Characteristics of the Binding of Tacrine to Acidic Phospholipids

Jukka Y. A. Lehtonen, Marjatta Rytömaa, and Paavo K. J. Kinnunen
Department of Medical Chemistry, Institute of Biomedicine, University of Helsinki, Helsinki, Finland

ABSTRACT Tacrine (1,2,3,4-tetrahydro-9-acridinamine monohydrate) is an inhibitor of acetylcholinesterase currently used in the treatment of the symptoms of Alzheimer's disease. The present study demonstrates preferential binding of this drug to acidic phospholipids, as revealed by fluorescence polarization, penetration into lipid monolayers, and effects on the thermal phase behavior of dimyristoyl phosphatidic acid (DMPA). A fivefold enhancement in the polarization of tacrine emission is evident above the main phase transition temperature (T_m) of DMPA vesicles, whereas below T_m only a 0.75-fold increase is observed. In contrast, the binding of tacrine to another acidic phospholipid, dimyristoylphosphatidylglycerol, did not exhibit strong dependence on T_m. In accordance with the electrostatic nature of the membrane association of tacrine, the extent of binding was augmented with increasing contents of egg PG in phosphatidylcholine liposomes. Furthermore, [NaCl] $>$ 50 mM dissociates tacrine (albeit incompletely) from the liposomes composed of acidic phospholipids. Inclusion of the cationic amphiphile sphingosine in egg PG vesicles decreased the membrane association of tacrine until at 1:1 sphingosine: egg PG stoichiometry binding was no longer evident. Tacrine also penetrated into egg PG but not into egg PC monolayers. Together with broadening of the main transition and causing a shoulder on its high temperature side, the binding of tacrine to DMPA liposomes results in a concentration-dependent reduction both in the combined enthalpy ΔH of the above overlapping endotherms and the main transition temperature T_m. Interestingly, these changes in the thermal phase behavior of DMPA as a function of the content of the drug in vesicles were strongly nonlinear. More specifically, upon increasing [tacrine], T_m exhibited stepwise decrements. Simultaneously, sharp minima in ΔH were observed at drug:lipid stoichiometries of approximately 2:100 and 25:100, whereas a sharp maximum in ΔH was evident at 18:100. The above results are in keeping with tacrine causing phase separation processes in the bilayer and may also relate to microscopic drug-induced ordering processes within the membrane.

INTRODUCTION

Alzheimer's disease is a major cause of progressive dementia in aging populations. Histologically, this disease is characterized by intracerebral amyloid fibril deposition, which has been identified to be amyloid β-protein, a proteolytic cleavage product of amyloid β-precursor protein (APP) (Sisodia and Price, 1995). Defective proteolysis of APP produces hydrophobic peptides 39 to 43 amino acid residues long, with a strong tendency to form aggregates (Soreghan et al., 1994). Aβ-Peptides bind to liposomes composed of acidic phospholipids, and their binding is driven by electrostatic attraction combined with a weak hydrophobic force (Terzi et al., 1994). Furthermore, this interaction facilitates the β-sheet formation by synthetic Aβ-peptides (Terzi et al., 1994). There are reports indicating that the membrane binding of Aβ may have a role in the pathogenesis of Alzheimer's disease (Yanagisawa et al., 1995). The only drug approved for the treatment of Alzheimer's disease is tacrine (Fig. 1). Its promising therapeutic efficiency has been attributed to the inhibition of acetylcholinesterase (Adem, 1992). However, recent studies have shown that the acetylcholinesterase-inhibiting efficiency of tacrine is rather poor, thus suggesting that it may exert its beneficial effects by other mechanism(s) (Adem, 1992; Xiao et al., 1993; Dell'Antone et al., 1995).

A large number of drugs possess as a common structural motif—a hydrophobic aromatic ring system, together with a short side chain containing a protonating amino group (Hanpft and Mohr, 1985). This class of compounds binds to phospholipids and in membranes probably interacts with their negatively charged phosphate groups, whereas the aromatic moiety resides either in the interfacial region (Tsai et al., 1987) or deeper within the membrane, the exact localization depending on the distance between phenyl rings and the amino group (Young et al., 1992). Several local and general anesthetics such as lidocaine (Barthel et al., 1988) and dibucaine (Seelig et al., 1988; Seelig and Ganz, 1991), as well as various neuroleptics, e.g., chlorpromazine (Lieber et al., 1984; Luxnat and Galla, 1986) and β-blockers such as propranolol (Herbette et al., 1983; Cao et al., 1991; Rhodes et al., 1992) can be allocated to this category. Likewise, amlodipine, a calcium channel blocker, resides in the inter-

Received for publication 1 May 1995 and in final form 5 February 1996.

Address reprint requests to Dr. Paavo K. J. Kinnunen, Department of Medical Chemistry, Institute of Biomedicine, P.O. Box 8, FIN-00014, University of Helsinki, Helsinki, Finland. Tel.: 358-0-1918237; Fax: 358-0-1918276; E-mail: paavo.kinnunen@helsinki.fi.

Abbreviations used: APP, amyloid β-precursor protein; tacrine, 1,2,3,4-tetrahydro-9-acridinamine monohydrate; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol; PPHPG, 1-palmitoyl-2-[10-(pyren-1-yl)]hexanoyl-sn-glycero-3-phosphoglycerol; PPDPC, 1-palmitoyl-2[6-(pyren-1-yl)]decanoyl-sn-glycero-3-phosphocholine; DMPA, 1,2- dimyristoyl-sn-glycero-3-phosphatidic acid; egg PG, egg phosphatidylglycerol; egg PC, egg phosphatidylcholine; Sph, sphingosine; egg PE, egg phosphatidylethanolamine; brain PS, bovine brain phosphatidylserine; plant PI, plant phosphatidyl inositol; DSC, differential scanning calorimetry; ΔH_m, main phase transition enthalpy; LUVs, large unilamellar vesicles; MLVs, multilamellar vesicles; PLA_2, phospholipase A_2; Aβ, amyloid β-peptide.

