.81x - 1.82$, $R^2 = 0.93$).

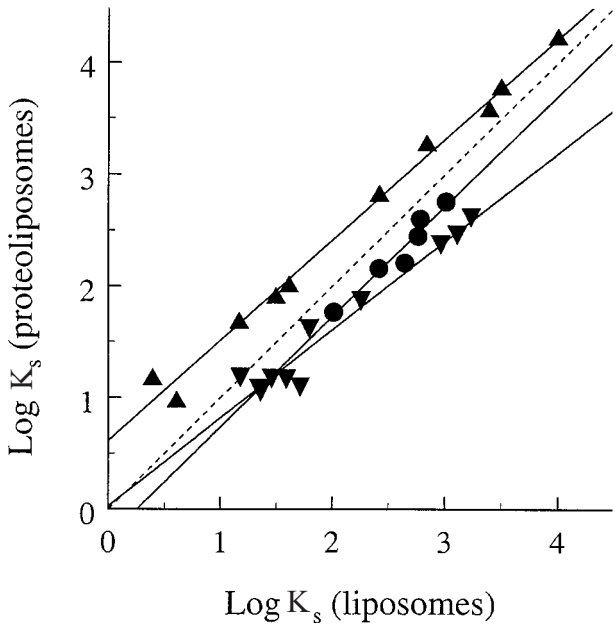

Figure 3 Effect of bacteriorhodopsin (21 weight%) combined with cholesterol (40 mol%) on drug partitioning into phospholipid bilayers. Log K_s values on bacteriorhodopsin–cholesterol proteoliposomes vs log K_s on liposomes, for positively (▲) and negatively (▼) charged or neutral (●) drugs, at pH 7.4. Hatched line: the no-effect-line $y = x$. The drugs are (from left to right) (▲): theophylline, atenolol, metoprolol, pindolol, oxprenolol, alprenolol, propranolol, loperamide, promethazine, chlorpromazine; (●): hydrocortisone, corticosterone, dexamethasone, phenytoin, diazepam, oxazepam; (▼): sulindac, ketoprofen, piroxicam, naproxen, warfarin, indoprofen, ibuprofen, flurbiprofen, indometacin, diclofenac and diflunisal.

Correlation between calculated permeability coefficient (P_{eff}) and log K_s

The calculated log P_{eff} values, used for modelling intestinal drug absorption, were compared with the log K_s values obtained on egg-yolk phospholipid liposomes (Figure 4). The calculated log P_{eff} values for ten positively charged drugs showed a fair correlation with the log K_s values (Figure 4A). However, for ten negatively charged drugs the range of the calculated log P_{eff} values was narrow in the tested log K_s interval (Figure 4B). Six neutral drugs showed large differences among the log P_{eff} values in a narrow log K_s range (Figure 4C). Five experimental permeability values (Figure 4A, B, open triangles) agreed well with the calculated values. The data for bacteriorhodopsin–cholesterol proteoliposomes showed similar correlations with the permeability values as the data for egg-yolk phospholipid liposomes did (not shown).

Discussion

The chromatographic drug retention normalized with regard to the phospholipid content in the column (K_s, Equation 1) reflects the drug partitioning into the lipid bilayers of (proteo)liposomes entrapped in gel beads. The drug is retarded in proportion to the partitioning and the amount (Beigi & Lundahl 1999) or volume (Yang et al 1999) of the bilayers. The partition coefficient can be calculated (Yang et al 1999).

Cholesterol decreased the drug partitioning into phospholipid bilayers (Figure 1) in agreement with earlier observations (Wright et al 1990; Xiang et al 1998, 2000; Balon et al 1999b). The planar and rigid ring system of cholesterol is thought to reside in the outer parts of the fatty acyl chain region where it tends to restrict the motion of the chains in liquid-crystalline bilayers (Kutchai et al 1983; Robinson et al 1995) and may sterically hinder the diffusion of drug molecules. The lowered effect on the partitioning at higher temperature

