Binding of Imipramine to Phospholipid Bilayers Using Radioligand Binding Assay

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Abstract. Binding of the tricyclic antidepressant imipramine (IMI) to neutral and negatively charged lipid membranes was investigated using a radioligand binding assay combined with centrifugation or filtration. Lipid bilayers were composed of brain phosphatidylcholine (PC) and phosphatidylserine (PS). IMI binding isotherms were measured up to IMI concentration of 0.5 mmol/l. Due to electrostatic attraction, binding between the positively charged IMI and the negatively charged surfaces of PS membranes was augmented compared to binding to neutral PC membranes. After correction for electrostatic effects by means of the Gouy–Chapman theory, the binding isotherms were described both by surface partition coefficients and by binding parameters (association constants and binding capacities). It was confirmed that binding of IMI to model membranes is strongly affected by negatively charged phospholipids and that the binding is heterogeneous; in fact, weak surface adsorption and incorporation of the drug into the hydrophobic core of lipid bilayer can be seen and characterized. These results support the hypothesis suggesting that the lipid part of biological membranes plays a role in the mechanism of antidepressant action.

Key words: Antidepressants — Binding — Liposomes — Phosphatidylcholine — Phosphatidylserine

Abbreviations: IMI, imipramine; DMI, desipramine; AMI, amitriptyline; CMI, clomipramine; DDMI, didesmethylimipramine; PC, phosphatidylcholine; PS, phosphatidylserine; CAD, cationic amphiphilic drug; TCA, tricyclic antidepressant; MLV, multilamellar vesicle; SUV, small unilamellar vesicle; LUV, large unilamellar vesicle; GC, Gouy–Chapman; B_{max}, maximum amount of drug bound to phospholipid (binding capacity); K, equilibrium association constant; $B_{max,app}$, apparent binding capacity; K_{app}, apparent association constant; K_d, equilibrium dissociation

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constant; IC_{50}, half-maximum displacement (cold ligand concentrations displacing 50% of bound radioligand); k_p, intrinsic (hydrophobic) partition coefficient; $k_{p,app}$, apparent overall partition coefficient; C_{eq}, equilibrium IMI concentration in the bulk aqueous phase; B_{IMI}, amount of IMI in milimoles bound *per* mole of phospholipid; σ, surface electric charge density; λ, Debye length; ψ_0, electrostatic surface potential; C_0, concentration of IMI in the aqueous phase at the membrane; PEI, polyethyleneimine.

Introduction

Antidepressants are amphiphilic molecules which are able to accumulate in lipid bilayer and alter its composition or physical characteristics. There is insufficient knowledge as regards the extent in which the action of antidepressants on lipid bilayers contributes to their therapeutic or adverse effects. Tricyclic antidepressants (TCAs) have been used in treatment of depression for almost 50 years and have been tested in many clinical and theoretical studies. Inhibition of serotonin and noradrenalin transporters in neuronal membranes is their primary biochemical effect leading to the therapeutic effect. Still it is unclear whether the TCA recognition takes place directly from the aqueous phase *via* binding to surface-exposed binding sites, or whether it is mediated by the lipid part of target membranes. The latter possibility was proposed on the basis of the amphiphilic properties of TCAs.

Liposomes are the best model of the lipid part of biological membranes and they enable determination of partition coefficients or binding parameters (Reith et al. 1984; Fišar et al. 1991; Mason et al. 1991; Seydel et al. 1992, 1994). Liposomes are simply vesicles in which aqueous solution is enclosed by a membrane composed of lipid molecules. Depending on the method of preparation, multilamellar vesicles (MLVs), small unilamellar vesicles (SUVs) or large unilamellar vesicles (LUVs) may be produced (New 1990). LUVs are the best model of cell membranes; however, it was demonstrated that MLVs are also a good model (Choi and Rogers 1991).

As a consequence of membrane heterogeneity, drugs are distributed non-uniformly in the lipid bilayer (Herbette et al. 1986; Müller et al. 1986) and multiple binding sites of cationic amphipaths with membranes have been described (Boulanger et al. 1980; Kelusky et al. 1986; Zachowski and Durand 1988). Although the lipid bilayer is a dynamic fluid structure, there are stable separate domains in which specific interactions can occur. Specific interactions of some ligands with phospholipid acyl chains may also occur (Bäuerle and Seelig 1991; Jørgensen et al. 1991).

At physiological conditions, TCAs in aqueous solution carry a positive charge ($pK = 9.4$). It seems that these drugs primarily act on the polar phase of lipid bilayers (Zimmer and Schulze 1981). The charged phosphate and carboxylic groups on phospholipid molecules are the main membrane binding sites for cations. Protonated amines can partition to a surprising extent into phospholipid bilayers; however, it has been found that this is not a consequence of simple ion pairing (Austin et al. 1995). Having their charge lost, nonpolar TCA molecules may be accumulated

in the hydrophobic core of lipid bilayers or can pass through membranes. Therefore, at equilibrium, TCA molecules are localized on the outer and inner membrane surfaces, in the hydrophobic core of lipid bilayers and in the aqueous phase. Indeed, TCAs are known to penetrate easily into the central nervous system (Seelig et al. 1994). In our initial experiments, we verified the assumption that permeability of lipid bilayers for TCAs enables a rapid and uniform distribution of the drugs in MLVs.

The tendency to refer to the result of drug-lipid bilayer interaction as "binding" has been subject to discussion because of the general meaning of this term in the classic enzyme-substrate or ligand-receptor interaction studies (Heirwegh et al. 1992). Binding into a bilayer is considered a restriction of dimensionality (Mosior and McLaughlin 1992; Beschiaschvili and Seelig 1992). It should be taken into consideration that lipid-TCA interactions can be purely electrostatic, purely hydrophobic, or a combination of both. In the case of a negatively charged membrane, not only the cationic drug, but also all other cations will accumulate at the lipid-water interface.

Partition coefficients have been found to be concentration-independent at low drug concentrations (Mason et al. 1989); yet they depend on temperature, pH and membrane lipid composition (Luxnat and Galla 1986; Zachowski and Durand 1988; Austin et al. 1995). A number of studies have shown that the surface partition coefficient, determined as the ratio of surface concentration of the drug to its actual bulk concentration in the immediate vicinity of the membrane, is independent on the bulk concentrations even at higher ligand concentrations (Bäuerle and Seelig 1991; Heirwegh et al. 1992).

The saturation of adsorption isotherms observed at higher concentrations of amphiphilic drugs has been explained by changes in electrostatic potential on the membrane surface due to incorporation of charged ligand molecules into the lipid bilayer. The effective ion concentration near the membrane surface differs from that in the bulk due to redistribution of the ions in the electrostatic, hydration, and steric-repulsion fields of the membrane. The electrostatics of charged lipid bilayers has been extensively discussed in a number of reviews (McLaughlin 1989; Cevc 1990, 1993; Langner and Kubica 1999). For long-range interactions, the electrostatic potential in the vicinity of the membrane surface can be approximated by the Gouy–Chapman (GC) theory. The electrophoretic mobility, profile of electrostatic potential and concentrations of small ions at the membrane surface are all described by the GC theory with sufficient accuracy. The GC theory has also been adopted to describe adsorption (incorporation) of drugs and relatively large molecules (hormones, peptides, proteins) onto the lipid bilayer surface (Stankowski 1991; Mosior and McLaughlin 1992; Seelig et al. 1996). After a correction for electrostatic effects by means of the GC theory, the binding isotherm (Langmuir or Stern) can be analyzed (Beschiaschvili and Seelig 1990). The binding parameters allow identification of one, two or more binding sites. The GC theory allows separation of the total binding to lipid bilayer into the hydrophobic and electrostatic contributions (Seelig et al. 1993).

