Interactions Between Tricyclic Antidepressants and Phospholipid Bilayer Membranes

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Abstract. Participation of electrostatic and other noncovalent interactions in the binding of tricyclic antidepressants (TCAs) to the lipid bilayers was estimated from pH-dependencies of imipramine, desipramine, amitriptyline and nortriptyline binding to the lipid bilayers prepared from different phospholipids, both electroneutral and acidic. The binding was studied using a radioligand binding assay. It was found that the membrane phospholipid composition and methylation of the acyl side chain of TCA has a decisive effect on participation of particular noncovalent interactions in the binding. Apparent high-affinity binding of TCAs to the phosphatidylcholine or phosphatidylethanolamine membranes are achieved mainly by incorporation of uncharged drug molecules into the hydrophobic core of the bilayers. Van der Waals forces and hydrophobic effect are responsible for this binding. Both charged and uncharged drug molecules bind to phosphatidylserine membranes, therefore coulomb- or ion-induced dipole interactions play a role in these binding. Different spatial distribution of charged residues within the interface causes different electrostatic interactions between charged TCAs and vesicles formed from phosphatidylserine and phosphatidylinositol. The data supports the hypothesis under which TCAs could have effect on affective disorders partially *via* binding to the lipid part of the membrane and following changes of lipid-protein interactions.

Key words: Antidepressants — Liposomes — Noncovalent interactions — pH-dependence — Phospholipids

Abbreviations: TCA, tricyclic antidepressant; IMI, imipramine; DMI, desipramine; AMI, amitriptyline; NOR, nortriptyline; PC, phosphatidylcholine; SM, sphingomyelin; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; GC, galactocerebroside; CH, cholesterol; CAD, cationic amphiphilic drug; pK, equilibrium constants for acids and bases; k_p, partition coefficient; K_d, equilibrium dissociation constant.

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Introduction

Adaptive changes in neurotransmitter transduction systems seem to be the key element in therapeutic effects of antidepressants (Stahl 2000). There are hypotheses of affective disorders supposing that both the disorder and therapeutic effects of antidepressants are connected with the alterations in the membrane lipid metabolism and with the changes in the physical state of the lipid bilayers (Mueller et al. 1970; Heron et al. 1980; Sengupta et al. 1981; Block and Edwards 1987; Hibbeln et al. 1989; Maes et al. 1994; Nakamura 1994; Rybakowski and Lehman 1994; Vinokur and Gubachev 1994; Hibbeln and Salem 1995; Penttinen 1995; Kunugi et al. 1997; Peet et al. 1998; Frasure-Smith et al. 2004). So, the study of the role of membrane lipids is necessary when understanding the nature of depression and adaptive mechanisms induced by antidepressants.

Membrane lipids and the lipid bilayer itself was recognized as a potent enhancer and a regulator of the membrane receptors, transporters, ion channels and enzymes (Srivastava et al. 1987; Cornelius 2001; Scanlon et al. 2001; Lee 2003). The number of different charged or uncharged lipids containing a broad spectrum of acyl chains and their distribution within the biological membrane suggests that the role of the membrane lipids is far from being known. Generally, lipid bilayers are heterogeneous both in the horizontal and vertical direction and into the bargain there are density fluctuations, regions with non-random lipid composition (domains, rafts) and nonbilayer structures (Jain 1983; Hong et al. 1988; Devaux 1991; Mouritsen and Jørgensen 1995; Mukherjee and Maxfield 2000; Barenholz 2002; Ohvo-Rekilä et al. 2002; Fielding and Fielding 2003).

A simplified four region model of the lipid bilayer (Fig. 1) is proposed (Bauer et al. 1990; Tieleman et al. 1997). The model includes the fact that the order of the hydrocarbon chains is relatively high in the region near to the lipid-water interface and decreases strongly towards the bilayer centre, however, the model does not account for charged residue distribution. The headgroups of phospholipids may be divided into two regions: i) a negatively charged phosphate group and ii) a positively, negatively, zwitterionic or uncharged group (Langner and Kubica 1999). Phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin (SM) are zwitterions with a negatively charged phosphate group and positively charged choline or ethanolamine group. Phosphatidylglycerol (PG) and phosphatidylinositol (PI) each have a single negative charge associated with the phosphate group; however, the phosphate group is screened stearically by glycerol or an inositol group. Phosphatidic acid contains a single negative charge associated with its phosphate group; a charge, unlike those of PI and PG, is not separated from the aqueous phase by any group. Phosphatidylserine (PS) has three residual charges; two negative charges are associated with phosphate and carboxyl group, one positive charge is located in the ammonium group; carboxyl group charge is easily accessible from the aqueous phase. The role of cholesterol (polar, noncharged molecule) can not be neglected both in direct action on the function of some membrane proteins (Scanlon et al. 2001; Fielding and Fielding 2003; Maekawa et al.

