0026-895X/93/010129-13$03.00/0

MOLECULAR PHARMACOLOGY, 44:129-141

Interaction of Tricyclic Drug Analogs with Synaptic Plasma Membranes: Structure-Mechanism Relationships in Inhibition of Neuronal Na^+/K^+-ATPase Activity

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Received March 11, 1993; Accepted April 23, 1993

SUMMARY

Perturbations of rat brain synaptic plasma membrane (SPM) bilayer structure and Na^+/K^+-ATPase activity were correlated for drugs that are structurally related and exhibit similar toxicological side effects but belong to different pharmacological classes. Na^+/K^+-ATPase IC_{50} values decrease linearly with increasing octanol/water partition coefficients (log-log plot) for a series of dimethylethylamine-containing drugs (i.e., chlorpromazine, amitriptyline, imipramine, doxepin, and diphenhydramine), emphasizing hydrophobicity in inhibition. However, nortriptyline and desipramine are 1.2 log units less hydrophobic than their *N*-methylated parent drugs but more potent inhibitors. To investigate this, bilayer surface structure was examined by the binding of the fluorophore 1-anilinonaphthalene-8-sulfonic acid (ANS) to SPMs. The dissociation constant and wavelength maximum of ANS are invariant with drug binding; however, the limiting fluorescence intensity of ANS (F_∞) is increased. Such data indicate that these cationic drugs bind to the membrane surface, increasing the number but not the polarity of ANS binding sites by canceling charge at anionic phospholipid groups. More importantly, there is a close linear correlation between the concentrations of drugs necessary to increase F_∞ by 40% and the IC_{50} values, with full compensation for the *N*-demethylated drugs. This correlation implies that drug-induced increases in SPM-bound ANS fluorescence are a better predictor of Na^+/K^+-ATPase inhibition than are octanol/water partition coefficients and that electrostatic interactions are also involved in inhibition. Furthermore, it points toward similar mechanisms of biomembrane surface interaction governing both inhibition and fluorescence change that are common to these drugs. K^+-dependent *p*-nitrophenylphosphatase activity is inhibited with the same potency as Na^+/K^+-ATPase activity, indicating that inhibition may involve drug interaction near the K^+ binding sites. Furthermore, chlorpromazine, diphenhydramine, and dimethylaminopropyl chloride alter K^+-activation of K^+-dependent *p*-nitrophenylphosphatase, progressing from noncompetitive through mixed to competitive inhibition as their hydrophobicity changes, and these mechanisms are consistent with steric hindrance of K^+ binding. In contrast to the ANS data, decreases in 1,6-diphenyl-1,3,5-hexatriene fluorescence anisotropy induced by these drugs do not correlate with Na^+/K^+-ATPase inhibition, and drug *N*-demethylation enhances inhibition without altering anisotropy; both findings indicate that Na^+/K^+-ATPase activity is not predominantly influenced by changes in bulk fluidity. Taken together, these data suggest that electrostatic interactions at the biomembrane surface between the protonated amino group of the drug and anionic groups on the enzyme and/or phospholipids near the K^+ binding sites are crucial to inhibition and that drug hydrophobicity modulates the number and orientation of these interactions.

Drug interaction with the biomembrane influences bilayer structure, which, in turn, can modulate processes ranging from membrane-bound enzyme activity and receptor binding to membrane permeability and transport. Membranes have been implicated as targets for the pharmacological actions of a variety of structurally diverse compounds, leading to one explanation for general anesthesia. Although proteins have also been considered as targets, there has been recent added evidence for the importance of an interaction involving the bilayer. Specifically, there is no pressure reversal of the effects of anesthetic agents in firefly luciferase, which is a well studied model for protein-targeted anesthetic inhibition (1). More generally, drugs may interact at multiple biomembrane sites differing substantially from their design targets (e.g., receptors), and such interactions may be important in eliciting many of the

This work was supported by National Institute on Alcohol Abuse and Alcoholism Grant AA06935 (B.B.M.), Individual National Research Service Award Predoctoral Fellowship AA05285 (M.A.C.), and institutional funds. Portions of this work were presented at the American Society for Biochemistry and Molecular Biology Meeting, New Orleans, LA (1990), and the Society of Toxicology Meeting, Miami Beach, FL (1990).

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ABBREVIATIONS: SPM, synaptic plasma membrane; ANS, 1-anilinonaphthalene-8-sulfonic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; IC_{50}, concentration of drug necessary to inhibit enzyme activity by 50%; K^+-*p*NPPase, K^+-dependent *p*-nitrophenylphosphatase; F_∞ 40%, concentration of drug necessary to increase the F_∞ of ANS by 40%.

toxicological side effects of the drugs (2). The significance of drug-induced membrane perturbation is often difficult to assess due to the lack of structural specificity that would be expected of drugs that bind stereochemically to receptor sites (3). However, the binding to biomembranes of a small mole fraction of drugs from many different pharmacological classes can cause isothermal shifts in the gel-fluid transition (4), changes in the bilayer dielectric constant (5), alterations of ion permeability (6), and/or modification of the annular boundary phospholipids (7). The latter modification has been linked to alteration in the function of membrane-bound enzymes, but the details of this link are poorly understood. An important approach to elucidating functional alteration is to investigate the effects of a series of structurally related drugs on a physiologically crucial membrane-bound enzyme and examine correlations between drug-induced changes in biomembrane structure and function. The present study correlates the effects of the structurally related tricyclic drug analogs[2] shown in Fig. 1 on SPM bilayer structure and on the activity of the SPM-bound Na^+/K^+-ATPase.

The group of drugs chosen for this study are linked by two important characteristics, i.e., similar chemical structure with systematic variation and similar toxicological side effects that differ from their primary pharmacological function. The tricyclic drug analogs are characterized structurally by a mostly planar, hydrophobic moiety separated by a linear chain of 5–6 Å from a charged secondary or tertiary amine. The most well studied example of these drugs is the phenothiazine chlorpromazine, which appears to bind to biomembranes through both hydrophobic and electrostatic interactions (8, 9), leading to impaired function (10). Although structural parallels among drugs from different pharmacological classes are not routinely emphasized in the literature, a molecular mechanism of biomembrane interaction based on such hydrophobic and electrostatic components may occur with more drugs than expected. For example, the antiarrythmic propranolol has structural characteristics related to those of the phenothiazines and binds somewhat similarly to biomembranes (11) but has a different primary pharmacological mechanism of action. The same mode of binding may also take place with the local anesthetic tetracaine (12). However, the relationships between the structure of these drugs and their molecular mechanism of biomembrane perturbation are still unclear.

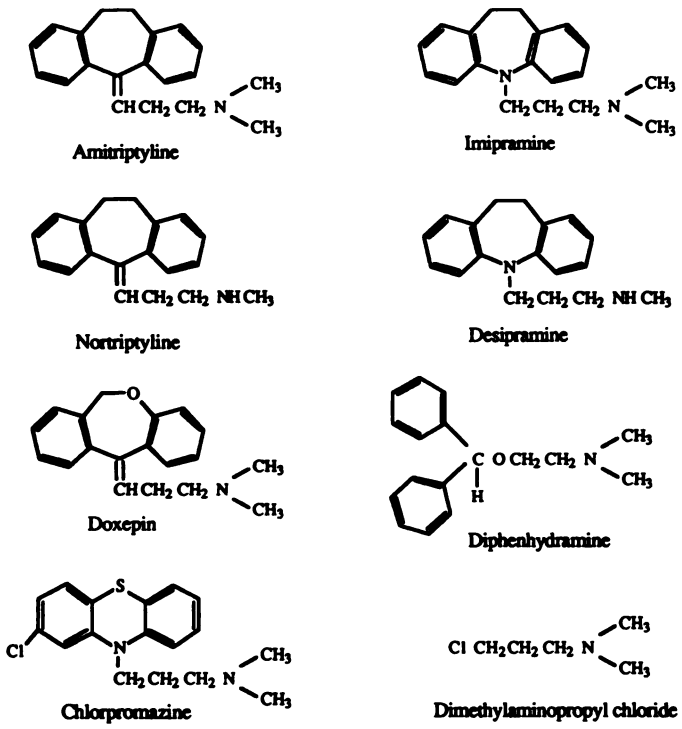


Fig. 1. Chemical structures of the tricyclic drug analogs investigated in this study and of dimethylaminopropyl chloride. The latter compound is similar in structure to the amine-containing portion of these drugs.

Often, amphipathic drugs from different pharmacological classes have similar chemical structures and exert similar, overlapping, toxicological side effects. Certain tricyclic antidepressant, phenothiazines, and antihistaminergics can produce pulmonary phospholipidosis, local anesthesia, acute sedation, and psychomotor impairment (13–15), apparently interacting independently of their primary pharmacological target sites. These drugs also exhibit membrane-stabilizing activity (16). Although it is not the goal of this study to relate the similar side effects to a specific membrane or enzyme perturbation, their existence emphasizes the importance of investigating structure-activity relationships among drugs of similar amphipathic, amine-containing structure.