Binding of TCAs to model membranes is apparently more affected by the bilayer lipid composition than by differences between individual TCAs (Cater et al. 1974; Fišar et al. 1991). This effect is mostly due to negatively charged phospholipids. Therefore, we used liposomes prepared both from the electroneutral phosphatidylcholine (PC) and from the negatively charged phosphatidylserine (PS). Imipramine (IMI) was used as a ligand representing the TCAs; in some experiments, however, we used other TCAs (desipramine, DMI; didesmethylimipramine, DDMI; clomipramine, CMI; amitriptyline, AMI).

The technique employed in our study was a radioligand binding assay analogous to that used in receptor studies (Bennett and Yamamura 1985). The advantage of this method is its ability to work with very low drug concentrations and to study high-affinity binding sites.

The total plasma levels of TCAs and their active metabolites during the therapy of depression are usually below 1 μmol/l (Razavi and Mendlewicz 1982), and up to 95 % thereof may be bound to plasma proteins. Consequently, the free TCA concentrations fall inside the nanomolar range (<50 nmol/l). So far, binding of IMI to lipid bilayers at nanomolar IMI concentrations has not been characterized.

The aim of our experiments was to give a description of different binding sites of IMI in lipid bilayers. Total binding of IMI to PC and PS liposomes was analyzed using the GC theory and the strong ("high-affinity") part of the total binding was characterized.

Materials and Methods

Chemicals and solutions

All samples were prepared in a buffer solution (120 mmol/l NaCl, 10 mmol/l KCl, 30 mmol/l Tris HCl, pH 7.4). Phospholipids in the chloroform/methanol solvent mixture (2 : 1, vol/vol; 5–20 mg/ml) were prepared in our laboratories. Crude extract of lipids from white matter of bovine brain was prepared (Folch et al. 1957; Koul and Prasad 1996), and PC and PS were isolated by column chromatography using alumina and DEAE-Sephadex. The resulting purity, determined by two-dimensional thin-layer chromatography, was over 95 %. The phospholipids were stored in a freezer under nitrogen atmosphere. The following stock solutions of tritium-labelled TCA ([³H]TCA) in methanol were used: 13.0 μmol/l [³H]IMI (specific activity 2.85 TBq/mmol, concentration 37 MBq/ml), 4.08 μmol/l [³H]DMI (2.0 TBq/mmol, 8.16 MBq/ml), 3.00 μmol/l [³H]AMI (4.07 TBq/mmol, 12.2 MBq/ml), 17.6 μmol/l [³H]DDMI (2.1 TBq/mmol, 37 MBq/ml); radiochemical purity >96 %; labelled in our laboratories (Krulík et al. 1991); 50 or 100 nmol/l of [³H]TCA solution with specific activity about 100 kBq/ml in buffer was used in a binding experiment. Tritium-labelled phospholipids, [³H]PC and [³H]PS (concentration 18.5 MBq/ml, radiochemical purity >96 %), were prepared by catalytic tritiation of double bonds in hydrocarbon chains of phospholipids (with a relatively great content of oleic acid) with gaseous tritium.

Preparation of liposomes

A modification of the Bangham method (Bangham et al. 1965; New 1990) was used to prepare PC and PS vesicles. Phospholipid concentrations were checked using phosphorus concentration determination (Bartlett 1959; Wagner et al. 1962). Liposomes were always prepared shortly before measurement. Brief summary of the method: An aliquot part of phospholipid solution containing less than 20 mg of phospholipid was introduced into a 20 ml vial and the liquid was completely evaporated from the solution by nitrogen stream at temperature about 40 °C. The vial was then placed in vacuum for at least one hour to remove residual solvent. After releasing the vacuum, 2 ml of buffer was added; the vial was filled with nitrogen, closed and incubated at 50 °C for 5 min. The sample was agitated until all lipid was removed from the walls of the vial and a homogeneous milky-white suspension arose. The vial was shortly sonicated (for 5 to 15 s) in an XL 2020 Sonicator (Misonix, Inc.) to release all lipids from the walls and to break down large clusters. Following incubation of the sample at 50 °C for 30 min, the suspension was left at room temperature for further 2 h and diluted by buffer solution to the required phospholipid concentration.

Radioligand binding assay

Binding equilibrium was studied by the radioligand binding assay (Bennett and Yamamura 1985). Tritium-labelled IMI was used as marker. Non-labelled (cold) IMI was used in the form of hydrochloride. Typically, liposome suspension was mixed with buffer and [^{3}H]IMI, and the sample was incubated at 20 °C, pH 7.4, for 30 min. The usual final phospholipid concentrations were 50–270 μmol/l for PS vesicles, 270–540 μmol/l for PC-MLVs, with the resultant concentration being about 5 nmol/l of [^{3}H]IMI. To separate bound and free ligand, centrifugation or rapid filtration were employed and total binding was measured. The specific binding of TCA to liposomes was calculated as the difference between the total and nonspecific binding, with nonspecific binding determined in the presence of excess cold ligand (50 μmol/l). Samples were measured in a scintillation cocktail using an LS 6000IC liquid scintillation counter (Beckman Instruments, Inc.).

Centrifugation

When working with biological or model membranes, the most common procedure to determine binding parameters or partition coefficients is centrifugation and determination of the solute remaining in the aqueous phase. The total volume of samples was typically 0.8 ml; 0.2 ml of the sample was removed after incubation to measure the total drug concentration and 0.6 ml was centrifuged either at 30,000×g at 20 °C for 15 min, or at 100,000 × g at 20 °C for 60 min; subsequently, 0.2 ml of supernatant was removed for free (bulk) ligand concentration determination.

The separation of liposomes from aqueous phase is not complete when using centrifugation. The amount of lipid vesicles remaining in the supernatant was determined using tritium-labelled phospholipids as markers. In more detail,

a trace amount (2 μl) of [³H]PC or [³H]PS was added to PC or PS in the chloro-form/methanol solvent mixture and lipid vesicles were prepared by the standard procedure. The percentage of liposomes in the pellet was determined. The amount of liposomes remaining in the supernatant strongly depends on duration of the centrifugation and rpm. We found that the percentage of phospholipid in pellet was $69 \pm 8\%$ ($n = 7$) for PC-MLVs and $54 \pm 9\%$ ($n = 7$) for PS vesicles, when samples were centrifuged at $30,000 \times g$ at $20\,°C$ for 15 min. When samples were centrifuged at $100,000 \times g$ at $20\,°C$ for 60 min, only 9.6% of PC-MLVs and 5.5% of PS-MLVs remained in the supernatant. In any case, a correction must be involved when the molar ratio of bound IMI to the molar amount of phospholipid (B_{IMI}) was calculated.