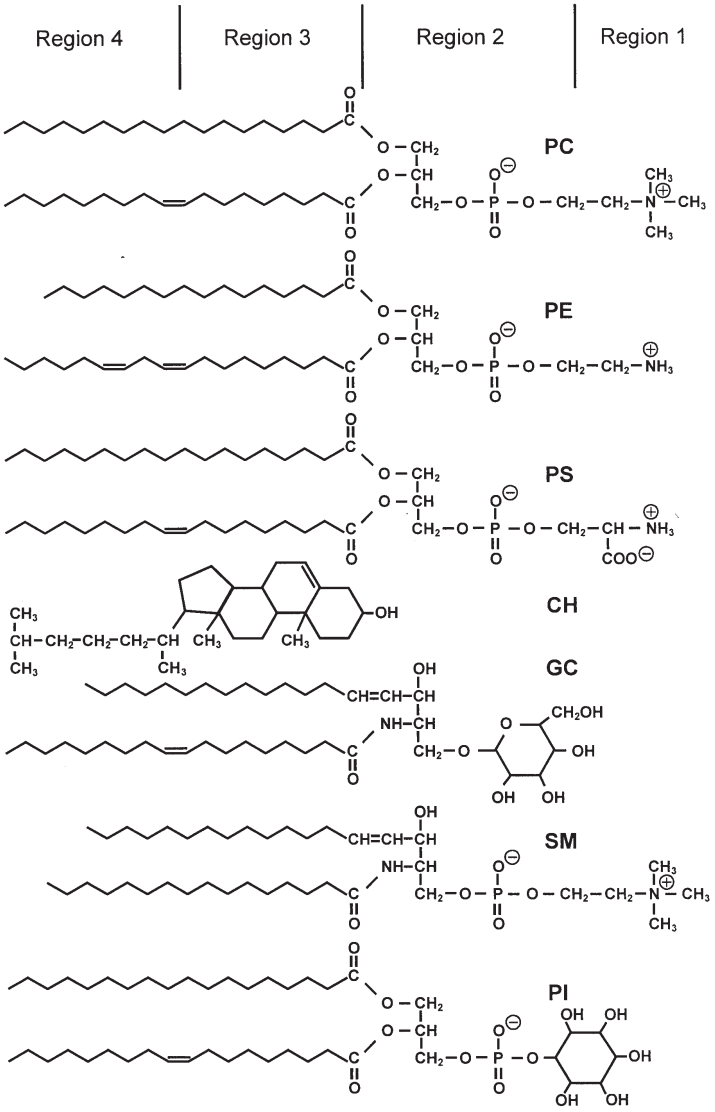

Figure 1. Schematic lipid bilayer division according to the four region model (Tieleman et al. 1997) and examples of the outer leaflet of the plasma membrane components (PC, SM, GC, and CH) and inner leaflet of plasma membrane (PE, PS, PI, and CH). Region 1 ("perturbed water") consists of a layer in which water structure is affected by the bilayer surface; this region ends where the lipid and the water densities are comparable. Region 2 ("interphase") includes headgroups and part of the tail methylenes; this region has the highest density in the bilayer. Region 3 ("soft polymer") consists of partially ordered acyl chains; it starts at the carbonyl groups and it is characterized by a high chain density and low free volume. Region 4 ("decane") is the centre of the bilayer which is characterized by a low density and high free volume. Regions 1 and 2 form the interfacial region.

2003; Pfrieger 2003) and in the membrane structure and fluidity (Shinitzky 1984; Yeagle 1985; Silvius 2003).

It has been shown in many previous studies that liposomes are a suitable model of the lipid part of the biological membranes for testing the potential biological activity of compounds (Reith et al. 1984; New 1990) and that there is a good agreement between the results obtained in the model systems and those obtained *in vivo*. Large unilamellar vesicles are the best model of cell membranes; however, it was demonstrated that multilamellar vesicles are also a good model (Choi and Rogers 1991; Fišar et al. 1991, 2004).

Multiple noncovalent bonds impart specificity both to the protein-protein or lipid-protein interactions and the drug-membrane interactions, so, it is remarkably difficult to quantify separate contributions of individual noncovalent interactions to the thermodynamics of drug-membrane interactions (Cooper 1999). There is known only a limited menu of noncovalent interactions: 1. electrostatic (ionic, Coulomb) interactions; 2. dispersion and repulsive van der Waals forces; 3. hydrogen bond, and 4. hydrophobic effect (interaction). Hydrophobic and polar interactions are collectively referred to as hydropathy. Van der Waals interactions rapidly decrease with an increasing distance, but Coulomb interactions between dipoles, especially between whole charges, are quite long-ranged.

The antidepressants belong to the group of the cationic amphiphilic drugs (CADs). It is not known if accumulation of antidepressants in the lipid part of biological membranes (Sikora et al. 1990; Krulík et al. 1991; Fišar et al. 1996) can be related to their therapeutic or side effects, but the protein-lipid-antidepressant interactions can influence the function of many membrane systems participating in the nerve signal transduction (Bevan et al. 1989; Mason et al. 1991; Seydel et al. 1992; Yang and Glaser 1995; Scanlon et al. 2001). Changes in the composition of the membrane lipids after the long-term administration of the antidepressants (Moor et al. 1988) indicate the role of membrane lipids in the mechanism of their action even if the phospholipids are not the specific target of the drug molecules. Many antidepressants may induce the generalized phospholipidosis, i.e. excessive accumulation of different phospholipids within the cell (Xia et al. 2000).

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 CADs with membranes were described (Boulanger et al. 1980; Kelusky et al. 1986; Zachowski and Durand 1988). The Gouy–Chapman theory has been adopted to describe adsorption (incorporation) of drugs, hormones, peptides and proteins onto the lipid bilayer surface (McLaughlin and Harary 1976; Eisenberg et al. 1979; McLaughlin 1989; Beschiaschvili and Seelig 1990; Cevc 1990, 1993; Stankowski 1991; Seelig et al. 1993; Langner and Kubica 1999; Averbakh and Lobyshev 2000). Thermodynamic analysis of the binding equilibrium for various amphiphilic or hydrophobic ligands led to the conclusion that the main driving force for the binding of these drugs into lipid bilayer is either a change in enthalpy, or enthalpy as well as entropy, it means van der Waals forces and a hydropho-

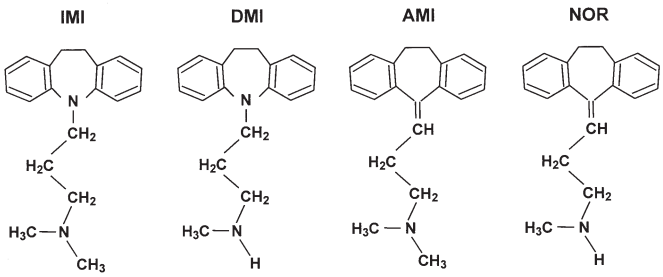

Figure 2. Chemical structures of tricyclic antidepressants used include IMI, DMI, AMI and NOR.

bic effect (Bäuerle and Seelig 1991; Wimley and White 1993; Seelig et al. 2000). Coulomb interactions are considered to explain surface adsorption of CADs.