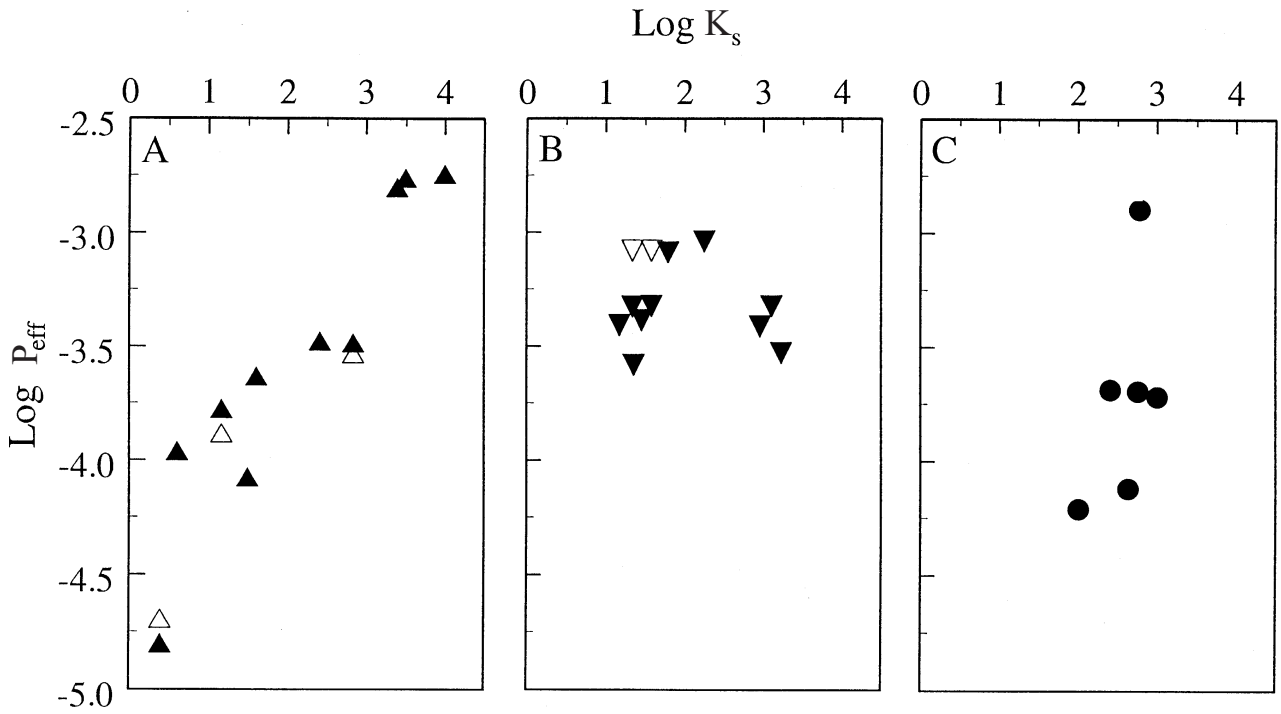

Figure 4 Calculated (filled symbols) and experimental human (open symbols; data from Winiwarter et al (1998)) log P_{eff} values vs log K_s values on egg-yolk phospholipid liposomes for positively charged drugs (A), negatively charged drugs (B) and neutral drugs (C). The drugs are (from left to right) (A) atenolol, theophylline, metoprolol, pindolol, oxprenolol, alprenolol, propranolol, loperamide, promethazine and chlorpromazine; (B) indoprofen, ketoprofen, sulindac, warfarin, naproxen, ibuprofen, flurbiprofen, indometacin, diclofenac and diflunisal; and (C) hydrocortisone, corticosterone, dexamethasone, diazepam, phenytoin and oxazepam.

(Figure 2) agrees with increased disorder of the fatty acyl chains.

Wang et al (1998) have reported that several sterol concentrations that allow the arrangement of the sterol molecules in superlattices (e.g. 20, 20.2, 25, 33.3, 40 and 50 mol%) gave partitioning minima of the antibiotic nystatin, whereas concentrations in between the above values provide higher partitioning. This effect may be specific for nystatin, which binds to sterols, or may represent a general effect on solute partitioning into membranes. Further analyses may elucidate this interesting issue.

The transmembrane proteins GLUT1 and bacteriorhodopsin affected the retention of the drugs by electrostatic interactions (Table 1) and, in the case of GLUT1, to some extent by a non-electrostatic effect. Drugs in the aqueous phase outside the membrane did not interact appreciably with charges on the hydrophilic faces of the proteins, as indicated by the weak drug–CM-Sepharose interactions. The electrostatic interactions between protein charges and charges of drug molecules can rather be expected to take place in the protein regions in contact with the polar headgroups of the phospholipids, or adjacent to these groups, in the way that other parts of the drug molecules can interact with the fatty acyl chains of the phospholipids or with hydrophobic amino-acid side chains of the protein (Figure 5). A lower permittivity (dielectric constant) in the headgroup region than in the aqueous phase enhances the effects of the charges. However, the total protein effect on drug partitioning in a natural membrane will depend on the protein composition and the pattern of protein–protein interaction. The additive nature of the cholesterol and protein effects agrees with the suggestion that these phenomena take place at different sites, as illustrated in Figure 5.

Natural membranes are composed of several types of membrane proteins and a variety of asymmetrically distributed lipid molecules which may form phase-separated domains. The partitioning of a drug into the membrane is thus determined by multifold interactions in different regions of the membrane and has not yet been mimicked by artificial membranes (see comparisons with red blood cell membranes in Results).