Filtration

Rapid filtration provides a very fast and efficient separation bound and free ligand. The filters used should maximally entrap particles, minimally bind the free radioli-gand and have a high permeability for the rinsing solution. We used Whatman glass microfiber filters (GF/F type) impregnated previously in 0.1% polyethyleneimine (PEI) (Sigma) to reduce nonspecific binding of radioligands to GF/F filters. Sam-ples were filtered and filters were washed rapidly three times with 3 ml of ice-cold buffer and, after adding the scintillation cocktail, they were assayed on a scin-tillation counter. We established that the washing procedure used is sufficient to remove the most part of unbound [³H]IMI, since repeated washing did not decrease the activity trapped on the filter. The measured activities of filters were corrected for the proportion of liposomes that pass through a GF/F filter.

Permeability of the GF/F filters for liposomes was tested using tritium-labelled phospholipids as markers. We found that $80\pm11\%$ ($n = 23$) of PC-MLVs or $82\pm5\%$ of PS vesicles ($n = 23$) was retained on GF/F filters impregnated in 0.1% PEI. The amount of MLVs trapped on filters did not depend on repeated washing of the filters.

The total duration of filtration and washing was about 10 s; it can be cal-culated that the filtration rate used allows correct determination of high-affinity binding processes with K_d of order 10^{-8} mol/l or less (Bennett and Yamamura 1985). The rapid, easy and efficient separation of free ligand is an advantage of the filtration technique, but the technique cannot be used for low-affinity binding processes ($K_d > 10^{-8}$ mol/l).

The percentage of radioligand bound to filters increases progressively at con-centrations below 4 nmol/l of [³H]IMI, regardless of whether cold IMI is present or not. Within the range of 5–30 nmol/l [³H]IMI, the percentage of [³H]IMI bound to filters was virtually constant ($0.67 \pm 0.18\%$, $n = 20$, $20\,°C$, pH 7.4). A higher per-centage of [³H]IMI binding to GF/F filters at very low radioligand concentrations (<4 nmol/l) could potentially distort the saturation curves and identification of high-affinity binding sites by K_d in the sub-nanomolar region. This explains why both total binding and displaceable binding, calculated as the difference of filter

activities with a sample incubated without and with excess cold IMI (50 μmol/l), were determined.

Data analysis

Binding model

Three regions are distinguished in the simplified model describing binding of an amphiphilic drug to a membrane (Seelig et al. 1993): (i) the bulk solution, (ii) the interface (aqueous layer immediately above the plane of binding), and (iii) the membrane. Both the protonated and unprotonated forms of the drug may be present in these regions. The fraction of the protonated IMI, F, depends on pH (Seelig et al. 1993) as follows:

$$F = (1 + 10^{\text{pH-p}K})^{-1} \tag{1}$$

where pK is the negative logarithm of the dissociation constant for the N-terminal amino group of IMI. It is well known that pK values in lipid membrane differ from those in the bulk solution (Cevc 1990; Miyazaki et al. 1992). The thermodynamic factors giving rise to shifts in pK also cause a difference in partitioning between the protonated and unprotonated forms of the amphiphile.

Analysis of binding isotherms

Analysis of binding isotherms of IMI follows the approach described for adsorption of monovalent cations (Eisenberg et al. 1979) or for binding of local anaesthetics (Lee 1978; Miyazaki et al. 1992), small peptides (Beschiaschvili and Seelig 1990; Seelig et al. 1993, 1996, 2000; Ziegler et al. 2003) and amphiphilic drugs (Bäuerle and Seelig 1991; Thomas and Seelig 1993) to lipid bilayers.

Membrane surface with an electric charge density σ gives rise to an electrostatic surface potential ψ_0. The relation between the two parameters is provided by the GC approximation, which was obtained from the Poisson equation provided that all structural membrane charges are confined to an infinitely narrow plane and that the ion distribution is governed solely by coulombic forces (McLaughlin and Harary 1976; Cevc 1990). The GC model predicts the electrostatic potential at the membrane surface to be

$$\psi_0 = (2kT/Ze)\text{arcsinh}\,(Ze\sigma\lambda/2\varepsilon\varepsilon_0 kT) \tag{2}$$

for symmetrical electrolytes with counter-ions and co-ions of similar valency, Z. ε is the dielectric constant of water, ε_0 is the permittivity of free space, k is the Boltzmann constant, λ is the Debye screening length, σ is the surface charge density, T is the temperature, and e is the elementary charge. The Debye length (in meters) relates ψ_0 to concentrations of ions in the bulk aqueous phase, and can be calculated from the following equation (Cevc 1990):

$$\lambda = [\varepsilon\varepsilon_0 kT/(10^3 N_{\text{A}}e^2 \sum(Z_i^2 c_i))]^{1/2} \tag{3}$$

where N_A is the Avogadro number and the summation goes over all ionic species of valencies Z_i and concentrations c_i (in mol/l). The surface charge density is defined as

$$\sigma = e/A_c \tag{4}$$

where A_c is the area *per* charge (in nm^2).

The effect of the surface potential is to change the ion concentration at the membrane-water interface (Seelig et al. 2000). If coulombic interaction is the only one included in the mean force potential, one obtains the ion concentration profile in GC approximation. The relation between the membrane active concentration of monovalent cations in the aqueous phase at the membrane, C_0, and the bulk equilibrium concentration, C_{eq}, is given by the Boltzmann equation

$$C_0 = C_{eq} \exp(-ze\psi_0/(kT)) \tag{5}$$

where z is the effective charge of adsorbed species.

The binding process is characterized by the transition of the drug from the layer of concentration C_0 into the membrane phase. Partitioning of cationic drugs into electrically neutral PC membrane gives rise to a positive surface potential of the lipid layer and the concentration of the cationic drug at the membrane surface decreases ($C_{eq} > C_0$). The negatively charged PS bilayers exhibit a negative surface potential that leads to accumulation of cationic drugs in the vicinity of the membrane surface ($C_{eq} < C_0$). Binding of amphiphilic drugs is thermodynamically driven by enthalpy and entropy changes and can be described by the Langmuir binding isotherm. Due to the ion concentration profile in the vicinity of the charged membrane surface, the degree of association is determined by the interfacial rather than by the bulk ion concentrations and the classical Stern adsorption isotherm is obtained (McLaughlin and Harary 1976; Cevc 1990). A description common in ligand-receptor studies (Bennett and Yamamura 1985) was used in our study

$$B = B_{max}C_0/(C_0 + 1/K) \tag{6}$$

where C_0 is the concentration of ligand in the aqueous phase at the membrane, B is the amount of drug in moles bound to mole of phospholipid at free drug concentration equal to C_0, B_{max} is the maximum amount of drug bound *per* mole of phospholipid (mol/mol), and K is the equilibrium association constant ($1/K = K_d$ is the dissociation constant) of the solute. The ratio B/B_{max} is the degree of association. The reciprocal value of B_{max} can be interpreted as the number of lipid molecules creating a specific binding site for IMI; however, it is a physically unrealistic picture of drug binding (Beschiaschvili and Seelig 1990; Ziegler et al. 2003). Some authors set the maximum number of binding sites equal to the number of lipid molecules (Lee 1978), i.e. $B_{max} = 1$.