Many biologically active molecules, including phospholipids and drugs, have multiple acidic or basic groups; it is essential to know the state of dissociation of each of these groups when their mutual interactions are studied. The Henderson–Hasselbach equation describes the relations between pH and equilibrium constants (pK) for acids and bases. The pK value of an ionisable drug in the membrane differs from those in the bulk solution. It was shown that partition coefficients (defined as ratio of molar concentration of drug in membrane to the concentration in aqueous phase) of charged (k_p^+) and uncharged (k_p°) forms of the drugs are different, which imply a pK shift of the partitioning compound (Lee 1978; Zachowski and Durand 1988; Cevc 1990; Mason et al. 1991; Miyazaki et al. 1992; Pauletti and Wunderli-Allenspach 1994; Hata et al. 2000; Schreier et al. 2000; Castro et al. 2001; Hunziker et al. 2001; Rodrigues et al. 2001). The pK shift is generally given by a polarity-induced shift (determined by thermodynamic differences in the ionization equilibrium of drugs at the water and membrane location) and electrostatic shift (determined by surface potential of charged membranes). Relation between the difference of pK of the drug in the membrane (pK_m) and in water (pK_w) on the one hand and k_p°/k_p^+ on the other can be readily derived at low drug concentrations (Lee 1978; Miyazaki et al. 1992):

$$\Delta \mathrm{p}K = \mathrm{p}K_m - \mathrm{p}K_w = \log(k_p^+/k_p^\circ) \tag{1}$$

This pK shift can be estimated from a pH dependence of the drug binding to the lipid membranes.

Tricyclic antidepressants (TCAs) are the most carefully searched group of antidepressants according to the fact that these drugs are therapeutically used almost 50 years. The primary biochemical effect of TCAs is the inhibition of the serotonin or norepinephrine membrane transporters (Hirschfeld 2000). Chemically they are dibenzazepines and dibenzcycloheptanodienes; e.g. imipramine (IMI), desipramine (DMI), amitriptyline (AMI) and nortriptyline (NOR) are included in the TCAs

group (Fig. 2). A molecular dynamics simulation of TCAs demonstrated considerable flexibility of the molecules, both in the side chain and in the ring system (Heimstad et al. 1991). It is generally thought that the TCAs are anchored at the lipid membrane *via* the hydrophobic moiety of the molecules (aromatic rings) and with their polar part (side chain inclusive charged amine group) being preferably localized in the vicinity of the phospholipid polar groups (glycerol backbone/phosphate), however, different interactions with the membrane at low and at higher TCAs concentrations were observed (Bauer et al. 1990; Freisleben and Zimmer 1991).

At a physiological pH, about 99 % of TCAs molecules carry a positive charge ($pK_{NH_3^+} = 9.4$) and their binding to membranes is strongly dependent on lipid composition of the membranes (Fišar et al. 2004). Over a wide range of pH values, both PC and PE molecules are completely electroneutral as the negative charge of the phosphate group ($pK_{PO_4^-} \leq 1$) is compensated by the positive charge of the choline head ($pK_{NH_3^+} = 11.25$); however, PC and PE become negatively charged at high pH. PS may carry, in addition to a negative charge on the phosphate ($pK_{PO_4^-} \leq 1$) and a positive charge of the amino group ($pK_{NH_3^+} = 11.5$), a negative charge on the carboxyl group ($pK_{COO^-} = 5.5$). As a result, PS will carry (under normal conditions) a negative net charge increasing at a higher pH while PS becomes electroneutral at a low pH. PI is negatively charged practically over the whole studied range of pH ($pK_{PO_4^-} = 2.7$; all pK values are adapted from Cevc (1990)).

Binding parameters of TCAs to lipid bilayers have been determined from saturation isotherms in our previous experiments (Fišar et al. 2004); data were analysed without and after correcting for electrostatic effects using the Gouy–Chapman theory. We confirmed that binding of IMI consists from at least two components differing by affinity and the strong part of the total binding (apparent high-affinity binding) can be characterized using filtration to separate bound and free ligand. The low-affinity component of the total binding can be related to the weak surface adsorption of charged TCA molecules. Diversity of low-affinity binding was described and characterized for various CADs (Zachowski and Durand 1988; Bäuerle and Seelig 1991; Austin et al. 1995); however, high-affinity binding was not identified and characterized in these studies. We supposed that both the Coulomb interactions between charged groups and the drug incorporation into hydrophobic core of lipid bilayer are responsible for apparent high-affinity binding.

The aim of the presented study is a more precise determination of the participation of different weak forces in the apparent high-affinity binding of TCAs to the lipid bilayers. The pH-dependencies could be analyzed to size up a proportion of Coulomb forces and other noncovalent interactions. To resolve between the effect of the antidepressant and the phospholipid, the measurements were carried out with 4 different TCAs, IMI, DMI, AMI and NOR, and with 4 different phospholipids, PC, PS, PE and PI; mixtures of phospholipids were used also. It is known that the mutual interactions tend to destabilize the lipid bilayer at high amphiphilic ligand concentrations (Zimmer 1984; Zachowski and Durand 1988; Balgavý and Devínsky 1996; Heerklotz and Seelig 2000; Sanganahalli et al. 2000; Schreier et al. 2000); that

is why very low (nanomolar) TCAs concentrations (i.e. a high molar phospholipid to antidepressant ratios) were used in our study.

Materials and Methods

Chemicals and solutions

All samples were prepared in a physiological saline (0.9 g/l NaCl, pH 7.4) and their pH were adjusted using 5 times concentrated buffers. The range of 2 to 12 was covered by three types of buffers obtained by mixing accurate volumes of following solutions: 0.5 mol/l citric acid and 1.0 mol/l disodium phosphate (pH 2, 3, 4); 0.33 mol/l monopotassium phosphate and 0.33 mol/l disodium phosphate (pH 5, 6, 7, 8); 0.5 mol/l glycine, 0.5 mol/l sodium chloride and 0.5 mol/l sodium hydroxide (pH 9, 10, 11, 12). Phospholipids in chloroform/methanol solvent mixture (2 : 1, vol/vol; 5–20 mg/ml) were isolated from total lipid extracts by column chromatography. Crude extract of lipids was prepared by Folch method (Folch et al. 1957; Koul and Prasad 1996). PC, PE and PS were isolated from the white matter of the bovine brain and PI from green peas. The resulting purity, determined by two-dimensional thin-layer chromatography, was over 95 %. Phospholipids were stored in a freezer under nitrogen atmosphere.