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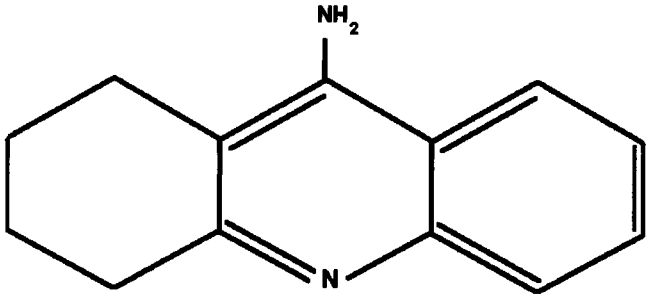

FIGURE 1 The chemical structure of tacrine.

facial region of phosphatidylcholine membranes and interacts with the lipid phosphate (Bäuerle and Seelig, 1991). One of the pharmacologically active compounds best characterized with respect to its lipid interactions is adriamycin, a widely used cytotoxic drug that binds to acidic phospholipids with a high affinity (e.g., Goormaghtigh and Ruysschaert, 1984; Mustonen and Kinnunen, 1991, 1993; Mustonen et al., 1993). It has been postulated to reside in two distinct binding sites in membranes: a superficial site and a site involving drug penetration into the hydrocarbon region of the membrane (Henry et al., 1985). Because of its chemical structure, i.e., a protonating amino group directly attached to an aromatic ringsystem (Fig. 1), tacrine should also favor partitioning into lipid bilayers. Accordingly, we investigated the interactions of this drug with phospholipids using fluorescence polarization and penetration into lipid monolayers, and studied by DSC its influence on phospholipid thermal phase behavior.

MATERIALS AND METHODS

Materials

HEPES, EDTA, DOPC, DMPC, plant PI, DOPA, egg PE, and porcine pancreatic PLA_2 were from Sigma, and egg PG, brain PS, and DMPA were from Avanti Polar Lipids (Alabaster, AL). DMPG was from Alexis (Läufelfingen, Switzerland). D-Sphingosine was purchased from Matreya (Pleasant Gap, PA). Tacrine was a generous gift from Parke-Davis Pharmaceuticals (Chicago, IL). PPHPG was from K&V Bioware (Espoo, Finland). The purity of lipids was checked by thin-layer chromatography on silicic acid-coated plates (Merck, Darmstadt, Germany) using a chloroform/methanol/water (65:25:4, v/v/v) solvent system. The concentrations of the phospholipids were determined by phosphorus assay (Bartlett, 1959) and those of cholesterol, sphingosine, and tacrine by dry weight.

Fluorescence measurements

For fluorescence polarization studies liposomes were prepared as follows. After mixing of the desired lipid compositions in chloroform the solvent was removed under a stream of nitrogen. The dry residues were then maintained under reduced pressure overnight and subsequently hydrated in 5 mM HEPES, 0.1 mM EDTA (pH 7.4) at least 10°C above the gel → liquid crystalline transition temperature (T_m) of the phospholipid in question. To obtain large unilamellar vesicles (LUVs) the dispersions were extruded through Millipore (Bedford, MA) 0.1-μm pore size polycarbonate filters (at a lipid concentration of 1.5 μmol/500 μl) using a LiposoFast low-pressure homogenizer (Avestin, Ottawa, Canada) essentially as described (Olson et al., 1979; MacDonald et al., 1991).

Fluorescence polarization measurements were carried out with an SLM 4800S spectrofluorometer equipped with Glan-Thompson calcite prism polarizers. Excitation at 330 nm and emission at 370 nm were selected with monochromators. Bandwidths of 1 and 16 nm were used for the excitation and emission beams, respectively. Emission was also monitored with a long-pass filter (350–410 nm). At the lipid:tacrine ratios used the addition of the drug did not cause changes in the absorbance of the samples. Values of steady-state fluorescence polarization P were calculated by the following equation (Lakowicz, 1983):

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}.$$

Differential scanning calorimetry

MLVs were prepared by mixing the appropriate amounts of the lipid in chloroform and tacrine in methanol. The drug-lipid mixtures were then evaporated to dryness under a stream of nitrogen, and traces of solvent were subsequently removed by evacuation under reduced pressure for at least 2 h. The dry residues were hydrated above the main transition temperature of the lipid components with 5 mM HEPES buffer (pH 7.4) containing 0.1 mM EDTA. The lipid concentration was 0.7 mM. The samples were then maintained on ice overnight before the heat capacity scans were recorded with a high-sensitivity adiabatic differential scanning calorimeter (DASM-4; Biopribor, Puschino, Russia) at a heating rate of 0.5°C/min. All samples had the same thermal history. Transition enthalpies are expressed in kilojoules per mole of phospholipid and were determined by integration of the peaks, using the internal electrical power calibration signal as a reference. The calorimeter was connected to a 486 PC via a DT01-EZ data acquisition board (Data Translation, Marlboro, MA), and data were analyzed and deconvoluted using the routines of Origin Software (Microcal, Northampton, MA). The enthalpy values given refer to the total enthalpy content of the main transition and the tacrine-induced shoulder. Deviation of the heat capacity from the baseline was taken as the beginning of the transition. Likewise, the point where the enthalpy returned back to the baseline was taken as the end of the transition. The deconvolution procedure used rests on the assumption that the observed enthotherms can be approximated as a linear combination of multiple, independent two-state transitions (Mabrey et al., 1978).

Monolayer experiments

Penetration of tacrine into egg PG monolayers was monitored using magnetically stirred circular wells (surface area, 31 cm^2; volume, 50 ml) drilled in Teflon. Surface pressure was monitored with a platinum Wilhelmy plate attached to a microbalance. Aqueous subphase was 5 mM HEPES containing 0.1 mM EDTA (pH 7.4). The lipid was spread on the air-buffer interface from a chloroform solution. All experiments were performed at ambient temperature (~24°C). The lipid monolayer was allowed to stabilize for 15 min before the injection of tacrine into the subphase. The output from the microbalance was connected to a 486 PC computer via a DT01-EZ data acquisition board, and data were analyzed by Origin Software.

Assay for phospholipase A_2

Phospholipase A_2 activity was determined by the kinetic assay described previously (Thuren et al., 1985). Liposomes were formed by rapidly injecting an ethanol solution of PPHPG (68 μl, 1.184 mM) into the buffer (1.6 ml) to yield a lipid concentration of 50 μM. Of this solution, 100-μl aliquots were pipetted into 2 ml of 5 mM HEPES buffer (pH 7.4) to obtain a final lipid concentration of 2.5 μM in the reaction mixture. The reactions were started by the addition of 50 ng of PLA_2, whereafter the progress of the phospholipid hydrolysis was followed by measuring pyrene monomer intensity at 400 nm using a Perkin-Elmer LS50 spectrofluorometer with a

magnetically stirred, thermostated cuvette compartment. The excitation wavelength was 344 nm, and the excitation and emission bandwidths were 5 nm. The assay was calibrated by adding known picomolar aliquots of (pyren-1-yl)hexanoate to the reaction mixture in the absence of the enzyme while detecting pyrene monomer emission.