Na^+/K^+-ATPase is a multisubunit enzyme responsible for neuronal homeostasis by the active transport of Na^+ and K^+ through biomembranes. The ionic gradient that is formed is crucial to cellular function and as much as 70% of available ATP may be utilized for its maintenance (17). Transport is affected by an incompletely characterized, complex reaction cycle including phosphorylation-linked extrusion of Na^+, dephosphorylation-linked uptake of K^+, and a rate-limiting step with a conformational ($E_2 \rightarrow E_1$) transition (18). Binding of K^+ is inhibited competitively by NH_4^+ and short-chain protonated amines, and their IC_{50} values are relatively high (19). Recent studies (20) have focused both on the gate mechanism of the K^+ binding sites, which involves ordered ion release, suggesting some level of site inequivalency, and on the gate-blocking effects of amines. The partial reaction cycle involving K^+-dependent dephosphorylation of the E_2 conformation is routinely studied using the substrate *p*-nitrophenylphosphate, yielding K^+-*p*NPPase activity (21). Although little is known concerning the details of Na^+/K^+-ATPase-phospholipid interactions, enzyme activity is also strongly influenced by changes in its phospholipid microenvironment (22–24) and by its association with negatively charged phospholipids (25). It has been suggested that the neurotoxic effects of amiodarone (26, 27), perhexiline (28), and Adriamycin (29) are through modulation of Na^+/K^+-ATPase activity caused by perturbation of SPM bilayer structure. Both the potential of Na^+/K^+-ATPase as a site for drug toxicity and its critical role in a number of physiological processes emphasize the importance of evaluating the effects of the tricyclic drug analogs on this enzyme.

ANS and DPH are fluorophores routinely used to quantitate drug-induced changes in biomembrane structure (30–33). ANS binds noncovalently, through mostly hydrophobic interactions, to the membrane surface near the level of the phosphate groups of the phospholipids (34), whereas DPH partitions into the interior of the bilayer (35). The fluorescence spectrum of ANS

[2] The drugs shown in Fig. 1 belong to three different drug classes (i.e., tricyclic antidepressants, phenothiazines, and antihistamines). However, in this study these drugs are collectively referred to as tricyclic drug analogs because of their structural similarities.

is extremely sensitive to changes in its microenvironment. More specifically, three parameters that can be monitored using ANS to investigate surface structure are 1) the wavelength maximum of membrane-bound ANS, which indicates the polarity of the fluorophore microenvironment, 2) the apparent dissociation constant of ANS from the biomembrane, and 3) the limiting fluorescence intensity extrapolated to infinite ANS concentration, which with certain generally accepted assumptions is proportional to the relative number of ANS binding sites on the biomembrane (36, 37). This proportionality has been found in a variety of studies with monovalent metal cations and amine-containing drugs and results from their binding to the membrane surface, which cancels negative charge on phospholipids, enhancing association of the anionic ANS (38, 39). At commonly used ANS concentrations, the fluorescence intensity of ANS bound to biomembranes is dominated by that portion originating from interaction with phospholipids versus proteins. This dominance originates with the larger number of ANS binding sites on the former, the low quantum yield of ANS bound to the latter, and the saturation of protein sites at low ANS concentrations [see Slavik (34) for an extensive review of ANS-biomembrane studies]. In contrast to investigation of the membrane surface, the anisotropy of DPH fluorescence is dependent upon the rotational mobility and extent of alignment of acyl groups in the membrane interior and is used to quantitate the "fluidity" of biomembranes (40). Drugs that perturb the biomembrane are known to cause structural alterations that modify the microenvironment of ANS, DPH, or both.

The important relationships between changes in biomembrane enzyme activity and bilayer structure have been difficult to ascertain, because membrane-bound enzymes such as Na^+/K^+-ATPase have little or no activity after complete lipid extraction (3) and because artificial phospholipid vesicles are not usually accurate models for drug binding to biomembranes (41). Psychotropic drugs may modulate both the activity of membrane-bound enzymes and the fluidity of biomembranes over the same concentration ranges (42–44). However, this parallel is not always observed (45), and little is known concerning its molecular mechanism or to what extent changes in fluidity may be secondary to more specific hydrophobicity- or charge-based interactions. Furthermore, most of the studies examining the effects of amphipathic drugs on bilayer structure were performed on artificial membranes (46, 47) and thus without simultaneous quantitation of enzyme activity. These difficulties emphasize the importance of the present study on SPMs, which focuses on an enzyme crucial to neuronal function that is bound to its native membrane. Here the tricyclic drug analogs are used as probes of a prototype enzyme that is transmembranous, pumps ions, and requires (acidic) phospholipids for maximal activity. What is found is a predictive correlation between drug-induced changes in Na^+/K^+-ATPase activity and membrane surface structure that suggests a similar molecular mechanism of biomembrane surface interaction governing both processes common to the tricyclic drug analogs. The potency of these drugs to cause inhibition and structural change is modulated by their regional hydrophobicity as well as the *N*-demethylation of their dimethylethylamino group. The detailed analysis given below suggests that electrostatic interactions between the protonated amino group of the drug and anionic groups located at the membrane surface are crucial to enzyme inhibition. However, the hydrophobicity of the aromatic rings of the drug strongly modulates the number of drug molecules bound to the membrane, their orientation, and the position of their amino groups with respect to the inhibition sites on the enzyme. Increased membrane fluidity, as quantitated by DPH anisotropy, does not appear to correlate directly with enzyme inhibition, and enhanced inhibition by drug demethylation does not alter DPH anisotropy. Molecular modeling and computational chemistry provide a clear picture of the structural relationships between the amphipathic drugs and phospholipids that supports the mechanism of fluorescence enhancement and enzyme inhibition on a molecular level.

Experimental Procedures

Materials. Male Sprague-Dawley rats weighing 190–225 g were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and fed a standard laboratory diet with water *ad libitum*. The animals were maintained in a room with constant temperature and a 12-hr light/dark cycle for at least 3 days before sacrifice. Chlorpromazine, amitriptyline, imipramine, doxepin, nortriptyline, desipramine, diphenhydramine, dimethylaminopropyl chloride (all as hydrochloride salts), Tris *p*-nitrophenylphosphate, and ouabain were purchased from Sigma Chemical Co. (St. Louis, MO). ATP was purchased from Boehringer Mannheim (Indianapolis, IN). ANS and DPH were obtained from Molecular Probes (Eugene, OR). All other chemicals were of at least reagent grade.

SPM preparation. Rats were sacrificed with CO_2 gas immediately before decapitation and the cerebral cortex was isolated. SPMs were prepared according to the flotation-sedimentation procedure of Jones and Matus (48). The SPMs obtained from the 28.5–34% sucrose interface were pelleted and resuspended in 5 mM Tris buffer (pH 7.5 at 22°) to give a final protein concentration of approximately 1.0 mg/ml. This purification procedure did not involve detergents or other perturbants routinely used for high levels of enrichment, in an attempt to preserve both a more native plasma membrane structure (annulus) and the usual population of neighboring proteins. Thus, the simultaneous exposure of the Na^+/K^+-ATPase to detergents and drugs did not occur in the present study. Protein concentration was determined by the method of Lowry *et al.* (49), using bovine serum albumin as the standard. SPMs were rapidly frozen and stored in liquid nitrogen until assayed. The 28.5–34% sucrose interface contained cleared membrane vesicles devoid of mitochondria and myelin (as verified by electron microscopy) and was enriched in SPM marker enzyme activity (Na^+/K^+-ATPase) 3.84 ± 0.16-fold over homogenate.

Assay of Na^+/K^+-ATPase and K^+-*p*NPPase activities. Na^+/K^+-ATPase activity in SPMs was measured at 37° using a modification of the method described by Forbush (50). The assay mixture contained 120 mM NaCl, 25 mM KCl, 4 mM $MgCl_2$, 60 mM Tris buffer (pH 7.40 at 37°), 1 mM EDTA, 4 mM Na_2ATP, and 16.2 μg (27.0 μg/ml) of membrane protein, in a total volume of 600 μl. The assay was initiated by the addition of ATP and was stopped after 10 min by the addition of an acidic solution of sodium dodecyl sulfate and ammonium molybdate. Inorganic phosphate was determined by measuring the absorbance of the phosphate-molybdate complex at 706 nm on a Hewlett Packard 8450A diode array spectrophotometer. Na^+/K^+-ATPase activity was calculated as the difference between total ATPase activity and that remaining after ouabain (2 mM) inhibition. The mean control Na^+/K^+-ATPase activity averaged over a large number of preparations was 75.1 ± 1.3 μmol of P_i/hr/mg of protein.

K^+-*p*NPPase activity was measured at 37° in a 600-μl assay mixture composed of 60 mM Tris buffer (pH 7.40 at 37°), 4 mM $MgCl_2$, 1 mM EDTA, 10 mM Tris *p*-nitrophenylphosphate, and 16.2 μg (27.0 μg/ml) of membrane protein (51). The assay was initiated by the addition of Tris *p*-nitrophenylphosphate and was stopped after 10 min. Inorganic phosphate was then assayed as described above. K^+-dependent activity was calculated as the difference between activities in the absence and

presence of 25 mM KCl. The mean control K^+-*p*NPPase activity was 15.1 ± 0.4 μmol of P_i/hr/mg of protein.