In the absence of sufficient calculated or experimental data for natural membranes, preliminarily prediction of

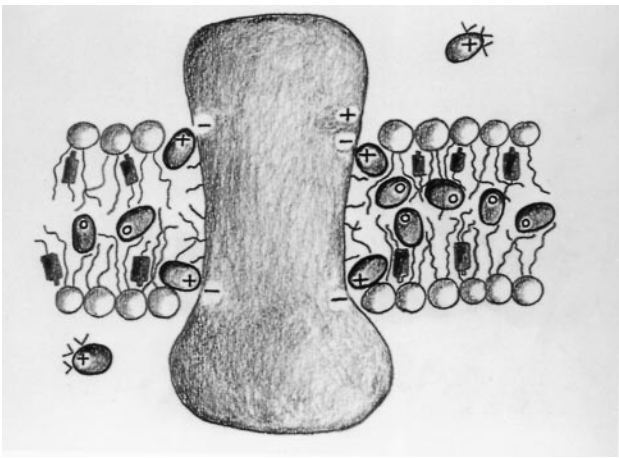

Figure 5 Schematic illustration of the distribution of a charged drug within a phospholipid bilayer containing cholesterol and a transmembrane proteins. Cholesterol molecules are depicted in the outer fatty acyl chain region (dark molecules with tails). Drug molecules (grey elliptical bodies) are shown positively charged (+) in the aqueous phase and at the protein surface and uncharged in the bilayer (0). The drug molecules prefer the centre of the hydrophobic bilayer region and are attracted to negative charges on the protein surface adjacent to the polar phospholipid headgroups (spheres). Negatively charged drug molecules would have been repelled. Neutral drugs are affected mainly by the cholesterol.

drug uptake in the body has in many cases relied on octanol–water partitioning data (log P_{oct}), which have been described to be poorly correlated with interactions between ionizable drugs and phospholipid bilayers (Balon et al 1999a). Neutral drugs show, on average, higher partitioning into phosphatidylcholine bilayers than positively charged drugs do compared with log P_{oct}, whereas negatively charged drugs exhibit the weakest bilayer interactions, as clarified by Österberg et al (2001). The correlation between calculated log P_{eff} values and the experimental log K_s values (Figure 4) was fairly good only for the positively charged drugs. Charged drugs also interact electrostatically with the polar regions of the bilayers themselves (Avdeef et al 1998; Österberg et al 2001). A negative charge on an oligopeptide impairs partitioning (Hjorth Alifrangis et al 2000) and positively charged solutes are strongly attracted to the negatively charged phosphatidylinositol membranes (Krämer et al 1998) which show a relatively high ζ-potential (the electric potential at the surface of shear of the charged particle). According to Krämer et al (1998), "the electrostatic interactions certainly influence drug accumulation in membranes, membrane effects of drugs and the activity of membrane protein active drugs". Phosphatidylcholine–phosphatidylserine (81:19) liposomes gave small electrostatic effects at physiological ionic strength (Beigi et al 1998). It has not been clarified to what extent a negatively or positively charged drug partitions as an ion into a membrane, after dehydration, or to what extent it attracts or sheds a proton, respectively, upon entering the hydrophobic regions of the membrane (Figure 5). When the effects of proteins on drug partitioning are combined with the effects of cholesterol we can imagine that the concentration of positively charged drug is increased adjacent to negatively charged proteins in the region of the polar phospholipid headgroups (whereas negatively charged drugs are depleted in the same region). Simultaneously, the drug concentration is decreased in the hydrophobic region of the membrane adjacent to the polar headgroup region by the presence of cholesterol there (Figure 5). For comparison of drug diffusion across different types of membranes, the decrease in bilayer area with increased content of transmembrane proteins should also be taken into account.

Conclusions

Inclusion of cholesterol in liquid crystalline lipid bilayers decreased the partitioning of drugs into the membrane, in agreement with previously suggested restrictions of the motion of the outer parts of the fatty acyl chains. Transmembrane proteins showed largely electrostatic effects on drug partitioning into the bilayers, presumably by attraction or repulsion of charged drugs within the outer regions of the bilayer. The cholesterol effect and the electrostatic protein effect were additive and thus seemed to take place in different regions of the membranes. The cholesterol effect differed between different drugs, in some cases also for drugs with similar partitioning into phospholipid bilayers, indicating that the drugs were differently distributed within the bilayers. The correlation between the calculated P_{eff} and experimental ILC values was fair only for drugs that were positively charged in the aqueous phase. More detailed experimental and theoretical analyses of drug and membrane features may allow calculation of drug partitioning into and permeation through membranes.

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