The apparent binding constant, K_{app}, and the apparent binding capacity, $B_{max,app}$ can be determined using the bulk IMI concentration (rather than C_0) in Eq. (6)

$$B = B_{max,app}C_{eq}/(C_{eq} + 1/K_{app}) \tag{7}$$

Binding of solutes to membranes can be analyzed in terms of either the binding or partitioning formalism. The partition coefficient formalism coincides with the linear part of the binding isotherm and some authors use the partition coefficients for a simplified description of ligand binding to membrane surface (Mason et al. 1989; Beschiaschvili and Seelig 1990; Seelig et al. 2000). The surface partition equilibrium can be formulated as

$$B = k_{\mathrm{p}}C_0 \tag{8}$$

where k_{p} is the hydrophobic partition coefficient. If the GC approximation is not considered, then

$$B = k_{\mathrm{p,app}}C_{\mathrm{eq}} \tag{9}$$

where $k_{\mathrm{p,app}}$ is the apparent overall partition coefficient.

Parameters of the binding isotherms (Eqs. (6) and (7)) were determined by nonlinear regression analysis (AccuFit Saturation Two-Site software, Beckman). Displacement curves were analyzed using the four-parametric logistic model (ImmunoFit EIA/RIA software, Beckman) to establish the values of cold ligand concentrations displacing 50 % of bound radioligand (IC_{50}).

Data is expressed as arithmetic mean $\pm$ standard deviation (S.D.). Mann–Whitney U-test (a nonparametric alternative to the t-test for independent samples) was used to determine the statistical significance of differences between means.

Results

Permeability of phospholipid bilayers

The purpose of the first experiment was to find whether multilamellar liposomes can be used as a model for determination of quantitative parameters of IMI binding to the lipid part of biological membranes. Problems in the determination of drug partition between liposomes and aqueous phase could arise due to the fact that only a small part of lipid bilayers in MLVs is exposed to the surrounding environment, because inner lamellae are confined by outer lipid bilayer(s).

The same technique was employed to prepare both PC and PS vesicles. However, because of the differences between the two phospholipid species (polar head size and charge), PC and PS liposomes are not the same. Unsonicated PC bilayers are characteristic by multilamellar packing. The fundamental structure of charged lipids dispersed in water is the unilamellar vesicle; and potential smaller particles entrapped in larger vesicles are also unilamellar (Hauser 1984). PS bilayers alone are very similar to those formed by other phospholipids, with only the headgroup segments being significantly different (Browning and Seelig 1980). It is therefore due to ask whether the lipid bilayers in PC or PS vesicles are sufficiently permeable for IMI. The problem was solved by comparison of fractions of bound IMI in three different samples differing by the time of addition of [³H]IMI to the sample (Fig. 1). Binding was measured using both filtration and centrifugation to separate bound and free [³H]IMI, and the percentage of bound ligand was calculated. The samples
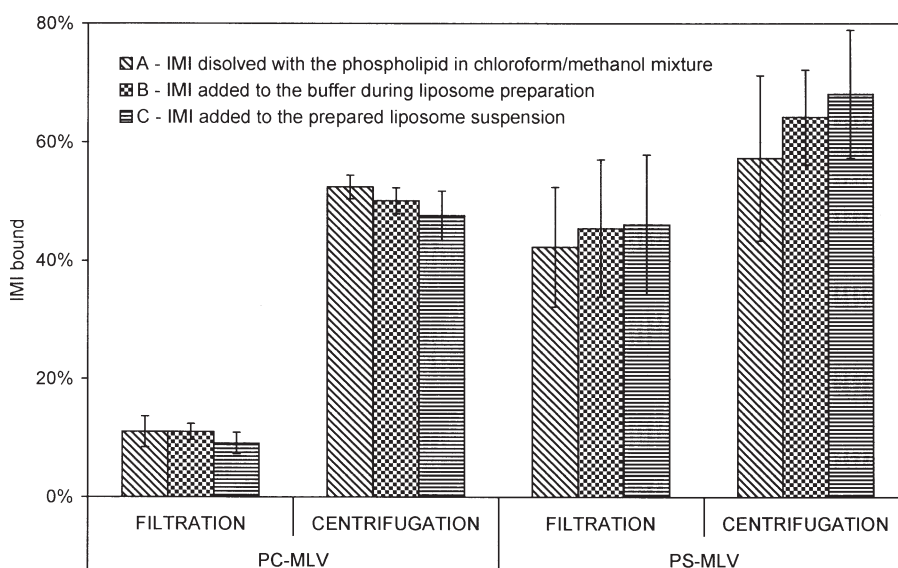
Figure 1. Binding of [³H]IMI to PC-MLVs and PS vesicles using filtration or centrifugation to separate bound and free radioligand. [³H]IMI was added to phospholipids in different steps of liposome preparation: (A) to chloroform/methanol solvent mixture prior to preparation of MLVs; (B) to the dried phospholipid film along with buffer; and (C) to the ready-to-use MLVs. Final concentrations were 1.33 mmol/l of PC, 0.27 mmol/l of PS and 7.8 nmol/l of [³H]IMI. Activities of samples were determined following a 30 min incubation period at 20 °C. Values are means ± S.D. ($n = 5$).

were measured in doublets. We found that the fraction of IMI bound to both PC and PS vesicles does not depend on the moment of [³H]IMI addition. The mean fraction of IMI bound to PC-MLVs equals $10.4 \pm 2.2\%$ ($n = 5$) when using the filtration separation, and $50.1 \pm 3.6\%$ when using centrifugation. The fraction of IMI bound to PS vesicles is significantly higher compared to PC-MLVs and amounts to $45 \pm 11\%$ when using filtration and $63 \pm 12\%$ when using centrifugation. These values are valid at our experimental conditions, i.e. 1.33 mmol/l PC or 0.27 mmol/l PS, 7.8 nmol/l [³H]IMI, 20 °C and pH 7.4.

We proved that a 30 min incubation at 20 °C is sufficient to restore equilibrium, i.e., a uniform distribution of IMI within liposomes and binding of IMI to both outer and inner lipid bilayers (Fig. 1). The implication is that repeated freeze-thaw cycles are not necessary in our experiments. A good agreement of the degree of binding of an amphiphilic drug to PC vesicles subjected and not subjected to several freeze-thaw cycles has been obtained previously (Bäuerle and Seelig 1991). As a result, the higher IMI binding to PS liposomes compared to PC-MLVs cannot be explained by a different availability of inner lipid bilayers. Sample filtration followed by rapid washing leads to removal of weakly bound molecules. Therefore,
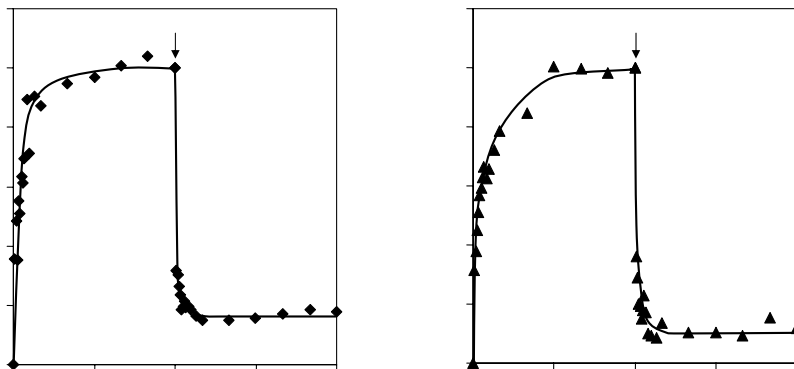
Figure 2. The time course of [³H]IMI association with liposomes prepared from (A) PC and (B) PS, and the dissociation following the addition of 50 μmol/l cold IMI at minute 60, determined at 20 °C, pH 7.4, at 4 nmol/l of [³H]IMI and phospholipid concentration of 0.66 mmol/l (PC) or 0.066 mmol/l (PS). Bound and free radioligand were separated by filtration and binding of [³H]IMI to the filter (equal to 1.1 % of total [³H]IMI) was subtracted from the total activity of the filter. Mean values of three (PC) or eight (PS) repetitions of the experiment are shown.

the filtration technique was found not to be useful for measurement of total binding of drugs to liposomes.