In both assays, SPMs were preincubated for 10 min at 37° either with or without drug in 60 mM Tris buffer before the reaction was started. Preliminary studies (not shown) established that Na^+/K^+-ATPase and K^+-*p*NPPase activities were linear with respect to protein concentration and incubation time. The SPM preparation did not possess latent Na^+/K^+-ATPase activity that could be unmasked with sodium dodecyl sulfate (0.01–0.9 mg of detergent/mg of SPM protein). Furthermore, none of the drugs interfered with the quantitation of inorganic phosphate over the concentration ranges examined in this study.

Enzyme kinetic analysis of K^+-*p*NPPase inhibition. The effect of various concentrations of K^+ (1.5, 2, 4, 6, and 10 mM) on chlorpromazine-, diphenhydramine-, and dimethylaminopropyl chloride-induced inhibition of K^+-*p*NPPase activity was measured using the assay described above. K^+ binds cooperatively to Na^+/K^+-ATPase, resulting in curved Lineweaver-Burk plots in both the absence and presence of drugs. The degree of apparent cooperativity (n) was calculated from the slope of the Hill plot obtained from $\log(v/(V_{max} - v))$ versus log $[K^+]$, where v is the initial velocity. The V_{max} used in the Hill plot was calculated from the y intercept of the second-order polynomial fit of the curved Lineweaver-Burk plot. The n value was then used to linearize the data by employing the following modified form of the Lineweaver-Burk equation (52):

$$\frac{1}{v} = \frac{K_m}{(V_{max}[K^+]^n)} + \frac{1}{V_{max}} \quad (1)$$

The plots of the kinetic data were fit to straight lines using regression analysis. The apparent V_{max} and K_m values were calculated from the y-intercept and slope of the regression line, respectively. The mechanism of enzyme inhibition was classified as either competitive, noncompetitive, uncompetitive, or mixed, based on the linearized plot intercepts. The effects of various concentrations of *p*-nitrophenylphosphate (1.0, 1.25, 1.7, 2.5, and 5.0 mM) on drug-induced inhibition of K^+-*p*NPPase activity was also quantitated. In this case, however, the Lineweaver-Burk plots were linear, with an n value of unity.

Measurement and quantitation of ANS fluorescence. The enhancement of fluorescence intensity caused by ANS binding to SPMs in the absence and presence of drugs was measured and analyzed using well established procedures (53). ANS was excited at 385 nm and emission spectra were recorded from 400 to 600 nm at 37° with a SLM 8000C spectrofluorometer. Fluorescence was measured in 60 mM Tris buffer (pH 7.40) with 81.0 μg (270 μg/ml) of membrane protein in 3 ml. Various concentrations of drugs were added to the cuvette with the same preincubation as used in the enzyme assays. Next, a 10-μl aliquot of a 3.0 mM ANS solution was titrated into the cuvette, the solution was mixed by inversion, and the emission spectrum was recorded after 5 min. This procedure was repeated with up to five additions of ANS, resulting in a series of final concentrations ranging from 10 to 50 μM. The maximum fluorescence intensity and its accompanying wavelength maximum (λ_{max}) were recorded after each addition of ANS. In the absence of SPMs, none of the drugs showed significant effects on the ANS spectrum. Fluorescence intensity was not time dependent from 1 to 15 min after mixing (data not shown). Fluorescence intensity measurements were corrected for dilution, background counts using buffer containing SPMs, and the fluorescence of ANS in the absence of SPMs.

The apparent dissociation constant (K_{app}) and limiting fluorescence intensity (F_∞) for ANS were determined by a Scatchard-like analysis using the following equation (54):

$$\frac{F}{[ANS]} = \frac{F_\infty}{K_{app}} - \frac{F}{K_{app}} \quad (2)$$

F is defined as the relative fluorescence intensity at a given ANS concentration and [ANS] is the molar fluorophore concentration. The Scatchard-like plot of $F/[ANS]$ versus F yields a slope that is equal to the negative reciprocal of K_{app} and a y intercept of F_∞/K_{app}. Multiple sets of binding sites with different values of K_{app} or interactions between sites are usually indicated by a departure from linearity. Drug-induced alterations in the binding of ANS to the SPMs are likely to be revealed as a change in K_{app}, F_∞, and/or λ_{max}, as has been shown in previous studies (30, 32).

Fluorescence anisotropy measurements. Changes in SPM-bound DPH fluorescence polarization induced by the addition of different drugs were quantitated as anisotropy at 37°. An SLM 8000C spectrofluorometer equipped with Glan-Thompson polarizers in the T format was used to simultaneously measure the parallel ($I_\parallel$) and perpendicular ($I_\perp$) components of the fluorescence emission of DPH. The emission beams passed through Schott KV long-pass filters with a cutoff of 418 nm. DPH was excited at 360 nm and the emission was read at 430 nm. The anisotropy ($\bar{r}$) of DPH was calculated (with the appropriate standard correction) using the equation (35):

$$\bar{r} = \frac{(I_\parallel - I_\perp)}{(I_\parallel + 2I_\perp)} \quad (3)$$

SPMs (81.0 μg) were suspended in 3 ml of 60 mM Tris buffer (27.0 μg/ml), and DPH was dissolved in tetrahydrofuran. A 1.5-μl aliquot of DPH was added to the membrane suspension, yielding a fluorophore concentration of 0.5 μg/ml (33). The suspension was incubated for 15 min in the dark at 37°, with occasional mixing and constant stirring, and then control anisotropy values were measured. Next, aqueous drug solution was added (5–50 μl) and equilibrated for at least 15 min with constant stirring before measurement of anisotropy. Subsequent drug additions were at the same time interval. Anisotropy did not change upon dilution, indicating no significant depolarization from light scattering or as an artifact of drug addition. The effect of chlorpromazine on anisotropy was not measured, because chlorpromazine quenches fluorescence and alters the lifetime of DPH (55).

Molecular modeling and computational chemistry. The relative orientation and interaction energy of tricyclic drug analogs and phospholipid molecules were examined using an IRIS 4D/220 GTX Silicon Graphics computer (Silicon Graphics Computer Systems, Mountain View, CA) running Quanta (Polygen, Waltham, MA) software. Particular attention was given to the question of whether electrostatic and hydrophobic interactions between drug and phospholipid would be sterically allowable and simultaneously maintained with energy minimization. The energy of the drug/phospholipid interaction was calculated using molecular mechanics (CHARMm force field), which evaluates the sum of the energy contributions from bond stretching, bond rotation, dihedral angles, improper angles, electrostatic interactions, and van der Waals forces (56). The drug was "docked" between two phosphatidylcholine molecules in a manner generally consistent with interactions and orientations implied by X-ray diffraction and NMR studies of propranolol and local anesthetics, respectively (11, 12). Energy minimization was then performed using 300 steps of conjugate-gradient and at least 500 steps of adopted Newton-Raphson algorithms leading to a low energy conformation. The drug was repeatedly repositioned and energy was again minimized until an apparent minimum energy conformation was obtained.

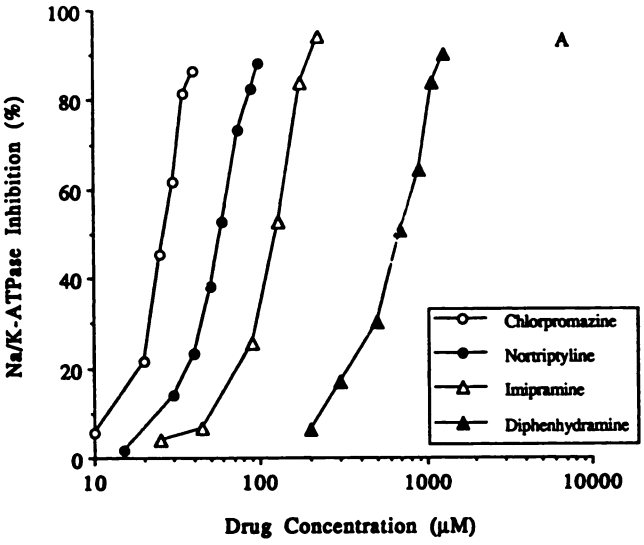
Statistical analysis. All experiments were performed with at least three different SPM preparations, and data are presented as the mean ± standard error of the mean. Correlation coefficients were calculated to compare Na^+/K^+-ATPase IC_{50} values, K^+-*p*NPPase IC_{50} values, F_∞ 40% values, and drug octanol/water partition coefficients. Student's t tests were used where appropriate. The level of statistical significance was chosen as $p \leq 0.05$.