RESULTS

Binding of tacrine to LUVs

Tacrine is intensively fluorescent, with excitation and emission maxima at 330 nm and 375 nm, respectively (Fig. 2). This allows its binding to liposomes to be monitored by following the increase in its emission polarization, *P*. Experiments were conducted by adding increasing concentrations of LUVs (up to a final concentration of 120 μM) to a 3.9 μM tacrine solution so as to increase the lipid:drug ratio. Association of the drug with acidic phospholipids results in a significant increase in *P* (Fig. 3). For LUVs composed of plant PI or egg PG, at a 1:30 drug:lipid molar ratio *P* increases ninefold, from 0.025 to 0.225. When the different lipids were compared, the values for *P* increased in the sequence DOPA < brain PS < egg PG < plant PI, whereas no change in emission anisotropy was seen for DOPC LUVs, indicating a lack of interaction with the latter lipid. As egg PE does not form bilayers at neutral pH, it was included in a DOPC matrix in increasing molar ratios (up to 75 mol%). However, as in the case of DOPC, no evidence for the association of tacrine with egg PE-DOPC mixtures was observed (data not shown). Likewise, the inclusion of increasing concentrations of palmitic acid (up to 75 mol%) in DOPC liposomes did not result in the membrane association of tacrine (data not shown).

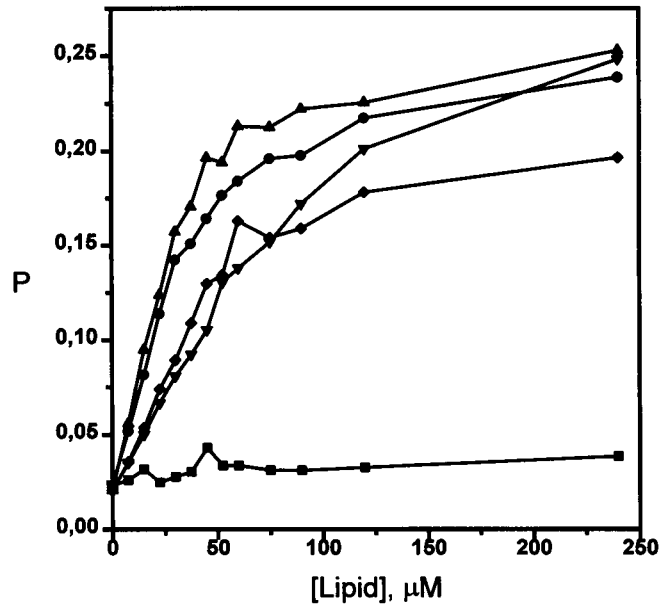

FIGURE 3 Increase in the fluorescence polarization of 3.9 μM tacrine due to increasing amounts of LUVs composed of plant PI (▲), brain PS (▼), egg PG (●), DOPA (◆), or DOPC (■). The medium was 5 mM HEPES, 0.1 mM EDTA (pH 7.4), and the temperature was 25°C.

Influence of NaCl and pH

Increasing ionic strength dissociates electrostatically membrane-bound molecules. As depicted in Fig. 4, 100 mM NaCl partly dissociates tacrine from vesicles composed of acidic phospholipids, and at 1:7.7 drug:lipid molar ratio a

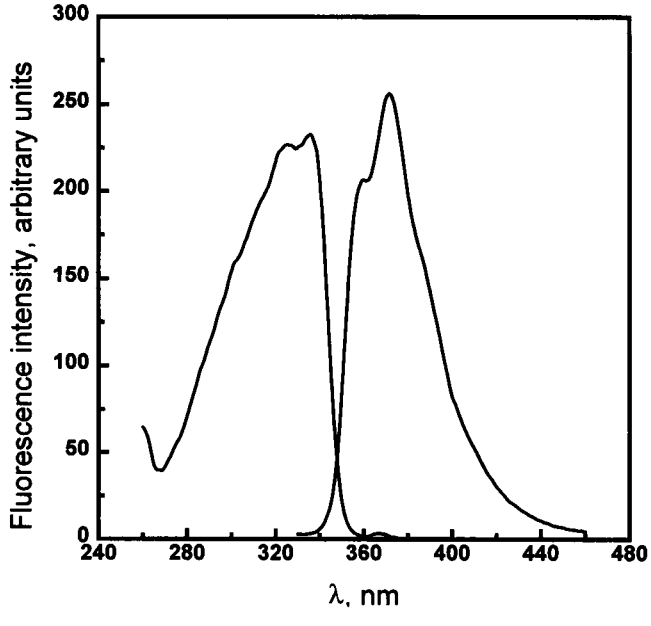

FIGURE 2 Fluorescence excitation (*left*) and emission (*right*) spectrum of 3.9 μM tacrine in 5 mM HEPES, 0.1 mM EDTA (pH 7.4). The temperature was 25°C.

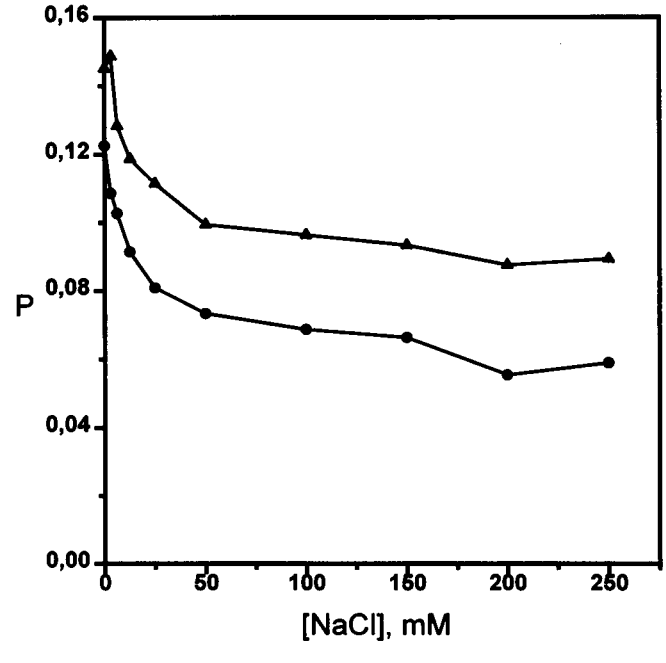

FIGURE 4 Reduction in fluorescence polarization upon increasing [NaCl]. The liposomes were prepared from egg PG (●) and plant PI (▲). Otherwise the conditions were as described in the legend for Fig. 3.

decrease from 0.123 to 0.059 in fluorescence polarization is seen. At higher salt concentrations (up to 250 mM) no further decrease in binding occurs. Changes in $[H^+]$ also influence the membrane binding of tacrine (Fig. 5). Lowering pH increases the protonation of egg PG, and the surface charge of liposomes is decreased. At pH 4.0 binding of tacrine to liposomes is diminished, whereas increasing the pH to 7.4 results in a twofold increase in fluorescence polarization, from 0.063 to 0.121. Inclusion of 150 mM NaCl decreases the membrane association of tacrine, and between pH 4 and pH 7.4 only a 1.5-fold increase is seen in P, from 0.039 to 0.060. Results similar to those above were obtained when brain PS was used instead of egg PG (data not shown).