The following stock solutions of tritium-labelled TCAs ([^{3}H]TCAs) in methanol were used: 80.37 μmol/l [^{3}H]IMI (specific activity 2.7 TBq/mmol, concentration 217 MBq/ml), 4.08 μmol/l [^{3}H]DMI (2.0 TBq/mmol, 8.16 MBq/ml), 3.00 μmol/l [^{3}H]AMI (4.07 TBq/mmol, 12.2 MBq/ml), 324 μmol/l [^{3}H]NOR (0.08 TBq/mmol, 25.9 MBq/ml); radiochemical purity >96 %; labelled in our laboratories (Krulík et al. 1991); 12.5 nmol/l of [^{3}H]TCA solutions in physiological saline were prepared just before measurement and used in binding experiments. Tritium-labelled phospholipids, [^{3}H]PC and [^{3}H]PS (concentration 18.5 MBq/ml, radiochemical purity >96 %) were prepared by catalytic tritiation of double bonds in hydrocarbon chains of phospholipids with gaseous tritium. Non-labelled (cold) TCAs were used in the form of hydrochlorides: 2 mmol/l stock solutions of IMI, DMI, AMI and NOR (all from Sigma-Aldrich Co., St. Louis, MO, USA) in physiological saline were used as displacement agents.

Preparation of liposomes

Lipid vesicles were prepared using Bangham method (Bangham et al. 1965; New 1990) shortly before measurement. Briefly, an aliquot part of phospholipid in chloroform/methanol solvent mixture containing less than 20 mg of phospholipid was introduced into a 50-ml vessel and the liquid was completely evaporated from the solution by nitrogen stream at temperature about 40 °C. The vessel 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 flask was filled with nitrogen, closed and incubated at 50 °C for 5 min. The sample was agitated until all the lipid was removed from the walls of the flask and a homogeneous milky-white suspension

arose. The flask was shortly sonicated (for less then 10 s) in an XL 2020 sonicator (Misonix Inc.) to release all lipids from the walls and to break down large clusters. After incubation of the sample at 50 ℃ for 30 min, the suspension was left at room temperature for further 2 h and diluted by buffer solution to the required phospholipid concentration (0.5 mg/ml). Phospholipid concentrations were checked by a phosphorus concentration determination (Bartlett 1959; Wagner et al. 1962). The same technique was employed to prepare PC, PE, PS, PI and mixed vesicles. However, because of the differences between the electroneutral and acidic phospholipids, liposomes are not the same: unsonicated PC bilayers are characteristic by multilamellar packing; pH-induced destabilization, aggregation and fusion of liposomes composed of PE was described (Ellens et al. 1984; Allen et al. 1990; Hazemoto et al. 1990); the fundamental structure of charged lipids dispersed in water is the unilamellar vesicle (Hauser 1984).

In our previous experiments (Fišar et al. 2004) we proved that the lipid bilayers in PC or PS vesicles were sufficiently permeable for TCAs and a 30 min incubation at 20 ℃ was sufficient to restore equilibrium, i.e., a uniform distribution of TCA within liposomes.

Radioligand binding assay

Radioligand binding assay was employed by analogy with the receptor studies (Bennett and Yamamura 1985; Fišar et al. 2004). The tritium-labelled TCAs were used, [^{3}H]IMI, [^{3}H]DMI, [^{3}H]AMI and [^{3}H]NOR. Bound and free radioligand was separated by a rapid filtration using a device harvester MB 18 (Brandel, Gaithersburg, MD, USA). The filtration time period (<10 s) was sufficiently short for the measurement of the high-affinity binding processes with equilibrium dissociation constant $K_d < 10^{-8}$ mol/l. Resulting concentrations were 5 nmol/l [^{3}H]TCA and 0.2 mg/ml of phospholipids. Brief summary of the method: the pH values of the liposome suspensions were adjusted (pH 2 to 12) using the 5 times concentrated buffers; the binding was started by the addition of 100 μl [^{3}H]TCA to 150 μl of the liposome suspension; following incubation at 20 ℃ for 30 min, samples were filtered through glass microfibre filters (GF/F type; Whatman International Ltd., Kent, UK) impregnated previously in 0.1 % polyethyleneimine (Sigma) and washed by 9 ml of saline and their activities were assayed in a scintillation cocktail using an LS 6000IC liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA, USA). It was proved that the buffer composition did not affect the binding of TCAs to vesicles. Samples were measured in doublets. Specific binding of TCAs to lipid vesicles was calculated as the difference between total and nonspecific binding with nonspecific binding determined in the presence of excess (50 μmol/l) of cold ligand.

Capture efficiency of liposomes on GF/F filters at different pH values was measured by the following technique. Trace amount (2 μl) of [^{3}H]PC or [^{3}H]PS was added to the phospholipid in a chloroform/methanol solvent mixture and liposomes were prepared by the standard procedure. The pH values of the liposome suspensions were adjusted, the total activity of 250 μl of the sample was measured and

250 μl of liposomes was filtered and washed. The percentage of vesicles captured on filters was calculated; samples were measured in doublets.

pH dependencies of investigated parameters were measured over a wide variety of pH (2 to 12) to evaluate interactions both of charged and uncharged drugs or lipid molecules.