The period required to achieve dynamic equilibrium between IMI in the aqueous phase and IMI bound to liposomes was determined in the next experiment as follows: The time course of [³H]IMI association with liposomes and of dissociation following addition of excess cold IMI is shown on Fig. 2. We found that the half-time of association of IMI with MLVs was 124 s ($n = 6$) for PC-MLVs and 108 s ($n = 9$) for PS vesicles. The equilibrium was fully restored during 30 min both for PC and PS-MLVs. The dissociation was started by addition of 50 μmol/l cold IMI. The half-time of dissociation of IMI from the MLVs was approximately 17 s for PC-MLVs and 6 s for PS-MLVs. The same results were found when using unlabelled CMI as displacement agent instead of IMI.

Total binding of IMI to liposomes

The isotherms of IMI binding/adsorption to neutral PC bilayers and to negatively charged PS bilayers were measured in the total IMI concentration range of 0.001 to 270 μmol/l (binding to PC) or 0.001 to 400 μmol/l (binding to PS) using centrifugation. The binding parameters were calculated by combination of Eqs. (2), (5), and (6) (Stern equation). The effect of both IMI and monovalent cations on the surface potential ψ_0 was considered (Fig. 3).
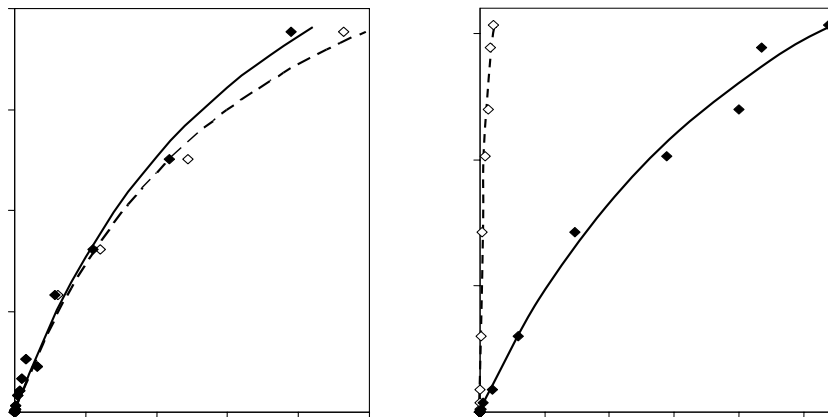
Figure 3. Binding of IMI to bilayer membranes composed of PC (A) or PS (B). The molar amount of IMI bound *per* mole of total lipid, denoted B_{IMI} (mmol/mol), is plotted versus the equilibrium concentration of IMI, denoted C (μmol/l), free in solution (empty symbols $\diamond$). The same data are plotted against the interfacial IMI concentration (filled symbols $\blacklozenge$) calculated using Eq. (5), i.e., taking into account electrostatic effects by means of the Gouy–Chapman (GC) theory. The dashed line is the theoretical binding curve calculated from Eq. (7) ($B_{\mathrm{max,app}} = 31$ mmol/mol, $K_{\mathrm{app}} = 6077$ l/mol for PC and $B_{\mathrm{max,app}} = 384$ mmol/mol, $K_{\mathrm{app}} = 8190$ l/mol for PS). The solid line is the theoretical binding curve calculated from Eq. (6) with binding parameters ($B_{\mathrm{max}} = 35$ mmol/mol, $K = 5596$ l/mol for PC and $B_{\mathrm{max}} = 608$ mmol/mol, $K = 93$ l/mol for PS) taking into account electrostatic effects by means of the GC theory and also Na^+ ($K_{\mathrm{Na+}} = 0.6$ M^{-1}) and K$^+$ ($K_{\mathrm{K+}} = 0.15$ M^{-1}) binding to PS in buffer (0.12 mol/l NaCl, 0.01 mol/l KCl, 0.03 mol/l Tris, pH 7.4, 20 °C).

Normally, inorganic monovalent cations do not bind to uncharged phospholipids such as PC (Eisenberg et al. 1979). The potential at the surface of a PC-vesicle in solution (containing 0.12 mol/l NaCl, 0.01 mol/l KCl and 0.03 mol/l Tris-HCl) should be 0 mV. The charged PS headgroups are assumed to be binding sites for Na$^+$ or K$^+$. Sodium and potassium binding to PS headgroups follows a Langmuir adsorption isotherm (at $B_{\mathrm{max}} = 1$); consequently, the mole fraction of these ions bound to PS, B_{NaK}, can be calculated as (Beschiaschvili and Seelig 1990):

$$B_{\mathrm{NaK}} = (K_{\mathrm{Na}}C_{\mathrm{Na}} + K_{\mathrm{K}}C_{\mathrm{K}})/(1 + K_{\mathrm{Na}}C_{\mathrm{Na}} + K_{\mathrm{K}}C_{\mathrm{K}}) \qquad (10)$$

where C_{Na} and C_{K} are concentrations of Na$^+$ and K$^+$. The representative ion-binding constants (intrinsic association constants) for PS membranes were $K_{\mathrm{Na}} = 0.6$ l/mol for Na$^+$ and $K_{\mathrm{K}} = 0.15$ l/mol for K$^+$ (Eisenberg et al. 1979; Cevc 1990). We determined $B_{\mathrm{NaK}} = 0.0685$ mol/mol in our experiment.

The surface charge density (Eq. (4)) of PC and PS vesicles in a buffer containing Na^+, K^+ and IMI can be written as (Beschiaschvili and Seelig 1990; Stankowski 1991):

$$\sigma = (e/A_L)[-B_{PS}(1 - B_{NaK}) + z_{IMI}B_{IMI}]/(1 + B_{IMI}A_{IMI}/A_L) \qquad (11)$$

where A_L is the surface area of lipid, A_{IMI} is the surface area of IMI, B_{PS} is moles of PS *per* mole of total lipid ($B_{PS} = 0$ for PC liposomes), B_{IMI} is the surface concentration of IMI defined as moles of IMI bound *per* mole of lipid, z_{IMI} is the effective charge of bound IMI.