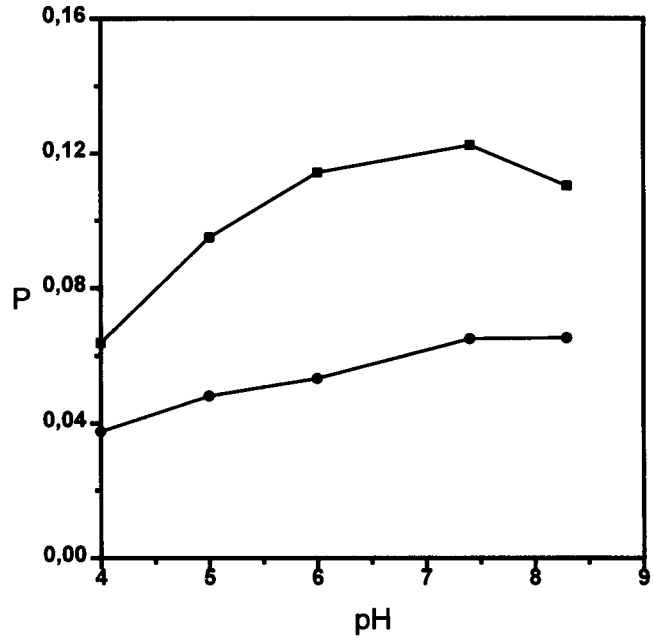

FIGURE 5 Influence of pH on the binding of tacrine to egg PG LUVs. Concentration of the drug was 3.9 μM and that of phospholipid 30 μM, corresponding to a drug:lipid ratio of 1:7.7. Binding was measured in both the presence (●) and absence (■) of 150 mM [NaCl]. Otherwise the conditions were as described in the legend for Fig. 3.

Effect of surface charge

To vary the negative surface charge density of DOPC LUVs, increasing amounts of egg PG were included. This is accompanied by an increase in the binding of the drug to liposomes, as revealed by the enhanced fluorescence polarization (Fig. 6 *A*). Compared to DOPC LUVs, and at a drug:lipid molar ratio of 1:7.7, tacrine polarization increases fivefold (to 0.14) upon association of this drug with DOPC:egg PG (1:7.7 molar ratio) LUVs. Under the same conditions as above, the inclusion of 150 mM NaCl results in a decrease in the membrane association of tacrine, and only a twofold increase in P was observed. To determine whether the glycero-phospholipid structure is essential for the membrane binding of tacrine, cholesteryl sulfate was utilized as the negatively charged lipid. Notably, for DOPC vesicles containing 50 mol% cholesteryl sulfate, the fluorescence polarization of tacrine increased to 0.155, in keeping with the membrane association of the drug (data not shown).

To neutralize the negative charge of egg PG, increasing amounts of the positively charged single-chain amphiphile sphingosine were included in LUVs. We have previously shown that Sph prevents the mainly electrostatic membrane association of cytochrome *c* and adriamycin (Mustonen et al., 1993). A similar observation was now made for the membrane association of tacrine. Addition of Sph to egg PG vesicles caused a gradual decrease in the membrane binding of tacrine, and at 1:1 egg PG:Sph ratio no binding was evident (Fig. 6 *B*).

Effects of the phospholipid phase state and cholesterol

Below the T_m of DMPA at 30°C an increase in fluorescence polarization of tacrine from 0.020 only to 0.035 was measured, whereas at 60°C (9.3°C above the T_m of DMPA) values for P increased fivefold (to 0.10) upon the membrane association of the drug (Fig. 7). This is compatible with the notion that increasing lipid surface density leads to solute exclusion (de Young and Dill, 1988). Reduced binding of several local anesthetics to DMPA membranes below T_m has been reported (Kaminoh et al., 1989). Interestingly, in contrast to DMPA, for DMPG the maximum value of P of tacrine polarization is 0.13 at both 10°C and 35°C, i.e., below and above the T_m of this phospholipid at 24°C. Tacrine did not bind to DMPC, either below (at 10°C) or above (at 30°C) its T_m at 24°C (data not shown). Cholesterol is a major constituent of cellular membranes and has profound effects on their physical properties. It has been shown to influence the membrane binding of several drugs (Auger et al., 1988). Accordingly, it was therefore of interest to study its effects on the membrane binding of tacrine. Interestingly, LUVs with increasing cholesterol:egg PG molar ratios did not differ from DOPC:egg PG vesicles in their binding of tacrine (data not shown).

Monolayer measurements

As a complementary technique allowing further insight to the binding of tacrine to phospholipids, we studied the association of this drug with monomolecular films residing on an air-water interface. The addition of tacrine to the subphase produced a rapid increase in the surface pressure ($\Delta\pi$) of egg PG monolayers (Fig. 8 *A*). Instead, no penetration of this drug into egg PC films was observed, even at low lipid packing densities, thus concealing the lack of interaction. In the range of surface pressures between 5 and 30 $mN \cdot m^{-1}$, the increase in the surface pressure due to tacrine penetration exhibited a maximum at approximately 15 $mN \cdot m^{-1}$; at higher surface pressures the penetration