Results

Inhibition of Na^+/K^+-ATPase activity. The effects of the series of structurally related tricyclic drug analogs shown in Fig. 1 (i.e., chlorpromazine, amitriptyline, imipramine, doxepin, diphenhydramine, nortriptyline, and desipramine) on SPM-bound Na^+/K^+-ATPase activity were determined. Representa-

tive concentration-response curves of Na^+/K^+-ATPase to chlorpromazine, imipramine, nortriptyline, and diphenhydramine are shown in Fig. 2A; all of the drugs inhibit Na^+/K^+-ATPase activity in a concentration-dependent manner. The Na^+/K^+-ATPase IC_{50} values range from 29 ± 2 μM (chlorpromazine) to 690 ± 15 μM (diphenhydramine). The SPM preparation used in this study was inhibited 4–9 times more potently (depending on drug) than found with the less highly purified synaptosomal preparations examined by others (57, 58).

For drugs with a fixed dimethylethylamino group and varying ring structure (i.e., chlorpromazine, amitriptyline, imipramine, doxepin, and diphenhydramine), there is a close linear relationship ($r = 0.99$, $p < 0.01$) between drug-induced inhibition of Na^+/K^+-ATPase activity (IC_{50}) and octanol/water partition coefficient, when both are plotted on a logarithmic scale (Fig. 2B). However, nortriptyline and desipramine deviate substantially from this relationship. *N*-Demethylation of amitriptyline and imipramine (resulting in nortriptyline and desipramine) causes the expected decreases in their octanol/water partition coefficients, by more than 1 log unit. However, contrary to the usual trends found with decreasing hydrophobicity (59), demethylation makes these two drugs much more potent inhibitors of Na^+/K^+-ATPase than would be expected from their octanol/water partition coefficients and even increases their inhibitory potency above that of their parent compounds. There is no correlation between drug pK_a and IC_{50} values. Octanol/water partition coefficients (measured at pH 7.4) were obtained from Moffat (60) and represent the ionized form of the drugs.



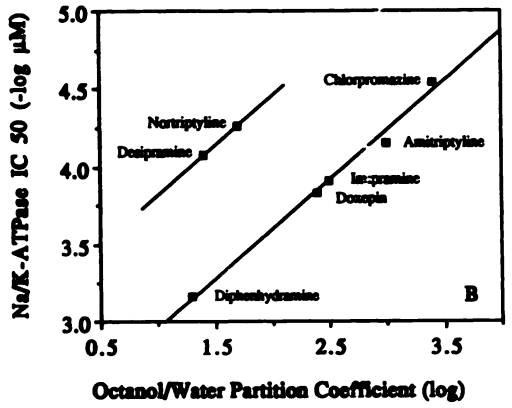
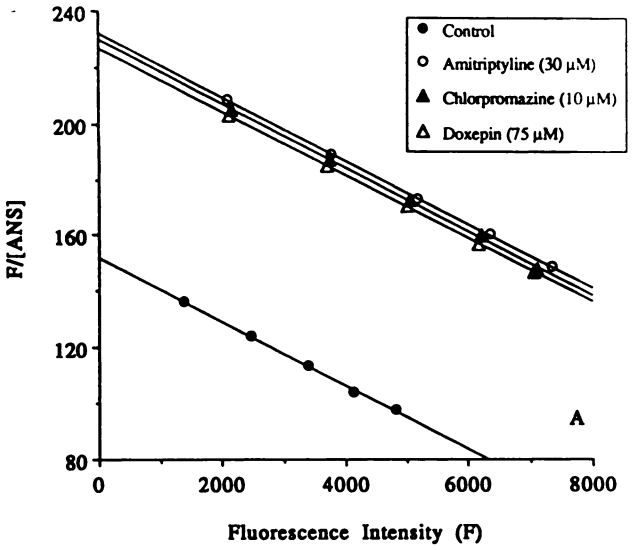


Fig. 2. Representative concentration-response curves for Na^+/K^+-ATPase inhibition by tricyclic drug analogs (A), and the relationship between drug IC_{50} values and octanol/water partition coefficients (B). The SPMs were preincubated with different drugs or with buffer alone before the Na^+/K^+-ATPase activity assay was initiated. Inorganic phosphate released was quantitated colorimetrically at 760 nm. These methods are described in detail in Experimental Procedures. Average Na^+/K^+-ATPase IC_{50} values were determined from at least three concentration-response curves from separate membrane preparations.

ANS fluorescence. The perturbation of SPM surface structure by the tricyclic drug analogs was quantitated through their effect on the equilibrium binding of the fluorophore ANS to the biomembrane. Titration of ANS into a solution of SPMs yields a hyperbolic increase in ANS fluorescence emission. More specifically, each drug increases the F_∞ of ANS without changing K_{app} (78 ± 4 μM) or λ_{max} (483 ± 2 nm) over the concentration ranges used in this study. Representative Scatchard-like plots of ANS titrations in the absence of drug (control) and in the presence of chlorpromazine, amitriptyline, and doxepin are shown in Fig. 3A. The concentration of drug necessary to increase the F_∞ of ANS 40% over control (F_∞ 40%) was determined for each drug. The increase in F_∞ induced by 30 μM amitriptyline was used as a positive control in all experiments involving the determination of F_∞ 40%. When F_∞ 40% and Na^+/K^+-ATPase IC_{50} values are compared, it is apparent that these drugs increase the number of ANS binding sites and inhibit Na^+/K^+-ATPase activity over the same concentration ranges. Furthermore, the relationship between F_∞ 40% and the octanol/water partition coefficient (Fig. 3B) shows correlations (for the dimethylethylamine-containing drugs) and deviations (for their demethylated forms) strikingly similar to those found with inhibition of Na^+/K^+-ATPase activity (Fig. 2B). To evaluate more precisely the relationship between drug-induced changes in membrane structure and Na^+/K^+-ATPase activity, values of F_∞ 40% were plotted versus those of Na^+/K^+-ATPase IC_{50} (Fig. 4). The result is a close linear correlation ($r = 0.99$, $p < 0.01$) for all drugs studied, with full compensation for the demethylated drugs.

Inhibition of K^+-*p*NPPase activity. All of the drugs inhibited K^+-*p*NPPase activity in a concentration-dependent manner. The K^+-*p*NPPase IC_{50} values were determined from the concentration-response curves and plotted versus those of Na^+/K^+-ATPase in Fig. 5. The linear least squares fit of the data ($r = 0.99$, $p < 0.01$) has a slope of 1.01 and a y intercept of nearly zero, showing that these drugs are equally potent inhibitors of K^+-*p*NPPase and Na^+/K^+-ATPase activities.

Enzyme kinetic analysis of K^+-*p*NPPase inhibition. Chlorpromazine, diphenhydramine, and dimethylaminopropyl chloride each inhibit K^+ activation of *p*NPPase activity by different mechanisms (Fig. 6). Typical of competitive inhibition, the addition of 10 mM dimethylaminopropyl chloride increases the K_m from 6.4 ± 0.3 to 8.0 ± 0.4 mM without altering V_{max} (18.4 ± 0.5 versus 18.2 ± 0.5 μmol of P_i/hr/mg). Chlorpromazine addition (25 μM) decreases V_{max} from 18.4 ± 0.5 to 10.8 ± 0.3 μmol of P_i/hr/mg without altering the K_m (6.4 ± 0.3 versus 6.7 ± 0.3 mM), indicative of noncompetitive inhibition. Furthermore, the presence of diphenhydramine (700 μM) causes a mixed type of inhibition by changing both the K_m from 6.4 ± 0.3 to 15.1 ± 0.6 mM and the V_{max} from 18.3 ± 0.5 to 7.3 ± 0.6 μmol of P_i/hr/mg. None of the compounds alter the apparent



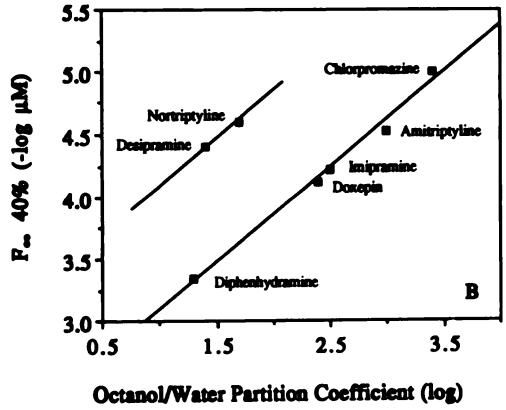


Fig. 3. Representative Scatchard-like plots of ANS binding to SPMs in the absence and presence of tricyclic drug analogs (A), and the relationship between the F_∞ 40% values and drug octanol/water partition coefficients (B). The SPMs were preincubated with different drugs or with buffer, in a manner paralleling the activity assays, before the ANS binding titration was started. Relative fluorescence intensities were measured at 480 nm and after appropriate correction were plotted as shown. Values of F_∞ were determined from the intercepts of the Scatchard-like plots in both the absence and presence of different drugs. Sufficient drug was added to increase F_∞ values 40% over control. Additional details about the experiment and calculations are given in Experimental Procedures. Average F_∞ 40% values were determined from at least three separate membrane preparations and were plotted versus drug octanol/water partition coefficients.