Data analysis

Data is expressed as the arithmetic mean ± standard deviation (S.D.). Selected pH curves were analyzed using the four-parametric logistic model (ImmunoFit EIA/RIA software, Beckman) to establish the pH values at which binding of TCA to lipid vesicles attains the one half of the maximal value. Statistical analyses were performed with the statistical package Statistica (StatSoft Inc.). Spearman R (non-parametric alternative to the Pearson product-moment correlation coefficient) was used to quantify relation between two quantitative parameters.

Results

Capture efficiency of filters

First, the capture efficiency of GF/F filters was determined using the tritium-labelled phospholipids as markers. We confirmed that the amount of vesicles captured on the filters did not depend on repeated washing of the filters. Rinsing of filters by volumes 3–15 ml of saline did not change the percentage of trapped vesicles, which was practically the same (about 80 % at pH 7.4) for PC, PS, PE and PI vesicles. The percentage of liposomes captured on the GF/F filter was found practically constant in the range of pH between 2 to 10 but marked a decrease of the capture efficiency was observed at pH above 10 (Fig. 3).

pH-dependencies of the binding

The radioligand binding assay was used to determine the pH-dependencies of the binding of IMI, DMI, AMI and NOR to liposomes prepared from PC, PE, PS, PI and mixtures (PC+PE) (1 : 1, w/w), (PC+PS) (1 : 1, w/w) and (PC+PI) (1 : 1, w/w). Displaceable high-affinity binding was determined and adjusted according to the percentage of phospholipid trapped on the filter; the corrected values were used in the pH curve. Only data from the range of pH 2 to 10 was interpreted due to low capturing efficiency of GF/F filters at higher pH (data points at pH 11 and 12 are shown in Figs. 4–6, but they are not used for curve fitting).

The binding at 5 nmol/l TCA concentration can be used to differentiate binding of charged from uncharged molecules (Fig. 4). The courses of pH-curves for PC or PE or PI vesicles corresponded with change of positive charge on IMI molecules both for binding to electroneutral PC or PE vesicles and for electronegative PI vesicles. However, the pH-dependence of IMI binding to the PC vesicles was shifted to lower pH. The course of the binding to the PS vesicles was more complex and reflected changes of positive charge on IMI and negative charges on PS molecules.

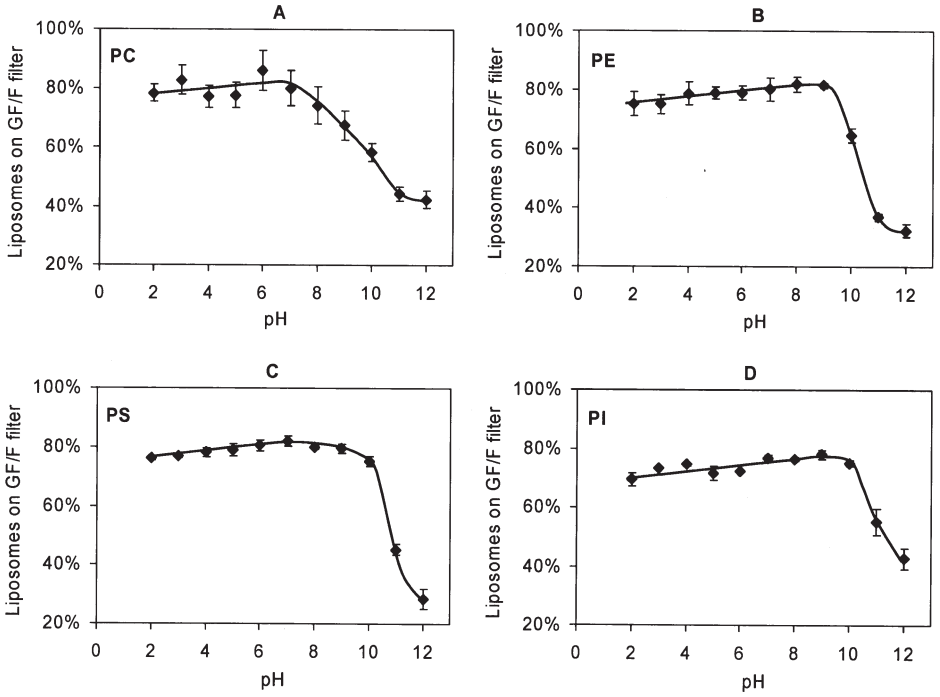

Figure 3. The percentage of liposomes collected on GF/F filter as a function of pH. Filters were impregnated in 0.1% polyethyleneimine and a saline was used as a rinsing solution. Tritium-labelled PC and PS were used as markers; final phospholipid concentration was 0.16 mg/ml. Values are reported as mean ± S.D. ($n = 3$).

The total binding of TCAs to lipid vesicles was dependent both on their lipid composition and on pH, whereas, the nonspecific binding (in presence of 50 μmol/l of cold TCA) was low and practically constant over the whole range of pH (Fig. 4). The high-affinity binding of TCAs to vesicles was displaced at high concentrations of unlabelled ligand; it is shown on Fig. 4 that inhibition of [^{3}H]IMI binding by cold IMI (0.5 μmol/l) was higher in vesicles prepared from electroneutral phospholipids (PC or PE) in comparison with acidic phospholipids (PS or PI). Similar results were found with liposomes prepared from mixtures of phospholipids, i.e. [^{3}H]IMI binding was displaced more markedly from (PC+PE) vesicles in comparison with (PC+PS) or (PC+PI) vesicles (data not shown).

The pH-dependencies of [^{3}H]TCA high-affinity binding to lipid vesicles prepared from different phospholipid classes are shown on Figs. 5 and 6. Corrected data were used in graphs, i.e. nonspecific [^{3}H]TCA binding to GF/F filters was subtracted and data were re-counted to agree with 100% collection of vesicles on GF/F filters. The pK of TCAs or phospholipids are marked in graphs (vertical dashed lines) and used in Discussion.