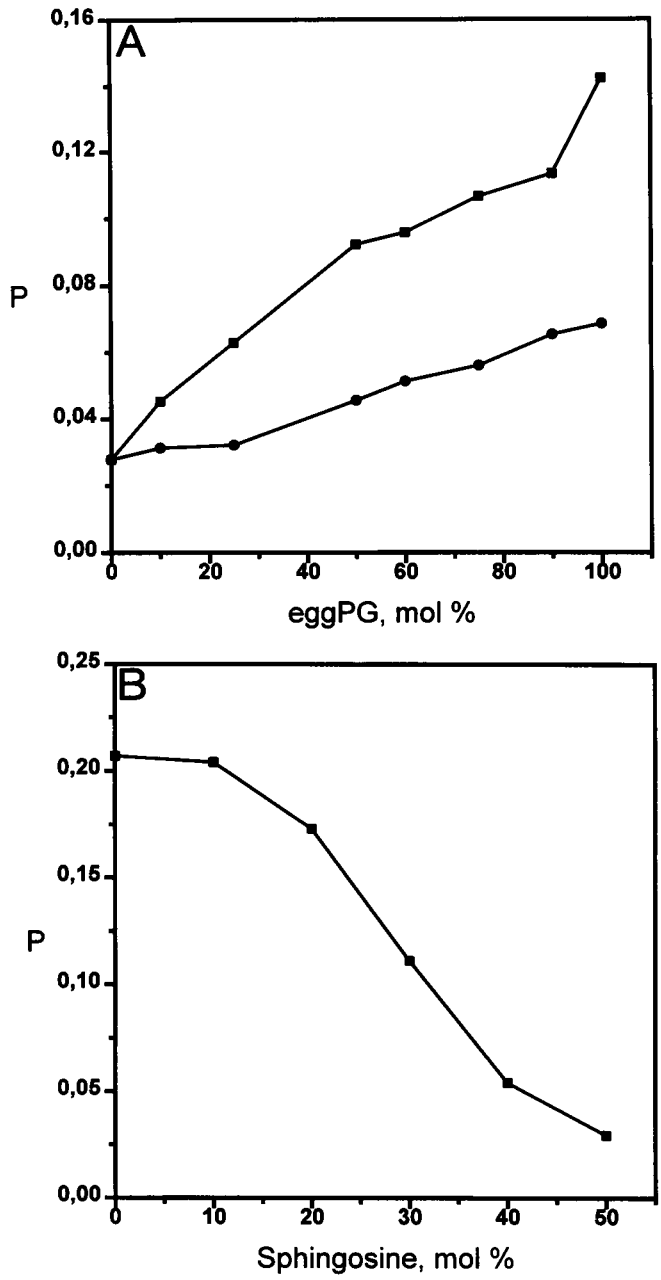

FIGURE 6 (*A*) The effect of increasing egg PG content in egg PC liposomes on the membrane association of tacrine, as measured by the increase in its fluorescence polarization. Binding was measured in both the presence (●) and absence (■) of 150 mM [NaCl]. (*B*) Reversal of membrane association of tacrine by increasing amounts of sphingosine included in egg PG LUVs. Tacrine and lipid concentrations were held constant at 3.9 μM and 120 μM, respectively.

appears to be hindered because of enhanced lateral packing of phospholipids (Fig. 8 *B*). Monolayer experiments thus support the notion of an electrostatic interaction of tacrine with acidic phospholipids, to be followed by penetration of the drug into the bilayer. In addition, these experiments clearly demonstrate that precipitation of the drug in the aqueous phase is unlikely under the conditions employed.

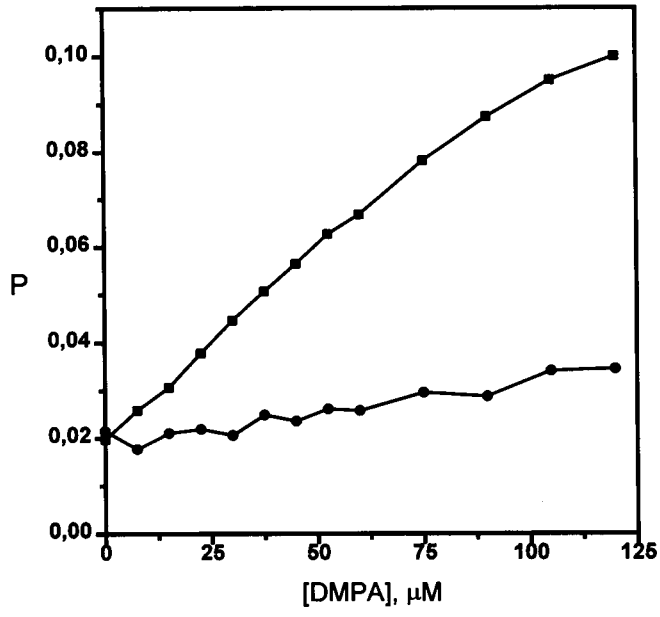

FIGURE 7 Influence of the phase state of DMPA on the membrane association of tacrine. The binding was measured both above (at 60°C, ■) and below (at 30°C, ●) the T_m of DMPA. Otherwise the conditions were as described in the legend for Fig. 3.

DSC measurements

The above data clearly show a strong binding of tacrine to acidic phospholipids. Accordingly, it was of interest to study the consequences of the drug-lipid interaction for the thermal phase behavior of the phospholipid membranes, as revealed by differential scanning calorimetry. Our data reveal the main transition of neat DMPA to be best fitted with a combination of three overlapping enthalpy peaks obeying Gaussian distribution (Fig. 9 *A*). Increasing concentrations of tacrine caused a nonlinear decrease in the main phase transition temperature T_m of DMPA (Fig. 9 *B*). A decrease in the transition enthalpy was accompanied by an increase in the half-width of the peak, in keeping with reduced cooperativity. In the presence of tacrine a shoulder on the high temperature side of the main transition peak becomes evident, thus suggesting that phase separation takes place (Fig. 9 *C*). This shoulder becomes more clearly evident at tacrine concentrations exceeding 19 mol%. Deconvolution of the peak suggests that its enthalpy content increases from 0.5 to 3.5 kJ/mol as the content of tacrine is increased from 24 to 40 mol%, respectively. The drug-induced shoulder represents a maximum of 16% of the total enthalpy ΔH. At drug:lipid ratios exceeding 1:4 a new transition at 35.2°C with an enthalpy of 1.2 kJ/mol was observed. The enthalpy of this transition increased and its temperature decreased linearly with increasing tacrine:DMPA molar ratio, and at 1:2 the transition was observed at 34.2°C with an enthalpy of 4.7 kJ/mol. In keeping with our fluorescence polarization data, increasing concentrations of tacrine up to 1:2 drug:lipid molar ratio did not influence the phase behavior of DMPC MLVs (data not shown).