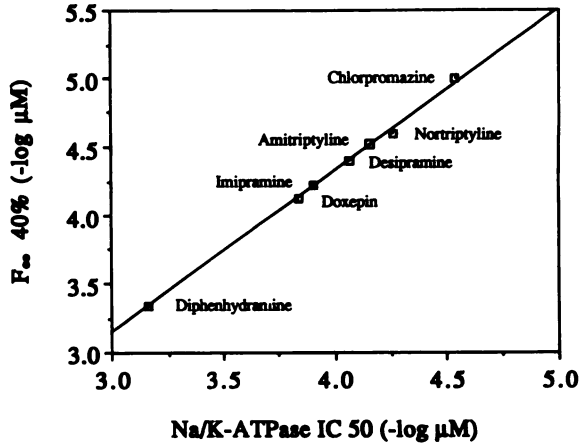


Fig. 4. Linear correlation between Na^+/K^+-ATPase IC_{50} and F_∞ 40% values for the tricyclic drug analogs. This plot includes nortriptyline and desipramine, which deviate substantially from the linear correlations in Figs. 2 and 3.

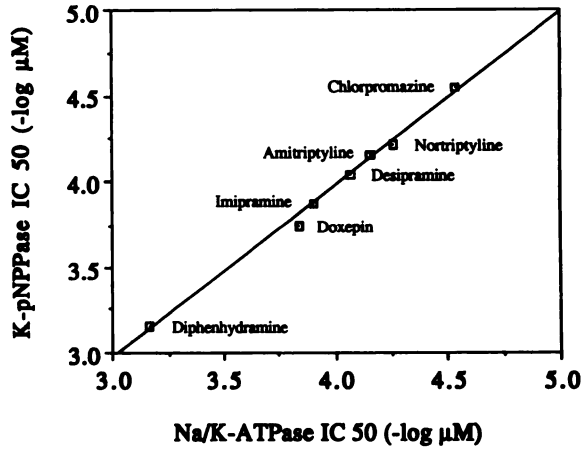


Fig. 5. Linear correlation between Na^+/K^+-ATPase and K^+-*p*NPPase IC_{50} values for tricyclic drug analogs. Na^+/K^+-ATPase IC_{50} values were taken from Fig. 2. K^+-*p*NPPase activity was assayed after preincubation with different drugs or with buffer alone by quantitating release of inorganic phosphate, as described in Experimental Procedures. All of the drugs inhibited K^+-*p*NPPase activity in a concentration-dependent manner, and average IC_{50} values were determined from at least three concentration-response curves from separate membrane preparations.

number of K^+ binding sites (n = 1.65 ± 0.05), as calculated from Hill plots. All observed mechanisms of inhibition are consistent with an inhibitor sterically hindering the binding of a substrate molecule (52). In addition, Fig. 7 shows that chlorpromazine, diphenhydramine, and dimethylaminopropyl chloride all inhibit *p*-nitrophenylphosphate activation of *p*NPPase activity noncompetitively and noncooperatively.

DPH anisotropy studies. The effect of the tricyclic drug analogs on the fluorescence anisotropy of SPM-bound DPH was quantitated as a measure of drug-induced alteration in the fluidity of the membrane interior. Amitriptyline, imipramine, doxepin, and diphenhydramine each decrease the anisotropy of DPH (i.e., increase fluidity) in a concentration-dependent manner (Fig. 8A) over concentration ranges similar to those that inhibit Na^+/K^+-ATPase activity. However, the order of potency in increasing fluidity (amitriptyline > doxepin > imipramine > diphenhydramine) is different from that of Na^+/K^+-ATPase inhibition, K^+-*p*NPPase inhibition, and enhancement of ANS fluorescence, which are the same. To investigate in more detail potential correlations between changes in membrane fluidity and inhibition, the anisotropy of DPH was measured for each drug at the concentration necessary to produce a 25% decrease in Na^+/K^+-ATPase activity. Fig. 8B shows that, when these anisotropy values are plotted versus Na^+/K^+-ATPase IC_{25} values for the undemethylated drugs (i.e., those differing in ring hydrophobicity only), no correlation is found.

The effect of drug demethylation on SPM-bound DPH anisotropy was also investigated. Table 1 shows that demethylation of amitriptyline and imipramine causes a 21 and 32% decrease, respectively, in Na^+/K^+-ATPase IC_{50} values. However, equal concentrations (70 μM) of amitriptyline and its

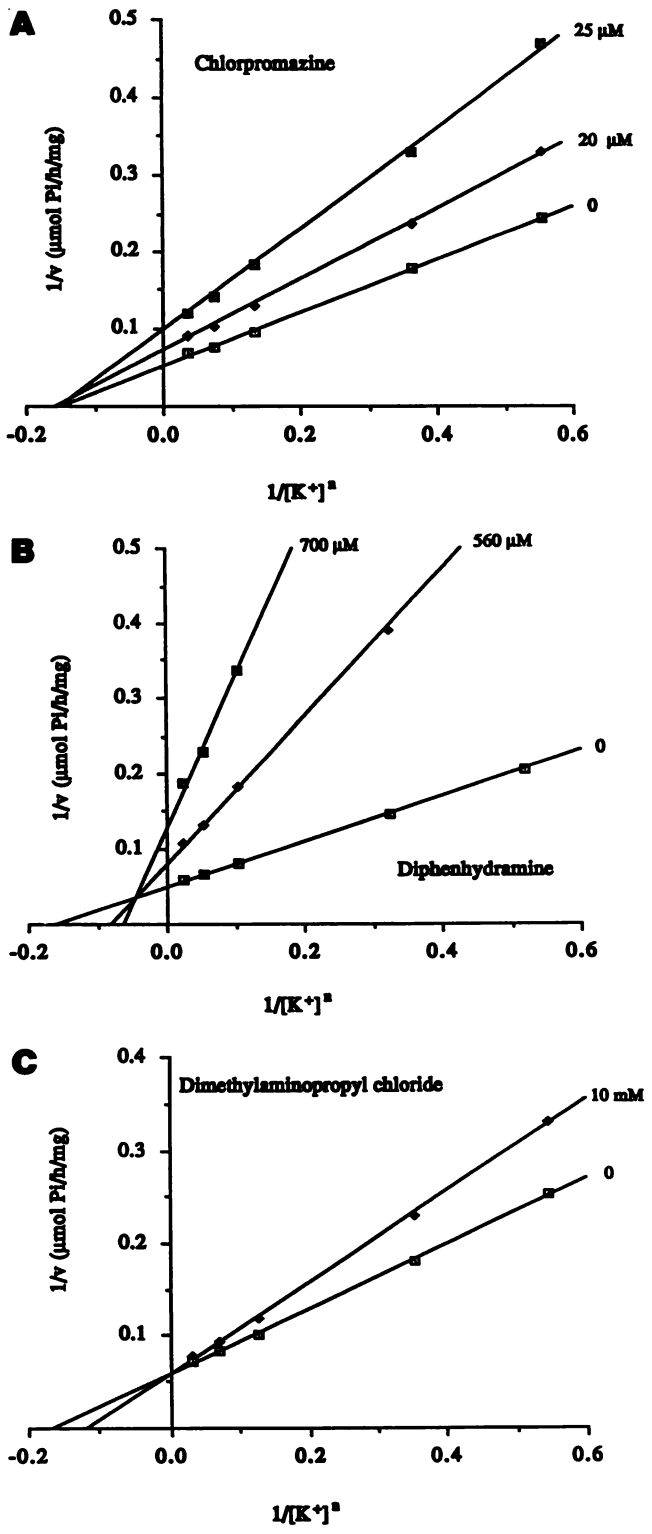


Fig. 6. Lineweaver-Burk plots of drug-induced inhibition of K^+-pNPPase activity in SPMs with variation of the concentration of the substrate K^+. K^+-pNPPase activity was assayed after preincubation with different compounds or with buffer alone, by quantitating release of inorganic phosphate. These plots were nonlinear unless the K^+ concentration was raised to the power $n = 1.65 \pm 0.05$. This average value was determined from Hill plots for the control data only and was found also to linearize data with different drug concentrations. Details of the assay procedure and data analysis are given in Experimental Procedures. Inhibition varies from noncompetitive for chlorpromazine (A) through mixed for diphenhydramine (B) to competitive for dimethylaminopropyl chloride (C).