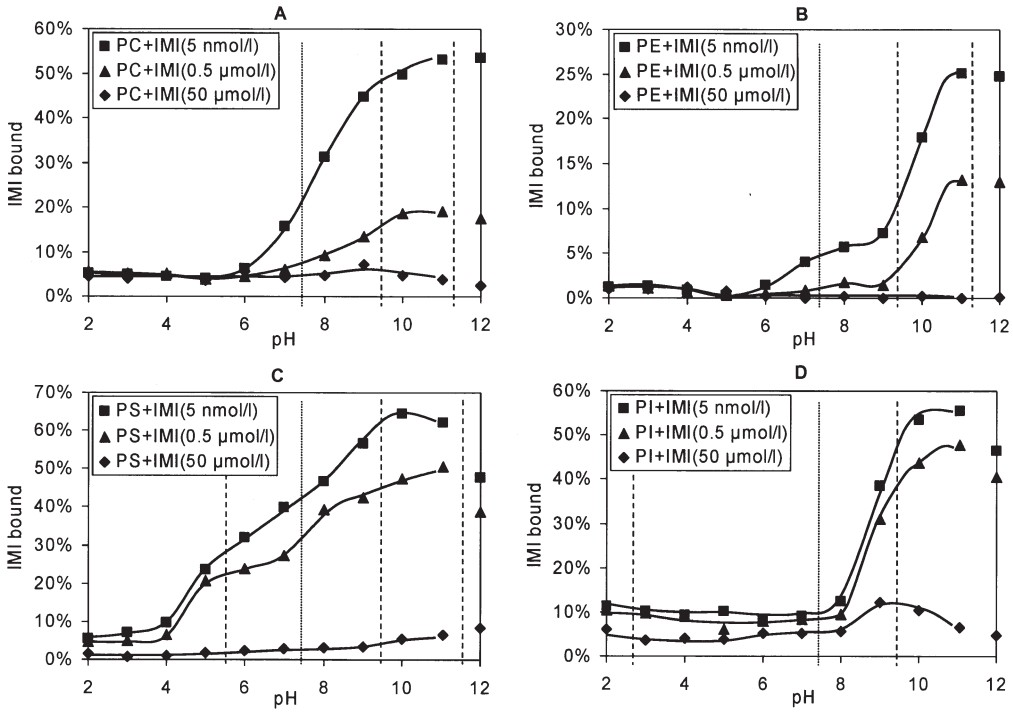

Figure 4. pH-dependencies of high-affinity IMI binding to the lipid vesicles prepared from PC (A), PE (B), PS (C) and PI (D) in the presence of 5 nmol/l [^{3}H]IMI at a phospholipid concentration about 0.2 mg/ml. Corrected data were used in graphs, i.e. nonspecific [^{3}H]IMI binding to GF/F filters was subtracted and data were re-counted to agree with 100% collection of vesicles on GF/F filters. Only data from the range of pH 2–10 was used in curve fitting due to low capture efficiency of GF/F filters at higher pH (data points at pH 11–12 are shown, but they were not interpreted). Displacement of [^{3}H]IMI binding following addition of 0.5 μmol/l or 50 μmol/l of cold IMI is depicted. Vertical doted lines indicate pH 7.4, vertical dashed lines represent pK values of TCAs (p$K_{NH_3^+}$ = 9.4) or pK values of phospholipids (Cevc 1990), i.e. PC (p$K_{NH_3^+}$ = 11.25), PE (p$K_{NH_3^+}$ = 11.25), PS (p$K_{NH_3^+}$ = 11.5, pK_{COO^-} = 5.5) and PI (p$K_{PO_4^-}$ = 2.7). The figure shows mean data from experiment repeated at least three times.

Similar curves were observed using [^{3}H]IMI, [^{3}H]DMI, [^{3}H]AMI or [^{3}H]NOR. Relation between bindings of different TCAs to the same phospholipid vesicles was determined as correlation coefficient (Spearman R). A significant positive correlations (at statistical significance $p < 0.01$) were discovered for PC, PE, PS, PI, (PC+PE), (PC+PS) and (PC+PI) vesicles, except for binding of DMI or NOR to the PC- and PI-derived vesicles.

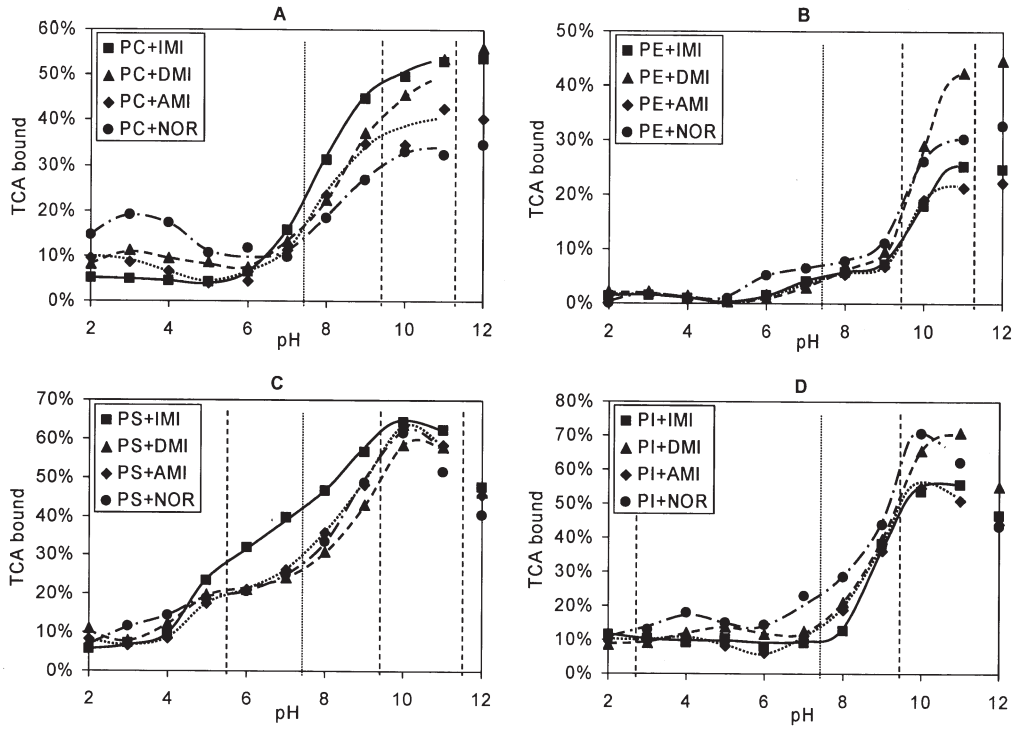

Figure 5. pH-dependencies of TCA binding to vesicles prepared from PC (A), PE (B), PS (C) and PI (D). High-affinity binding of tritium labelled IMI (■), DMI (▲), AMI (◆) or NOR (●) was measured; final concentrations were 5 nmol/l of TCA and 0.2 mg/ml of phospholipid. Corrected data were used; the meaning of the vertical dotted and dashed lines is the same as in Fig. 4. Values are means calculated from experiments repeated 3 to 9 times. S.D. are not shown due to graph lucidity.