In the present study, we measured total binding of IMI to lipid membranes using centrifugation for separation of bound and free ligand at 20 ℃ and pH 7.4; i.e., we did not resolved binding of the charged and uncharged forms of IMI. Therefore, the proportion of charged IMI molecules bound to membranes was calculated according to previously published data. Since the pK of the IMI amino group is 9.4, it follows from Eq. (1) that about 99.0 % of IMI in bulk solution bear positive charge. The interfacial pK shifts of different tertiary amine drugs vary somewhat, but have a mean value of Δp$K = -0.95$ (Miyazaki et al. 1992). Assuming that the interfacial pK shift of IMI is similar to that in tertiary amine local anaesthetic analogues, 91.8 % of IMI bound to the membrane carry a positive charge, i.e. the effective charge, z_{IMI}, of the N-terminal amino group is about $+0,92$.

In model membranes, IMI is preferably localized with its charged side-chain near phospholipid polar head groups; the nonpolar tricycle ring system of IMI is incorporated into the acyl chain (hydrophobic) region of the lipid bilayer (Bauer et al. 1990; Freisleben and Zimmer 1991). Therefore, a correction for membrane expansion due to IMI penetration into the bilayer was included. The lateral packing densities and molecular areas of charged and uncharged lipids in the fluid phase are approximately the same; the lipid cross sectional area of $A_L = 0.68$ nm^2 (Beschiaschvili and Seelig 1990; Cevc 1990; Ziegler et al. 2003) was assumed in all our calculations. A considerable flexibility of the TCA molecules, both in the side chain and in the ring system (Heimstad et al. 1991) has been demonstrated; we estimated for IMI that $A_{IMI} \approx 0.5$ nm^2. Since loading of membranes with the drug was rather low ($B_{IMI} \leq 0.2$), the accuracy of the value of A_{IMI} is not critical and σ can be evaluated using Eq. (11).

The surface charge density σ gives rise to electrostatic potential at the membrane surface, ψ_0, which can be calculated using Eqs. (2) and (3). The Debye length (Eq. (3)) was determined ($\lambda = 0.83$ nm at concentration of monovalent electrolyte in the bulk aqueous phase equal to 0.13 mol/l, 20 ℃) and surface potentials of PS or PC vesicles were calculated (Eq. (2)) both in absence of IMI and in presence of different IMI concentrations. Finally, the concentrations C_0 (Eq. (5)) were determined; the data was evaluated using Eq. (6), and parameters B_{max} and K were calculated. The results are summarized in Fig. 3 and in Tables 1 and 2. Experimental data (empty symbols in Fig. 3) deviate markedly from linearity, with B_{IMI} being smaller or bigger than predicted by the GC theory (filled symbols in

Table 1. Binding of imipramine to phosphatidylcholine membranes

C_{eq} (μmol/l)	B_{IMI} (mmol/mol)	σ (μC/m^2)	ψ_0 (mV)	C_0 (μmol/l)	k_p (l/mol)	$k_{p,app}$ (l/mol)
0	0	0	0	0		
0.0008	0.0012	0.00027	0.00032	0.0008	1602	1602
0.0014	0.0016	0.00036	0.00043	0.0014	1214	1214
0.0019	0.0020	0.00043	0.00052	0.0019	1037	1037
0.0023	0.0021	0.00046	0.00056	0.0023	940	940
0.0039	0.0036	0.00078	0.00094	0.0039	931	931
0.0069	0.0058	0.0013	0.0015	0.0069	844	844
0.013	0.011	0.0024	0.0029	0.013	836	836
0.026	0.014	0.0030	0.0036	0.026	527	527
0.052	0.032	0.0070	0.0084	0.052	614	613
0.10	0.049	0.011	0.013	0.10	474	474
0.16	0.057	0.012	0.015	0.16	362	362
0.27	0.11	0.024	0.028	0.27	407	406
0.48	0.13	0.029	0.035	0.48	280	280
0.88	0.34	0.074	0.089	0.88	386	385
2.0	0.81	0.18	0.21	2.0	410	407
3.7	1.1	0.23	0.28	3.7	291	288
5.4	1.7	0.36	0.44	5.3	314	309
8.0	2.6	0.57	0.68	7.8	337	329
16	2.3	0.49	0.59	16	144	141
30	5.8	1.2	1.5	29	202	191
60	8.1	1.7	2.1	56	145	134
122	12.5	2.7	3.2	109	115	102
232	18.8	4.0	4.8	195	97	81

PC liposomes were incubated in buffer (pH 7.4) at 20 °C for 30 min with different IMI concentrations. The final volume of samples was 0.8 ml, and the final concentrations was 0.36 mmol/l of PC; 0.7–20 nmol/l of [^{3}H]IMI was used as marker. 0.2 ml of the sample was removed following incubation to measure the total sample activity and 0.4 ml was centrifuged (30,000 × g, 20 °C, 15 min) and the activity of 0.2 ml of the supernatant was subsequently measured. C_{eq}, the equilibrium IMI concentration in the bulk aqueous phase; B_{IMI}, the amount of IMI in milimoles bound *per* mole of PC; σ, the electric charge density (Eq. (11)); ψ_0, the electrostatic surface potential (Eq. (2)); C_0, the concentration of IMI in the aqueous phase at the PC membrane (Eq. (5)); k_p, the hydrophobic partition coefficient (Eq. (8)); $k_{p,app}$, the apparent overall partition coefficient (Eq. (9)). The samples were measured in doublets. Mean values were determined from 4 independent measurements.

Fig. 3), indicating that there is either an electrostatic repulsion of IMI as the PC membrane surface becomes positively charged, or electrostatic attraction of IMI as the PS bilayer is negatively charged.

"High-affinity" binding of TCAs to liposomes

Saturation experiment

The purpose of the following experiment was to determine the binding parameters

Table 2. Binding of imipramine to phosphatidylserine membranes

C_{eq} (μmol/l)	B_{IMI} (mmol/mol)	σ (μC/m^2)	ψ_0 (mV)	C_0 (μmol/l)	k_p (l/mol)	$k_{p,app}$ (l/mol)
0	0	-219	-119	0		
0.0002	0.0029	-219	-119	0.017	169	13008
0.0005	0.0038	-219	-119	0.037	103	7884
0.0008	0.0071	-219	-119	0.065	109	8392
0.0012	0.0097	-219	-119	0.093	104	7977
0.0016	0.013	-219	-119	0.13	103	7893
0.0021	0.017	-219	-119	0.16	109	8381
0.0029	0.027	-219	-119	0.22	119	9169
0.0040	0.028	-219	-119	0.31	89	6870
0.0060	0.040	-219	-119	0.46	86	6632
0.0078	0.043	-219	-119	0.60	72	5510
0.012	0.056	-219	-119	0.89	62	4798
0.054	0.22	-219	-119	4.1	54	4170
0.15	1.0	-219	-119	12	90	6902
0.44	2.5	-218	-119	33	74	5659
1.5	7.3	-216	-118	110	66	4974
5.4	18	-212	-117	392	45	3245
19	60	-195	-113	1174	51	3167
64	142	-165	-105	2931	49	2237
156	203	-146	-99	5782	35	1297
248	239	-135	-95	8014	30	966
323	289	-122	-90	8692	33	894
428	307	-117	-89	10766	29	716

PS liposomes were incubated in buffer (pH 7.4) at 20 °C for 30 min with different IMI concentrations. The final volume of samples was 0.8 ml, and the final concentrations was 0.26 mmol/l of PS; 0.7–20 nmol/l of [^{3}H]IMI was used as marker. Symbols for quantities have the same meaning as in Table 1. The samples were measured in doublets. Mean values were determined from 7 independent measurements.

characterizing strong ("high-affinity") binding of IMI to PC or PS membranes. PC and PS liposomes were incubated with 20 different [^{3}H]IMI concentrations. The final volume of samples was 0.25 ml; the final concentration was about 0.23 mmol/l of phospholipid. The binding experiment started by addition of [^{3}H]IMI in concentration of 0.7–60 nmol/l, and the samples were incubated at 20 °C, pH 7.4, for 30 min. Rapid filtration was used to separate bound and free ligand. Nonspecific binding was measured at the same experimental conditions, but excess cold ligand (50 μmol/l) was added before [^{3}H]IMI. The samples were measured in doublets. Displaceable binding was used to determine the binding parameters B_{max} and K_d using Eqs. (6) or (7).