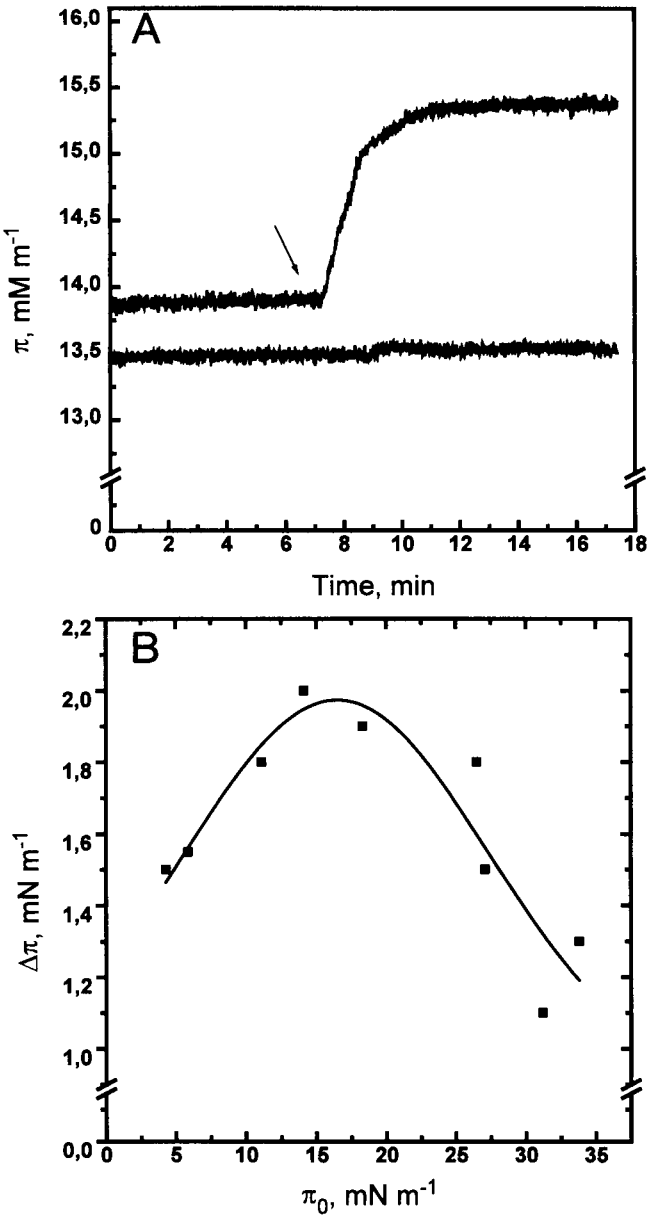

FIGURE 8 (*A*) Time course of the increase in surface pressure induced by the addition of 1 μM tacrine (*arrow*) to the buffer underneath either an egg PG (*upper trace*) or an egg PC (*lower trace*) monolayer initially at 13.8 and 13.5 mN·m^{-1}. The magnetically stirred aqueous subphase was 5 mM HEPES, 0.1 mM EDTA (pH 7.4). (*B*) Increase in the surface pressure ($\Delta\pi$) of egg PG monolayers due to the addition of 1 μM tacrine to the subphase, plotted as a function of the initial pressure π_0.

Compilation of the data obtained by DSC is shown in Fig. 10. Interestingly, in the presence of increasing contents of tacrine a strongly irregular variation both in T_m as well as in the combined enthalpy of the main transition and the drug-induced shoulder was evident. With increasing [tacrine] T_m exhibited stepwise decrements at approximately 15:100, 30:100, and 42:100 drug:lipid molar ratios (Fig. 10 *A*). Simultaneously, sharp minima in ΔH were observed at drug:lipid stoichiometries of approximately 2:100 and 25:100, the enthalpy finally plateauing

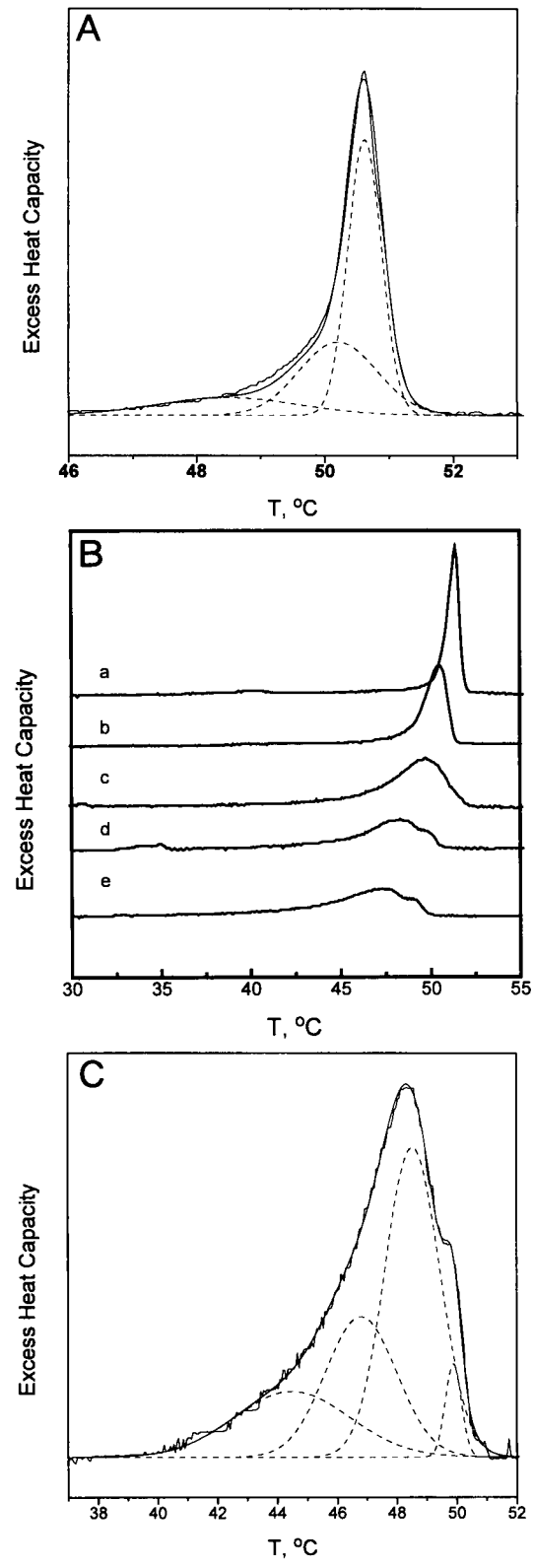

FIGURE 9 (*A*) Deconvolution of the main transition of neat DMPA by a combination of three overlapping Gaussian enthalpy peaks. (*B*) DSC thermograms for 0.7 mM DMPA in 20 mM HEPES (pH 7.4) containing 0.1 mM EDTA in the absence (*a*) and in the presence of 11.5 (*b*), 19.5 (*c*), 32 (*d*), and 45 (*e*) mol% of tacrine. (*C*) Deconvolution of the main transition of DMPA containing 28.5 mol% tacrine, using the same method as described for *A*.