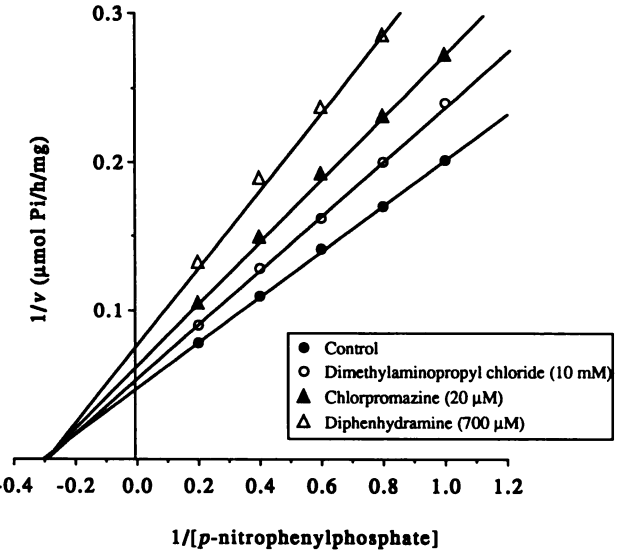


Fig. 7. Lineweaver-Burk plots of drug-induced inhibition of K^+-pNPPase activity in SPMs with variation of the substrate p-nitrophenylphosphate. K^+-pNPPase activity was assayed after preincubation with different compounds or with buffer alone, by quantitating release of inorganic phosphate. Details of the assay procedure and data analysis are given in Experimental Procedures. In all cases examined the inhibition is noncompetitive.

demethylated form (nortriptyline) produce the same percentage of decrease in DPH anisotropy. Similarly, equal concentrations (125 μM) of imipramine and desipramine induce the same membrane-disordering effect. Demethylation of the dimethylpropylamino group of the drug apparently changes enzyme activity through a perturbation at the membrane surface and not its interior. Furthermore, the addition of 10 mM dimethylaminopropyl chloride does not change the anisotropy of DPH ($\bar{r} = 0.1991 \pm 0.002$ versus the control value of 0.2002 ± 0.0015) but produces a 25% inhibition of Na^+/K^+-ATPase activity, which shows that inhibition can occur without DPH-detected perturbation of the bilayer interior.

Molecular modeling and computational chemistry. The total energies of several possible conformations of the diphenhydramine/phosphatidylcholine complex were calculated using the CHARMm force field. The lowest energy conformation is given in Fig. 9 and shows the protonated amine of the drug close to the anionic oxygen of the phospholipid, with the hydrophobic rings projecting into the lipophilic acyl chains. The linear chain connecting the rings and the amine is relatively extended by these electrostatic and hydrophobic interactions. The inclusion of specific water molecules would likely attenuate somewhat the electrostatic interaction and thus enhance the relative importance of the hydrophobic interaction in drug binding. A similar conformation was found for the entire series of tricyclic drug analogs.

Discussion

Many drugs are known to inhibit a variety of physiological and enzymatic processes with potency that varies linearly with octanol/water partition coefficient (58, 59). Na^+/K^+-ATPase activity is inhibited in this manner for a series of tricyclic drug analogs that differ only in ring hydrophobicity, as shown in the present study with rat SPMs (Fig. 2B) and by Roufogalis (58) using the nondemethylated forms of some of the same drugs

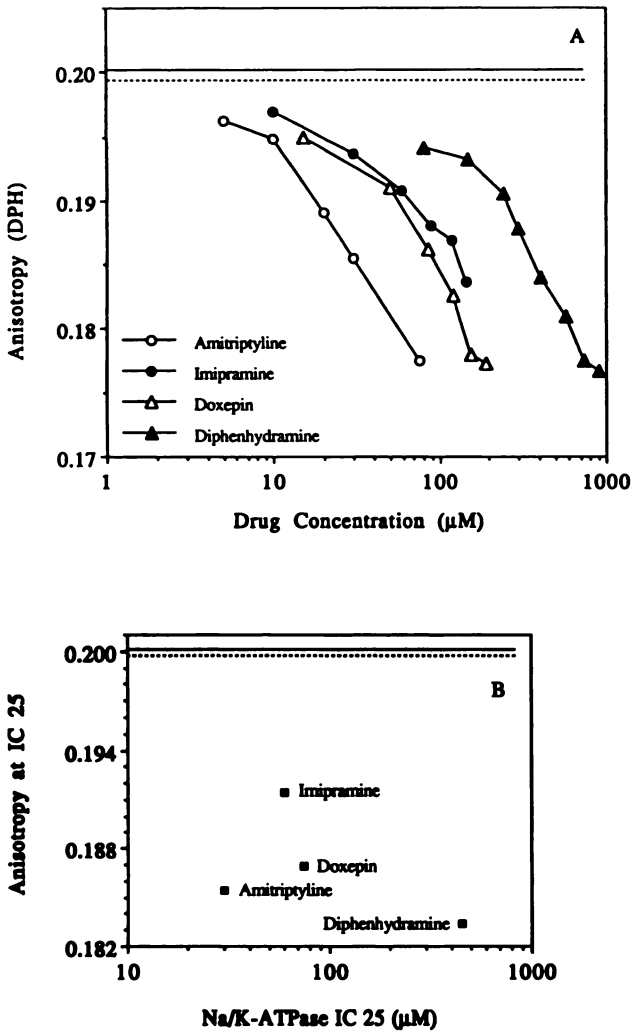


Fig. 8. Representative concentration-response curves of SPM-bound DPH anisotropy for tricyclic drug analogs (A), and the relationship between DPH anisotropy and Na^+/K^+-ATPase inhibition (B). DPH was incorporated into SPMs by incubation for 15 min at 37° before titration with drugs. The intensities of the parallel and perpendicular components of DPH fluorescence were measured at 430 nm and used to calculate anisotropies. Details of the assay procedure and data analysis are given in Experimental Procedures. The anisotropy of DPH was measured at the Na^+/K^+-ATPase IC_{25} concentration for each drug and plotted versus the IC_{25} value itself. ——, Mean control anisotropy; - - -, standard error of the mean. Anisotropies are an average from at least three separate membrane preparations.

with a crude microsomal fraction of bovine brain cortex. This linear relationship emphasizes the importance of drug hydrophobicity in enzyme inhibition. Furthermore, using this relationship, the decrease in octanol/water partition coefficient of 1.2 log units with drug demethylation predicts a nearly 10-fold decrease in inhibitory potency for nortriptyline versus amitriptyline and desipramine versus imipramine. However, the *N*-demethylated forms of amitriptyline and imipramine are more potent inhibitors of Na^+/K^+-ATPase activity than are their methylated forms (Table 1). Indeed, the effect of demethylation differed from that predicted by merely a shift in the octanol/water partition coefficient axis equivalent to the loss of one methyl group (Fig. 2B). These data indicate that inhibition of Na^+/K^+-ATPase activity is dependent not only on drug octanol/water partition coefficient but also on factors such as regional hydrophobicity and localized versus dispersed charge distribution. In agreement with the latter, a limited investigation of Na^+/K^+-ATPase inhibition with small aliphatic amines showed that secondary amines were less potent than primary amines (61), and the quaternary ammonium form of chlorpromazine was found to be a less potent inhibitor of dopamine binding to the D-1 receptor than was the unmodified form (62). Furthermore, increases in pharmacological and toxicological activities of nortriptyline and desipramine over their parent compounds follow the same trends (10, 63, 64). Finally, the tricyclic drug analogs are protonated at pH 7.4, which also implies a nonspecific role for charge, in that these drugs should not penetrate fully into the hydrophobic interior of the bilayer. Indeed, tetracaine binds to artificial membranes at different depths, depending upon the presence or absence of its cationic charge (65). Thus, it is likely that the tricyclic drug analogs inhibit Na^+/K^+-ATPase activity through perturbation at or near the biomembrane surface. Such perturbation may include the simultaneous interactions of one drug molecule with both anionic and hydrophobic groups on the enzyme as well as the phospholipids (i.e., within the annulus). In addition, it is unlikely that the tricyclic drug analogs inhibit Na^+/K^+-ATPase activity through permanent disorganization of SPMs, lipid peroxidation, or free radical generation, because previous studies indicated that inhibition is reversible upon washing of biomembranes exposed to psychotropic drugs (43, 58).

The interaction of the tricyclic drug analogs with the SPM surface was investigated using equilibrium binding of the fluorophore ANS. A number of factors can modulate ANS fluorescence in a membrane suspension. Drug-induced alteration of the ANS emission spectrum could originate with changes in the binding affinity of ANS for the biomembrane (K_{app}), in the relative number of binding sites available (F_∞), and/or in the microenvironment of the bound fluorophore (λ_{max}). The drugs in this study produced a concentration-dependent increase in F_∞, whereas K_{app} and λ_{max} remained unchanged. The invariant K_{app} indicates no change in ANS binding strength. However, the increase in F_∞ along with the unchanged λ_{max} indicates that these drugs perturb SPM bilayer surface structure by increasing the number but not the polarity of ANS binding sites. A tricyclic drug analog-induced increase

TABLE 1
Effects of amitriptyline and imipramine *N*-demethylation on Na^+/K^+-ATPase inhibition and SPM fluidity

	Amitriptyline	Nortriptyline	Imipramine	Desipramine
Na^+/K^+-ATPase IC_{50} (μM)	70 ± 3	55 ± 5	125 ± 5	85 ± 5
DPH anisotropy at the IC_{50} values of the nondemethylated drugs (i.e., 70 and 125 μM)[a]	0.1760 ± 0.002	0.1756 ± 0.003	0.1843 ± 0.003	0.1842 ± 0.002

[a] The control DPH anisotropy value was 0.2002 ± 0.0015.