The same method was used to determine the binding of different tritium-labelled TCA ([^{3}H]IMI, [^{3}H]DMI, [^{3}H]DDMI, [^{3}H]AMI) to liposomes prepared from crude lipid extract from white matter of bovine brain. The results are summarized

Table 3. Parameters of "high-affinity" binding of tricyclic antidepressants to lipid bilayers prepared from PC, PS, or from the total lipids from white matter of bovine brain

lipid	ligand	K_d (nmol/l)	B_{max} (μmol/mol)	$K_{d,app}$ (nmol/l)	$B_{max.app}$ (pmol/mg)	n
PC	IMI	9.8 ± 3.6	3.1 ± 2.0	9.8 ± 3.6	4.0 ± 2.6	10
PS	IMI	1262 ± 568	34.5 ± 9.5	16.4 ± 7.4	45.3 ± 12.4	10
crude lipid extract	IMI DMI DDMI AMI			20.0 ± 10.0 28.9 ± 3.9 19.3 ± 10.4 26.6 ± 2.8	3.3 ± 2.1 4.0 ± 1.0 3.4 ± 2.5 5.1 ± 2.1	3 3 3 3

Isothermal saturation curves were measured following incubation of liposomes composed of phoshatidylcholine, PC, phosphatidylserine, PS, and of crude lipid extract from white matter of bovine brain with tritium labelled imipramine, IMI, desipramine, DMI, didesmethylimipramine, DDMI, and amitriptyline, AMI, at 20 °C, pH 7.4, for 30 min. The final volume of samples was 0.25 ml, the final phospholipid concentrations was about 0.23 mg/ml, and the radioligand concentration was between 0.7 and 60 nmol/l. Filtration was used to separate bound and free radioligand. The samples were measured in doublets and nonspecific binding was deducted. Binding to PC and PS membranes was evaluated using the GC theory, and the dissociation constant, $K_d = 1/K$ (nmol/l) and the amount of IMI in micromoles bound *per* mole of phospholipid, B_{max}, were determined from Eq. (6). The apparent dissociation constant, $K_{d,app} = 1/K_{app}$ (nmol/l) and the apparent binding capacity, $B_{max.app}$, in picomoles of TCA bound *per* milligram of lipid, were calculated using Eq. (7) to characterize binding to liposomes prepared from lipid mixture. Values are reported as mean $\pm$ S.D.; the means were calculated from n values. Both K_d and B_{max} were found to be significantly higher in PS membranes than in PC membranes; as determined by Mann–Whitney U-test.

in Table 3. No statistically significant differences were found between individual antidepressants.

Competition curves

Competition curves were produced by incubating liposomes at a fixed concentration of [3H]IMI with increasing concentrations of unlabelled ligand. The concentration of unlabelled ligand displacing 50 % of [3H]IMI binding (half-maximum displacement, IC_{50}) was determined and its relation to phospholipid concentration was studied. We tested the efficiency of unlabelled IMI, CMI or DMI to displace [3H]IMI. The same procedure was used to determine IC_{50} at different [3H]IMI concentrations (1.25–18 nmol/l) or different phospholipid concentrations (0.23–1.06 mmol/l of PC, 0.013–0.23 mmol/l of PS).

We found that major part of IMI binding to MLVs is displaceable and IC_{50} values are practically the same when using different TCAs as competitors. An increase in IC_{50} values from 0.20 μmol/l to 3.4 μmol/l was found at the concentrations range of 0.23–1.06 mmol/l of PC, and IC_{50} increased from 0.22 μmol/l to
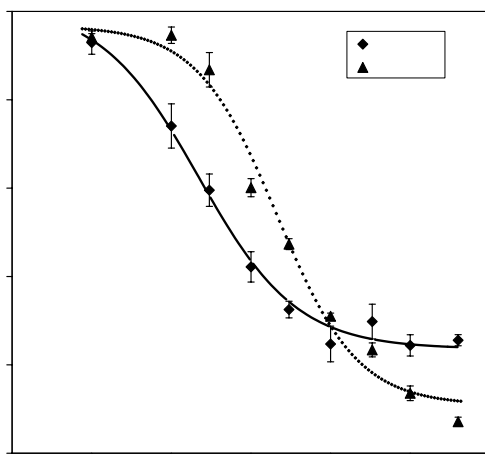
Figure 4. Inhibition of [³H]IMI binding by clomipramine (CMI). Binding was determined in liposomes prepared from PC or PS. The final volume of samples was 0.25 ml, the final concentrations was 0.23 mmol/l of lipid, 5 nmol/l of [³H]IMI and 0–400 μmol/l of unlabelled CMI. The binding was started by the addition of [³H]IMI and samples were incubated at 20 °C, pH 7.4 for 30 min. Rapid filtration was used to separate bound and free ligand. The figure shows a typical experiment repeated four times. S.D. of the means ranged between 0.7 and 5.0 %. The IC_{50} calculated under these conditions was 0.20 μmol/l of CMI for PC liposomes and 2.4 μmol/l of CMI for PS liposomes.

2.4 μmol/l at the concentration range of 0.013–0.23 mmol/l of PS. No significant dependence of IC_{50} on [³H]IMI concentration was observed and no marked differences in displacement efficiencies of IMI, CMI, and DMI were observed. Sample displacement curves are shown on Fig. 4 (0.23 mmol/l of PC or PS and 5 nmol/l of [³H]IMI, with CMI used as a competitor to displace [³H]IMI binding).

Discussion

Equilibrium binding of IMI to PC and PS membranes was studied by a radioligand binding assay, using centrifugation to separate bound and free ligand. Binding of IMI was described in terms of surface partition equilibrium and a partition constant (Eqs. (8), (9)), or using the parameters of the Langmuir or Stern adsorption isotherms (Eqs. (6), (7)). Electrostatic effects were taken into account by means of the GC theory (Cevc 1990; Stankowski 1991).