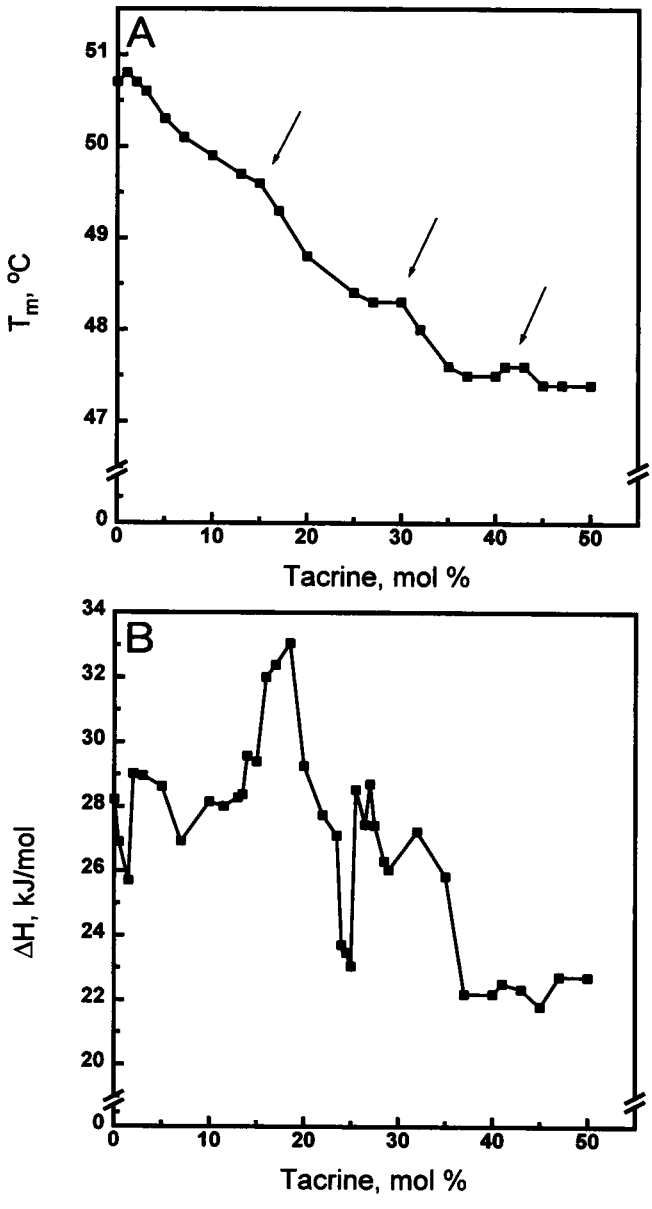

FIGURE 10 (*A*) The dependence of the main transition temperature T_m of DMPA on the concentration of tacrine. Arrows indicate the T_m steps at distinct tacrine:DMPA molar ratios. Lipid concentration was maintained constant at 0.7 mM in 20 mM HEPES (pH 7.4) containing 0.1 mM EDTA. All samples had the same thermal history. (*B*) Influence of increasing concentrations of tacrine on the total enthalpy ΔH of DMPA.

between 37:100 and 1:2. A sharp maximum in ΔH was evident at 18:100, together with less distinguished peaks at about 3:100, 26:100, and 32:100 (Fig. 10 *B*). To determine the standard error of enthalpy, measurements of 0, 13, 25, 27, 30, and 40 mol% of tacrine were studied. The standard deviation calculated from five separate DSC runs for each sample varied between 1.7% and 5.6%, and was ~1% for neat DMPA. Accordingly, the enthalpy changes shown cannot be explained by a large experimental variation.

Influence of tacrine on PLA_2 activity

The chemical structure of tacrine resembles those of chlorpromazine and lidocaine, which are both known inhibitors of PLA_2 (Kunze et al., 1976). It was thus of interest to also determine whether tacrine would affect the activity of this enzyme. Ethanol-injected PPHPG vesicles were used as a substrate, and PLA_2-catalyzed phospholipid hydrolysis was measured by monitoring the liberation of pyrene-labeled fatty acid. However, at least under these conditions the presence of tacrine (up to 1:1 drug:lipid molar ratio) had no effect whatsoever on phospholipase A_2 activity (data not shown).

DISCUSSION

The chemical structure of tacrine, a drug used in the treatment of the symptoms of Alzheimer's disease, readily suggests that this compound binds to lipids. Accordingly, we investigated this possibility by fluorescence polarization, by measuring restriction of its motion upon binding to liposomes, by the monolayer technique, as well as by quantitating drug-induced changes in the thermal phase behavior of DMPA by differential scanning calorimetry. Strong association of this drug to acidic phospholipid-containing membranes could be demonstrated, whereas little selectivity between different monovalent acidic lipids was evident. The concentration of membrane-bound tacrine increased monotonically as a function of increasing acidic phospholipid: tacrine molar ratios, as revealed by fluorescence polarization. Instead, tacrine did not bind to vesicles formed from zwitterionic lipids, such as PC. Notably, upon mixing of increasing amounts of PE into PC matrix, no binding of tacrine could be seen, suggesting that the hydration layer of PC does not provide an energy barrier preventing tacrine penetration into the bilayer. Tacrine is an amphipathic molecule, and it is thus expected to reside in the interfacial region of the bilayer. For DOPC, for instance, this zone has been estimated to account for ~50% of the total thermal thickness of the membrane (White and Whimley, 1994). However, the lack of interaction with zwitterionic phospholipids also reveals that hydrophobicity alone is not sufficient for the membrane binding of tacrine. Evidence for an electrostatic interaction with acidic phospholipids is provided by the enhanced binding of tacrine to DOPC vesicles upon increasing their egg PG content. Likewise, increasing the ionic strength of [NaCl] dissociates, although incompletely, tacrine from the acidic egg PG, thus suggesting two different types of tacrine-binding sites in the membrane, as is the case for adriamycin. In keeping with electrostatic forces being important, charge neutralization of egg PG in the plane of the membrane by the positively charged amphiphile sphingosine completely reverses the membrane association of tacrine. The penetration of tacrine into egg PG monolayers is in accordance with the location of the drug in the interface. Notably, the glycerophospholipid structure is inessential for the electrostatic association of tacrine to

liposomes, and efficient binding is also observed with DOPC membranes containing cholesteryl sulphate.

Interestingly, the binding of tacrine to DMPA is temperature dependent and is significantly reduced below T_m. This can be understood to result from the increased lateral packing of DMPA in the gel state. Diminished penetration into lipid monolayers has been shown for a number of compounds (e.g., adriamycin) at increasing lipid packing densities (Mustonen and Kinnunen, 1991). In contrast to DMPA, tacrine binds to DMPG equally effectively, both below and above T_m, thus suggesting that below T_m the association of tacrine with membranes also has specificity with respect to the polar headgroup structure. This difference might be due to the different orientation of the headgroup phosphate moieties of PG and PA. On the other hand, effective and tight hydrogen-bonded networks have been suggested to be formed by PA (Eibl and Woolley, 1979), whereas similar tight association of the headgroups due to hydrogen bonding should not be possible for PG. Such hydrogen bonding should be augmented below T_m of DMPA and result in diminished membrane association of tacrine, whereas binding to DMPG would not be similarly affected by the transition.