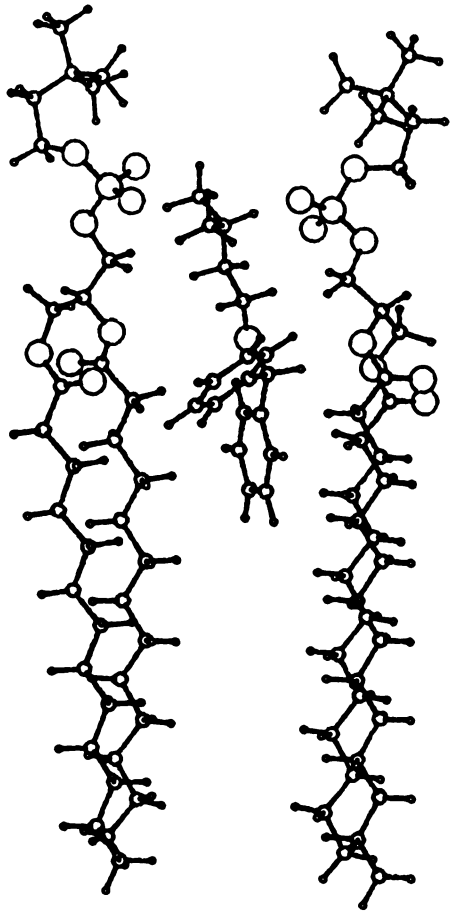


Fig. 9. Interaction of the tricyclic drug analog diphenhydramine with phosphatidylcholine simulated by molecular modeling and computational chemistry techniques. The drug was positioned between two phosphatidylcholine molecules in an initial configuration generally consistent with propranolol and local anesthetic binding to bilayers. The energy of the molecules was then minimized using molecular mechanics calculations performed with a Silicon Graphics 4D/220 GTX computer running Quanta software. Details of the positioning and minimization are given in Experimental Procedures. A similar conformation was calculated for the entire series of tricyclic drug analogs.

in the number of ANS binding sites without alteration of the quantum yield is in agreement with a large number of previous studies that have characterized the effects of monovalent metal cations, NH_4^+, local anesthetics, and chlorpromazine on binding of ANS to membranes (30, 38, 54). ANS preferentially binds to phosphatidylcholine molecules with the nonpolar portion of the fluorophore submerged toward the acyl chains and the sulfonate group projecting outward at the level of anionic oxygens of the polar head groups (66, 67). The drug-induced increase in F_∞ is governed by drug binding to the membrane, allowing its protonated amino group to partially cancel phospholipid negative charge. Molecular modeling and other techniques strongly implicate electrostatic interactions between phosphate oxygen atoms and the drug (see below). This charge cancellation, in turn, increases the number of negatively charged ANS molecules that bind to the bilayer surface. The association of cationic metal and NH_4^+ ions with the membrane surface is both weak and electrostatically driven (38), but the binding of amine-containing drugs can have an increasingly important hydrophobic component, depending upon their structure of the drugs. Specifically, the tricyclic drug analogs are approximately 2–4 orders of magnitude more potent in increasing F_∞ than is NH_4^+ (F_∞ 40% = 100 mM), and creation of ANS binding sites varies linearly with ring hydrophobicity (Fig. 3B). These two factors emphasize the importance of the hydrophobic rings of the drugs in anchoring the protonated amino group to the biomembrane, which, in turn, results in enhanced surface charge cancellation that creates more binding sites for ANS. Anchoring and orientation are also implied by the observed drug-induced change in SPM fluidity, whereas dimethylethylaminopropyl chloride does not induce such a change.

The scenario just described is supported strongly by molecular modeling and computational chemistry techniques. Fig. 9 is representative of the tricyclic drug analogs and shows that they bind to phospholipids in a minimum energy conformation that aligns their protonated amino group close to the anionic oxygen of the phospholipid and projects their hydrophobic rings into the acyl chains. These two fundamental interactions provide the basis for the enhanced ANS fluorescence as well as the variation of F_∞ 40% with drug hydrophobicity. Furthermore, this calculated conformation is generally consistent with NMR studies of the binding of similar amphipathic drugs to artificial membranes, which show both drug-induced immobilization of the polar head groups by electrostatic interactions and expansion of the membrane surface at the level of the second or third methylene carbon (8, 68). Direct interaction between phospholipid phosphate oxygens and the quaternary ammonium group of a tricyclic antidepressant analog was found with NMR spectroscopy (9), and the same was evident from neutron diffraction studies of propranolol in phospholipid bilayers (11).

The changes in F_∞ 40% values induced by these drugs show precisely the same correlations and deviations, with respect to octanol/water partition coefficient, as do changes in Na^+/K^+-ATPase IC_{50} values (Figs. 2B and 3B). Apparently, both membrane surface perturbation and enzyme inhibition are enhanced by increased drug hydrophobicity and by localized versus dispersed positive charge on, and/or *N*-demethylation of, the dimethylethylamino group of the drugs. Furthermore, there is a close linear correlation between F_∞ 40% and Na^+/K^+-ATPase IC_{50} values for all drugs studied, with full compensation for their *N*-demethylated forms (Fig. 4). This correlation provides three important pieces of information; 1) tricyclic drug analog-induced increases in ANS fluorescence are a better predictor of Na^+/K^+-ATPase inhibition than is the octanol/water partition coefficient, 2) similar combinations of hydrophobic and electrostatic interactions modulate both the drug-induced increase in the binding of ANS to phospholipids and the inhibition of this enzyme, and 3) regional hydrophobicity is more important than overall hydrophobicity in prediction of inhibition. It has been shown that drug hydrophobicity can be a major determinant of binding to artificial membranes; however, those studies had less systematic structural variation in the drugs that were examined and larger deviations from linearity than are found in the present study (69, 70). In addition, the increase in both ANS binding and enzyme inhibition with increasing ring hydrophobicity (for a fixed concentration of drug) is consistent with additional drug binding to the membrane and thus a net increase in the number of interactions between protonated dimethylethylamino groups and anionic phosphate oxygens, as well as any properly positioned anionic groups on the enzyme (see below).

The reaction sequence of Na^+/K^+-ATPase involves sequen-

tial partial reactions, including Na^+-dependent phosphorylation and a subsequent K^+-dependent dephosphorylation (18). Thus, in parallel with studies of Na^+/K^+-ATPase inhibition, the effects of tricyclic drug analogs on the K^+-*p*NPPase reaction were also quantitated. K^+-*p*NPPase activity is inhibited with the same potency as is Na^+/K^+-ATPase (Fig. 5), which indicates that inhibition may involve disruption of the K^+-dependent phosphatase reaction, thus shifting the rate-limiting step from the $E_2 \rightarrow E_1$ conformational change. Indeed, it would be fortuitous if different drugs had dominant interactions at two different sites (e.g., the K^+ and Na^+ binding sites) and produced this same linear relationship over such a broad concentration range. A recent study strongly implicates one or two carboxyls as being essential in metal cation binding (71), and the tricyclic drug analogs are cationic. Furthermore, the kinetics of enzyme inhibition by chlorpromazine, diphenhydramine, and dimethylethylaminopropyl chloride are all consistent with steric hindrance of K^+ binding. This result is in agreement with the suggestion by others (20, 72) that inhibition of Na^+/K^+-ATPase activity by less complex, protonated amines is steric and due to their competition with K^+ for extracellular binding sites on the enzyme. This competition requires high concentrations of amines, is reversible upon washing, and is believed to occur without the amines becoming occluded (20), which together imply a weak interaction at the biomembrane surface. Thus, the fluorescence and enzyme inhibition data with tricyclic drug analogs suggest an extension of weak, amine-based inhibition to amphipathic drugs that contain an amino group separated from a hydrophobic moiety by the appropriate distance such that both electrostatic and hydrophobic interactions can occur simultaneously. This extension, together with the proven ability of drugs to bind to the membrane surface, is consistent with similar mechanisms of interaction common to the tricyclic drug analogs governing both enzyme inhibition and membrane surface perturbation. Specifically, it is hypothesized that the K^+ binding sites of the enzyme are at or near the level of the phospholipid phosphate groups (above the hydrophilic head group/hydrophobic acyl interface) and that the crucial factor in inhibition is hydrophobic ring-stabilized electrostatic interactions between the protonated amino group of the drug and anionic groups on the enzyme and/or phospholipids near the K^+ binding sites. Indeed, the cation binding sites on Na^+/K^+-ATPase may even involve both protein- and phospholipid-donated groups. Such a mechanism is consistent with the formation of Ca^{2+} bridges between protein kinase C and acidic membrane phospholipids (73). Apparently, metal ion coordination is a shared function, with phospholipids being required to form the complete ion binding site in protein kinase C and other proteins. (A drug-protein interaction independent of phospholipids and a more generalized, cumulative, drug perturbation of the protein annulus will be discussed in more detail below.)