Discussion

Our study was directed to the characterization of the apparent high-affinity binding (characterized by dissociation constant $\leq 10^{-8}$ mol/l) of TCAs to the model lipid bilayers prepared from different phospholipids. It was shown, that apparent high-affinity binding of TCAs to bilayer membranes is strongly dependent both on their phospholipid composition and on the pH of the aqueous phase. Short range van der Waals, electrostatic forces and the hydrophobic effect participate in these bindings. To interpret the obtained results in the terms of weak interactions participating in this binding, heterogeneity of lipid bilayer structure and the spatial distribution of charged residues within the interface must be considered.

The study of interactions of TCAs with undisturbed lipid bilayers and the differentiation of binding of charged *vs.* uncharged molecules was found useful only

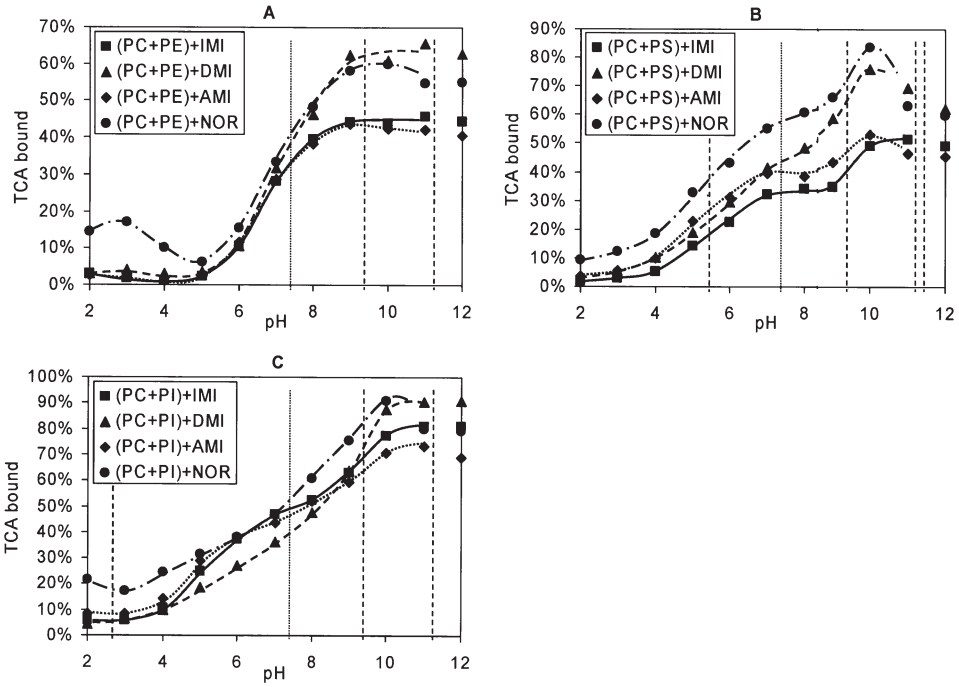

Figure 6. pH-dependencies of TCA binding to vesicles prepared from mixture (1 : 1, w/w): A. (PC+PE), B. (PC+PS) and C. (PC+PI). High-affinity binding of tritium-labelled IMI (■), DMI (▲), AMI (◆) or NOR (●) was measured; final concentrations were 5 nmol/l of TCA and 0.2 mg/ml of phospholipid. Corrected data were used; the meaning of the vertical dotted and dashed lines is the same as in Fig. 4. Values are means calculated from experiments repeated 3 to 6 times. S.D. are not shown due to graph lucidity.

at sufficiently low (nanomolar) drug concentrations (Fig. 4). So, the method used in this paper provided the determination of binding at very low antidepressant concentrations, i.e. near to the free TCA concentrations in plasma during the pharmacotherapy (<50 nmol/l).

A decrease in the displaceable binding in [^{3}H]TCA to the lipid bilayers towards very low values corresponded with decrease in pH values from 10 to 2 (Figs. 5, 6). The courses in pH-curves depended strongly on the phospholipid composition of liposomes; however, they were very similar for different TCAs. As PC and PE molecules are practically electroneutral over the whole studied range of pH values, and as the positive charge of TCAs disappears with pH elevation, these pH-dependencies (Figs. 5A,B and 6A) can be interpreted as a facilitated incorporation of uncharged TCA molecules into the hydrophobic core of the PC, PE or (PC+PE) bilayers. It can be stated that the [^{3}H]TCA binding to liposomes prepared from

electroneutral phospholipids follows the pH-dependent degree of dissociation of the drug. However, the binding to the PC liposomes attained one half of the maximal value at a pH about 7.7 for IMI or AMI and at pH about 8.3 for DMI or NOR (Fig. 5A), i.e. 1.7 and 1.1 pH unit lower in comparison with TCA in aqueous medium ($pK_w = 9.4$), which corresponds to a 50- and 13-fold higher partition coefficient of the uncharged drug compared with that of the charged form (Eq. (1)). So, the k_p°/k_p^+ of methylated TCAs (IMI, AMI) to PC bilayers is significantly higher than k_p°/k_p^+ of demethylated drugs (DMI, NOR). It seems that both the charge and methylation of the acyl side chain of TCAs affect the apparent high-affinity binding of TCAs to PC membranes. This result is valid only at nanomolar TCA concentrations.