In the present analysis, the use of interfacial concentrations rather than bulk concentrations of IMI did not lead to a simple partition model (Eq. (8)) for IMI binding. The last two columns of Tables 1 and 2 demonstrate that the B_{IMI}/C_0

and $B_{\mathrm{IMI}}/C_{\mathrm{eq}}$ ratios vary considerably even in the low concentration range. Thus, binding of IMI to PC or PS membranes cannot be described by a simple surface partition equilibrium over the whole concentration range measured. The concentration dependence of the partition coefficients (k_{p}, $k_{\mathrm{p,app}}$) can not be explained by self-association of IMI in solution, because the critical micellar concentration of IMI is 47 mmol/l (Schreier et al. 2000). The concentration dependence of partitioning at high IMI concentrations (>10 μmol/l) can be attributed to the low lipid-to-IMI molar ratio; however, this does not explain the changes in the partition coefficients at low IMI concentrations.

The hydrophobic partition coefficients were found to be significantly higher in PC membranes compared to PS membranes in the whole concentration range (Tables 1 and 2); this finding can be interpreted as better accessibility of the hydrophobic core of PC bilayers to IMI. The apparent partition coefficients determined for PC membranes were in good agreement with the hydrophobic partition coefficients calculated using the GC theory (in particular, at low IMI concentrations). Hence, the effect of bound IMI on the surface charge of PC membranes is negligible at IMI concentrations below 1 μmol/l. To the contrary, the apparent partition coefficients for PS membranes were found to be much greater than the hydrophobic coefficients. These results show that the electrostatic interaction between IMI and PS membrane is prevailing; however, the interaction is not purely electrostatic in nature and van der Waals forces and hydrophobic effects contribute to the total binding.

The parameters of the Stern adsorption isotherm, B_{max} and K, (Eq. (6)) provide a better description of IMI binding to lipid membranes than a simple partition coefficient. A significantly higher binding capacity and a lower association constant were found for binding to PS vesicles ($B_{\mathrm{max}} = 608$ mmol/mol, $K = 93$ l/mol) compared to binding to PC liposomes ($B_{\mathrm{max}} = 35$ mmol/mol, $K = 5596$ l/mol). Therefore, nature of the phospholipid strongly affects the drug partition. Both a higher $B_{\mathrm{max,app}}$ and a higher K_{app} were found for binding to PS vesicles (384 mmol/mol, 8190 l/mol, respectively) compared to PC membranes (31 mmol/mol, 6077 l/mol, respectively). This demonstrates the role of the surface charge on PS membranes in the binding process and the necessity of application of interfacial (rather than bulk) cationic drug concentration in analyses of binding to negatively charged lipid bilayers.

In simple terms, the reciprocal value of B_{max} could be interpreted as the number of lipid molecules constituting a "specific" IMI binding site in PC membranes ($1/B_{\mathrm{max}} = 28.6$) or PS membranes ($1/B_{\mathrm{max}} = 1.6$). In the first model, i.e. in the terms of receptor binding studies, our results can be interpreted by referring to low-affinity binding sites in the lipid bilayer. The second model is based on a non-specific electrostatic accumulation of IMI near the membrane surface followed by hydrophobic adsorption. Such electrostatic/chemical partition model is physically more realistic (Ziegler et al. 2003).

The lipid binding constants are small compared to receptor binding constants, which are of order 10^{8}–10^{10} l/mol. However, assuming that each receptor molecule

is surrounded by some 10^6 lipid molecules, the product of "number of lipids" and "binding capacity" and "binding constant" is close to a receptor binding constant. Under these conditions, a significant proportion of IMI could be bound to lipid bilayer and thus become lost for receptor binding if the receptor binding site was accessible from the aqueous phase (Heirwegh et al. 1992; Seelig et al. 1993). This effect can be compensated by the presence of negatively charged groups in the vicinity of the receptor binding site. If the receptor binding site was localized in the hydrophobic part of the lipid bilayer, the binding parameters would be calculated using the intramembrane drug concentration rather than its concentration in the aqueous phase (Heirwegh et al. 1992).

To discover a potential interference between IMI binding to lipid bilayer and to a specific protein binding site in a biological membrane, we assayed the "high-affinity" part of the total IMI binding to PC or PS membranes in more detail. Filtration and nanomolar IMI concentrations (corresponding to free IMI in the plasma at therapeutically active drug concentrations) were used in a set of experiments designed in analogy with the procedures used in receptor binding studies (saturation experiments, time course of association and dissociation, displacement of ligand bound). Accumulation of IMI molecules in the vicinity of negatively charged PS membranes was taken into account using the GC theory.

We found that the filtration technique can yield misleading results, because pure PC membranes show a saturable displaceable (specific) high-affinity binding ($B_{\max} = 3.07$ μmol/mol and $K = 1.02 \times 10^8$ l/mol), which is physically unrealistic. The apparent binding constant for membranes containing $100\,\%$ PS was much greater ($K_{app} = 6.09 \times 10^7$ l/mol) than the intrinsic (hydrophobic) binding constant ($K = 7.93 \times 10^5$ l/mol). In the latter case, binding was due to electrostatic attraction of the cationic IMI to the negatively charged surface of a PS membrane. Consequently, interference between binding to a specific protein binding site and binding to a lipid bilayer may occur in receptor binding studies when IMI or a similar amphiphilic ligand is used. The filtration assay is useful in the study of the strong part of total binding of an amphiphilic drug to a lipid bilayer.

The difference between the total binding values, established by centrifugation and filtration (Fig. 1), corresponds to the amount of bound ligand that can be removed during sample filtration and washing, i.e., to the amount of very weakly bound molecules. These molecules are probably adsorbed to the surface of MLVs mainly through the van der Waals forces and long-range electrostatic interactions. This weak surface adsorption can be regarded as non-specific, being common to different amphiphilic ligands; hence, it cannot be related to specific therapeutic effects of the drug. As the weakly bound molecules may affect the membrane electrostatic parameters, their contribution to side effects of the studied drug cannot be ruled out.

Interactions of CADs with lipid bilayers were intensively studied in recent years; it is therefore difficult to find something new in this field. We attempted to characterize both the total and "high-affinity" binding of IMI, representing antidepressant drugs, to liposomes prepared from electroneutral or negatively charged

phospholipids. The radioligand binding assay, which is currently seldom used for such experiments, enabled us to obtain some new results and the method described can be used in other experiments with CADs and liposomes. The centrifugation technique for separation of bound and free ligand can be used to determine binding parameters of total binding of IMI to lipid membranes; the filtration technique provides characterization of "high-affinity" binding. Specification of different types of forces participating in the "high-affinity" binding of TCAs to lipid membranes is the subject of our following paper, where pH-dependence of this binding is analyzed.

Due to electrostatic attraction, binding of the positively charged IMI to the negatively charged surface of PS membranes was augmented compared to binding to neutral PC membranes. After correction for electrostatic effects by means of the GC theory, the binding isotherms were described by binding parameters.

To sum up, there is a marked accumulation of antidepressants in lipid membranes, and binding of IMI consists from at least two components differing by affinity. The capacity of both the total and "high-affinity" binding is significantly higher in PS membranes than in PC membranes. This can be explained by the high-capacity of surface binding of IMI to PS vesicles caused by the Coulomb interactions between charged groups. A significant part of the surface adsorption of IMI to PC liposomes is very weak and can be easily released. It can be assumed that the nature of "high-affinity" binding of IMI to PS vesicles is mainly electrostatic, whereas the "high-affinity" component of the IMI binding to PC liposomes comprises molecules incorporated into the hydrophobic core of the lipid bilayer.

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