Binding of tacrine (drug:lipid stoichiometry $< 1:2$) to membrane decreased both T_m and ΔH, thus indicating weakened intermolecular interactions between DMPA molecules. However, membranes of different tacrine-DMPA stoichiometries exhibit strongly nonlinear thermal behavior as a function of the content of drug. More specifically, at critical drug:lipid molar ratios, structures with either higher or lower melting enthalpies are formed. Likewise, stepwise decrements in T_m were evident at well-defined tacrine:DMPA stoichiometries. As far as we know irregular thermal phase behavior such as that reported here for tacrine:DMPA complexes has not been observed previously for drug-lipid interactions.

The appearance of the shoulder on the high temperature side of the main transition is indicative of phase separation. The shoulder becomes more pronounced at tacrine concentrations exceeding 19 mol%, and its enthalpy increases upon increasing tacrine concentration, so as to constitute a maximum of approximately 16% of the total transition enthalpy ΔH. Accordingly, it cannot explain the irregular variation in ΔH seen as a function of tacrine concentration.

The relationship between a global thermodynamic quantity such as heat capacity and phase diagrams and membrane microstructure is not trivial. The observed changes in T_m and total enthalpy content of the endotherms upon varying the drug:lipid stoichiometry relate to the "melting" of the lattices formed. The fact that these changes take place abruptly at fairly well-defined drug:lipid molar ratios could indicate the presence of structured drug:lipid alloys, the actual organization being dependent on the relative proportions of the constituents. Yet, the DSC data do not give any insight into the actual structures formed. Notably, a high degree of ordering in binary membranes would not be without precedent.

Evidence for the formation of superstructures in liquid crystalline cardiolipin/phosphatidylcholine membranes was reported by Berclaz and McConnell (Berclaz and McConnell, 1981; Berclaz and Geoffrey, 1984). Membrane partial specific volumes as well as lipid lateral diffusion rates are altered in a strongly nonlinear manner by varying cholesterol:phospholipid stoichiometry (Rubenstein et al., 1979, 1980; Recktenwald and McConnell, 1981; Carruthers and Melchior, 1986). To this end, regular distribution of sterols in liquid crystalline phosphatidylcholine vesicles has recently been demonstrated (Chong, 1994; Tang et al., 1995). Our laboratory provided evidence for the regular distribution of the pyrene-labeled phospholipid analog PPDPC in binary liquid crystalline lipid mixtures and Langmuir-Blodgett films (Somerharju et al., 1985; Kinnunen et al., 1987). Likewise, dips in the pyrene excimer-to-monomer fluorescence emission intensity ratio observed for DMPC liposomes containing increasing amounts of PPDPC (Tang and Chong, 1992; Chong et al., 1994) were interpreted to result from concentration-dependent organization of pyrene-labeled acyl chains into hexagonal superlattices at critical mole fractions (Kinnunen et al., 1987; Virtanen et al., 1988; Sugar et al., 1994). Regular distribution is believed to arise from the long-range repulsive interaction between PPDPC molecules due to elastic deformation of the lipid matrix around the bulky pyrene moiety acting as a substitutional impurity (Kinnunen et al., 1993). The existence of regular patterns is determined by the balance between the energy minimization due to maximum separation of the impurities and their entropy-driven randomization. Somewhat analogously to the above, we interpreted our resonance energy transfer measurements on the membrane association of cytochrome *c* in terms of the formation of hexagonal arrays of protein on liposome surfaces (Mustonen et al., 1987).

As is the case for pyrene- and cholesterol-induced perturbation of lipid packing in phosphatidylcholine liposomes, the binding of tacrine to the DMPA polar headgroup and its partial penetration into the bilayer should cause both steric perturbation as well as disruption of the hydrogen-bonded network of DMPA. Accordingly, the different alloys formed at the different drug:lipid stoichiometries by tacrine and DMPA (the former residing in the interface) should each posses different melting temperatures and enthalpies. However, at this stage of our studies the above should be considered as a working hypothesis only, and further data are clearly warranted to substantiate this possibility. Notably, phase separation and possible formation of structured alloys due to membrane-associated drug are not mutually exclusive.

The preferential association of tacrine with acidic phospholipids demonstrated in the present study opens novel possibilities of elucidating its therapeutic mechanism of action. Unfortunately, although drug-lipid interactions are abundant, their importance has remained open. Yet, the in vitro evidence accumulated on membranes and drug-membrane interactions during the last 10 years implies that

membrane binding could mediate a wide range of effects. For instance, for pathophysiological processes such as multidrug resistance of cancer cells, a role for drug-lipid interactions has been suggested (Escriba et al., 1990; Canaves et al., 1991; Callaghan et al., 1993; Wadkins and Houghton, 1993). Likewise, the mechanism of action of adriamycin has been related to its lipid interactions, as it is cytotoxic without entering the cell (Tritton and Yee, 1982). This effect could be explained by the activation of phospholipases A_2 by adriamycin (Mustonen and Kinnunen, 1991). Mobility of lipids in membranes is important for the function of several membrane-associated enzymes and has been shown to be altered by a number of drugs (Siegfried et al., 1983; Wright and White, 1986). Likewise, thickness of the membrane has emerged as an important parameter modulating the structure and function of several integral membrane proteins (Cornea and Thomas, 1994). To this end, it is of interest that high concentrations of chlorpromazine have been demonstrated to increase the thickness of erythrocyte membranes (Lieber et al., 1984). Membrane might serve also as a concentrator, facilitating the binding of drugs and peptide hormones to their receptors (Rhodes et al., 1985; Herbette et al., 1986; Sargent and Schwyzer, 1986).

Binding of drugs to phospholipids could also modulate the membrane interactions of peripheral proteins, as shown for chlorpromazine, which prevents the membrane association of vinculin (Ito et al., 1983). The pathophysiology of Alzheimer's disease and its progression centrally involves amyloid formation, resulting from the accumulation of a hydrophobic peptide Aβ of a known amino acid sequence (Marotta et al., 1992). Strong enhancement of the aggregation of this peptide by acidic phospholipids has recently been observed (Terzi et al., 1994). Accordingly, binding of tacrine to acidic phospholipids might interfere with the interaction of Aβ-peptide with negatively charged phospholipids. This possibility is currently being investigated in our laboratory.

The authors thank Birgitta Rantala for skillful technical assistance. We also wish to thank M.D./Ph.D. student Juha Holopainen for a comprehensive literature survey of Alzheimer's disease.

This study was supported by the Finnish State Medical Research Council, Biocentrum Helsinki, the Sigrid Juselius Foundation, and the Faculty of Medicine of the University of Helsinki (PKJK), and the Finnish Medical Foundation and the M.D./Ph.D. program of the University of Helsinki (JYAL), as well as by the 350 Years Anniversary Foundation of the University of Helsinki (MR).

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