The pH-dependence of TCA binding to the PE vesicles (Figs. 4B, 5B) was markedly different from binding to PC vesicles. It can be explained by differences of PE from PC; the headgroup of PE is smaller than the phosphocholine of PC and PE can readily constitute a nonbilayer structures. Therefore, mixture PC and PE (1 : 1) was used to prepare lipid bilayers. It was demonstrated that pH-dependencies of TCAs binding to (PC+PE) liposomes (Fig. 6A) are very similar to the curves obtained with PC liposomes (Fig. 5A); however, the pK shift is greater. The binding to the (PC+PE) vesicles attained one half of the maximal value at a pH about 6.7 for IMI or AMI and at pH about 7.0 for DMI or NOR, i.e. 2.7 and 2.4 pH unit lower in comparison with TCA in aqueous medium, which corresponds to a 500- and 250-fold higher partition coefficient of the uncharged drug compared with that of the charged form. This could be related to the existence of nonbilayer structures in PE membranes.

The pH-dependence of TCA binding to the PS vesicles (Figs. 4C, 5C) was markedly different from binding to PC vesicles. It is done by the fact that both the drug and the PS charges vary over the used range of pH values. The course of the binding to the PS vesicles is clearly affected both by protonation and deprotonation of interacting molecules. The initial increase in the binding with the rising of pH from value 2 to 7, correlates with the addition of a negative charge to PS ($pK_{COO^-} = 5.5$); hence, this increase in binding of TCAs to the PS vesicles is caused predominantly by Coulomb interactions between charged TCA^+ and serine group of PS. For pH value between 7 and 10, the binding increases, the implication being binding of an uncharged ligand to a charged PS is also significant. The data suggest approximately equal fractional contributions of the charged and uncharged TCAs to the PS membranes at physiological pH. A certain decrease in the binding for pH > 10 is probably associated with the loss of the positive charge of the drug and, consequently, a decrease in Coulomb interactions. These results confirm the significant role played by Coulomb interactions in apparent high-affinity TCA binding to the PS vesicles at physiological pH. The magnitude of binding to the PS vesicles even at a marked decrease in Coulomb interactions (at pH > 9) could mean that both ion-induced dipole interactions and van der Waals forces and hydrophobic effect play a role.

The difference between TCAs binding to PC *vs.* PS vesicles could reflect a

greater representation of nonbilayer structures in PS membranes. Therefore, binding to liposomes prepared from mixture PC and PS (1 : 1) was also measured. The course of the pH-dependence of the high-affinity binding of TCAs to the mixed (PC+PS) vesicles (Fig. 6B) was found very similar to the course obtained for PS vesicles; so, eventual nonbilayer structures do not play significant role. It seems that the binding to PS overlays the binding to the PC at lower pH and the contribution of the charged TCAs to the binding to (PC+PS) vesicles (i.e. electrostatic interaction) is multiple higher than that of uncharged TCAs at physiological pH.

The pH-dependence of TCA binding to the vesicles prepared from PI was executed to discover if binding of TCA to the PS vesicles is caused by a total charge of phospholipid only, or if there exists some specificity of TCAs binding to the PS membranes, i.e. if spatial distribution of charged groups and their masking by noncharged groups play a role in the binding. It was found that the course of TCAs binding to the PI vesicles (Figs. 4D, 5D) is very different from binding to PS vesicles; it seems that binding is realized mainly by incorporation of uncharged drug into PI vesicles. The difference between TCAs binding to PS and PI vesicles may be caused by nonbilayer structures in PI vesicles rather than by different spatial distribution of charged groups; this was confirmed by using mixture PC and PI (1 : 1) to prepare liposomes. The pH-dependencies of TCAs binding to the liposomes prepared from (PC+PI) (Fig. 6C), PS or (PC+PS) (Figs. 5C, 6B) are similar. So, the binding of TCAs to (PC+PI) vesicles is realized both by charged and non-charged form of TCAs. However, binding of positively charged TCA^+ at pH < 4 was very low, although PI is negatively charged over the whole studied range of pH ($pK_{PO_4^-} = 2.7$). Probable cause is that a negatively charged phosphate group of PI is shaded off stearically by an inositol group and Coulomb interaction is too weak to constitute the high-affinity binding. This result implies the existence of partial specificity in TCAs binding to the PS membranes, which is not determined by a total negative charge of PS polar heads. It is obvious that different spatial distribution of charged residues within the interface causes different electrostatic interactions between charged TCAs and surfaces formed from PS and PI. Diverse composition and conformation of uncharged groups may also contribute to the specificity of TCAs binding to the PS vesicles.

It was shown in this study that both PS and PI play an important role in the binding of TCAs to the lipid bilayers at physiological pH and there is some specificity of TCAs-serine group interaction. Because TCAs easily permeate through the membrane, they can interact also with the PS or PI on the inner membrane surface. PS is known as an acidic phospholipid affecting the activity of many membrane enzymes (including Na^+K^+-ATPase; phospholipases; protein kinase C etc.) taking part in a signal transduction. It follows that phospholipid mediated action of TCAs on nerve signal transduction need not be quite nonspecific as was supposed and that the role of the lipid phase in the cell membrane is different, but no less important than the role of membrane proteins.

It can be concluded that the proportion of Coulomb interactions, van der Waals forces, ion-induced dipole interactions and the hydrophobic effect in apparent high-

affinity binding of TCAs to the model lipid membranes is strongly dependent on the phospholipid composition of lipid membranes; so, a various charged residue arrangement within phospholipid headgroups is the determining factor. Acidic phospholipids participate markedly in the binding at physiological pH. There is dependence of TCAs binding to lipid bilayers on the type of TCA also; the pK shifts clearly reflect effect of methylation/demethylation of the acyl side chain of TCAs. It can be supposed that the findings presented in this paper could be common to most of the CADs.

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