The present study shows that inhibition of *p*NPPase K^+ activation progresses from noncompetitive by chlorpromazine through mixed by diphenhydramine to competitive by dimethylaminopropyl chloride. Dimethylaminopropyl chloride does not alter DPH anisotropy at concentrations that inhibit the enzyme, implying it does not interact with the membrane interior. Furthermore, the chlorine to amino group distance is insufficient for the compound to simultaneously penetrate into the acyl region and interact with phospholipid phosphate oxygens or anionic binding sites on the enzyme that are at this distance from the hydrophobic surface. Thus, the orientation of dimethylaminopropyl chloride is apparently not controlled by hydrophobic interactions with the membrane, as with chlorpromazine and diphenhydramine, and the former can interact in an unconstrained manner but weakly with the K^+ binding sites, causing competitive inhibition. In contrast, the hydrophobic rings of chlorpromazine and diphenhydramine, when anchored appropriately in a hydrophobic region, can impose steric constraints on the interaction of the amino group of the drug with the K^+ binding sites of the enzyme to varying extents, resulting in a progression to noncompetitive inhibition. More specifically, it is speculated from the molecular modeling and the progression in enzyme kinetic data that the difference between inhibition of K^+ activation by chlorpromazine and diphenhydramine is related to the depth to which the drugs penetrate into the acyl region of the membrane bilayer, modulated by the large difference in their octanol/water partition coefficients. It is clear from Fig. 9 that the overall extent and specific geometry of interaction between the anionic phosphates and protonated amino groups can depend substantially on this depth of penetration. Binding of the tricyclic drug analogs in such a manner to the annular phospholipids of Na^+/K^+-ATPase could align the amino group of the drug at or near the level of the K^+ binding sites on the enzyme and thus interfere with K^+ binding. Furthermore, a change in depth of drug penetration into the bilayer would also likely alter the position or orientation of its amino group with respect to the K^+ binding sites on the enzyme, affecting the mechanism of inhibition. In support of this scenario, it has been shown that the charged forms of the local anesthetics tetracaine and procaine are localized at different depths within phospholipid bilayers, based on their hydrophobicity (74). In addition, it is likely that localization of the K^+ binding sites near the phospholipid phosphate groups could facilitate access to higher K^+ concentrations than found in bulk solution and those concentrations could be further increased by the high density of negatively charged phospholipids in the annulus of the enzyme (75). Finally, chlorpromazine, diphenhydramine, and dimethylaminopropyl chloride all inhibit *p*-nitrophenylphosphate activation of K^+-*p*NPPase in a noncompetitive manner, suggesting that they interact with the enzyme at a position distinct from the *p*-nitrophenylphosphate binding site.

Na^+/K^+-ATPase activity is sensitive to the identity and organization of its surrounding phospholipid microenvironment. There is considerable evidence suggesting that change in "membrane fluidity" is the "mechanism" by which the function of membrane-bound Na^+/K^+-ATPase is modulated by drugs (26, 76), and this may well be the case in certain instances. The lipophilic characteristics of the tricyclic drug analogs suggest that they could differentially perturb the bilayer, thus altering its fluidity. In this study, amitriptyline, imipramine, doxepin, and diphenhydramine were found to inhibit Na^+/K^+-ATPase activity and decrease the anisotropy of DPH (i.e., increase fluidity) over similar concentration ranges (Fig. 8A), generally consistent with a relationship between membrane fluidity and enzyme activity. However, at least in the case of doxepin and imipramine, the fluidity-activity relationship is apparently not the dominant factor, in that imipramine inhibits enzyme activity more potently than does doxepin but increases fluidity less potently. This discrepancy was investigated in more detail by

searching for a correlation between the drug-induced changes in the anisotropy of DPH in SPMs at concentrations equal to their Na^+/K^+-ATPase IC_{25} values versus the IC_{25} values themselves. Specifically, if inhibition of Na^+/K^+-ATPase activity and changes in membrane fluidity were directly related, all of these drugs should decrease the anisotropy of DPH with equal potency at these concentrations. As shown in Fig. 8B, there is no clear relationship between anisotropy and IC_{25} values, indicating that there are intrinsic differences in the ability of the drugs to perturb the membrane interior that are not directly related to inhibition of Na^+/K^+-ATPase activity. This lack of relationship supports the data outlined previously suggesting that ANS is probing more closely than is DPH the inhibitory site(s) of the tricyclic drug analogs. Also in agreement with these data is a study by Kuroda and Fujiwara (12) in which the relative order of anesthetic potency for a series of local anesthetics could be correlated with their ability to bind to phospholipid polar head groups and not with their ability to affect membrane fluidity.

The importance of perturbation of the membrane surface versus interior for Na^+/K^+-ATPase activity was investigated further by comparing the effects of drug *N*-demethylation on DPH anisotropy and enzyme inhibition. Both F_∞ 40% and IC_{50} values were altered in a proportional manner with demethylation of the dimethylethylamino group of amitriptyline and imipramine, but anisotropy was unchanged (Table 1). These factors again imply that increasing the fluidity of the membrane interior (as quantitated with DPH) does not predominantly modulate enzyme activity. Furthermore, because there was no change in anisotropy with demethylation, this modification apparently does not alter the number of drug molecules bound to the membrane for a fixed concentration of drug in solution. It follows that the increase in Na^+/K^+-ATPase inhibition with demethylation does not originate from an increase in the number of drug molecules bound to the membrane, which should alter fluidity, but originates with a change in charge localization on the dimethylethylamino group, which does not influence the membrane interior. Furthermore, because anisotropy was unchanged, the dominant factor in drug binding to the membrane is hydrophobic and not electrostatic. As an alternative to these arguments, the loss of the methyl group itself might remove some unelucidated steric factor that enhances inhibition.

The biomembrane-based mechanism of tricyclic drug analog-induced Na^+/K^+-ATPase inhibition presented above is straightforward, uses the smallest number of assumptions concerning the availability and occupation of drug binding sites, and is in line with the current focus on specific interactions at the K^+ binding sites on the enzyme. However, there are alternative mechanisms requiring additional assumptions that also appear consistent with the data. First, the inhibition could originate independently of the bilayer with a relatively well defined, hydrophobic ring-binding pocket on the enzyme surface that is the appropriate distance from the K^+ binding sites such that the sites are blocked with the charged amino group. The change in mechanism of inhibition, as given by enzyme kinetics, could also result from interactions independent of the bilayer, with ring binding constraining the extent of charged amino group interaction with the K^+ sites. In this case it must be assumed that ANS binding to the phospholipids reports in a proportional manner on drug binding to the enzyme, which still implies that the same contributions of hydrophobic, electrostatic, and possibly spatial factors elucidated in this study govern drug binding to the latter. A second alternative is that the specific electrostatic interactions between the protonated drugs and anionic phospholipids or enzyme groups are less important than a general reduction in K^+ concentration near the membrane surface and it is this reduction that inhibits the enzyme. The lack of correlation between changes in membrane fluidity and Na^+/K^+-ATPase activity would support this model; however, it is then not clear how diphenhydramine and chlorpromazine would differentially inhibit K^+ activity kinetically. A third alternative is a generalization of the annular phospholipid model to emphasize the localization of many protonated drug molecules in the annulus and the resulting cumulative disruption of electrostatic as well as hydrophobic interactions at the bilayer-enzyme interface. This mechanism, however, also lacks specificity.

In summary, this study finds a predictive correlation between tricyclic drug analog-induced changes in Na^+/K^+-ATPase activity and bilayer surface structure that suggests a similar molecular mechanism of biomembrane surface interaction governing both processes. Variation in drug hydrophobicity and charge localization modulate this interaction. The extent of drug-induced perturbation of the membrane interior does not appear to correlate with Na^+/K^+-ATPase inhibition. Furthermore, the study suggests that this membrane-based interaction is common to drugs from different pharmacological classes that have a positively charged amine and substantial hydrophobic region separated by an appropriate distance, and it emphasizes the significance of this interaction in modulating the activity of at least one physiologically important enzyme. Indeed, this kind of surface interaction may modulate inhibition of other membrane-bound proteins as well. Certain other ATPases share structural homology and aspects of their catalytic cycle with the Na^+/K^+-ATPase (77). The Na^+ channel, like the Na^+/K^+-ATPase, has a high density of negative surface charge in its phospholipid annulus (75, 78), which may make it susceptible to the simultaneous electrostatic and hydrophobic interaction described above. Thus, of additional significance is the possibility that this kind of membrane surface interaction alters the activity of other membrane-bound systems, which may lead to additional investigations, including studies of the overlapping toxicological side effects of drugs with similar structures.

References

1. Moss, G. W. J., W. R. Lieb, and N. P. Franks. Anesthetic inhibition of firefly luciferase, a protein model for general anesthesia, does not exhibit pressure reversal. *Biophys. J.* **60**:1309–1314 (1991).
2. Kinter, W. B., and J. B. Pritchard. Altered permeability of cell membranes. *Handb. Physiol. Sect. 9* 563–576 (1977).
3. Jain, M. K. *Introduction to Biological Membranes.* John Wiley and Sons, New York (1988).
4. Hill, M. W. The effect of anesthetic-like molecules on the phase transition in smectic mesophases of dipalmitoyllecithin. *Biochim. Biophys. Acta* **356**:117–124 (1974).
5. Hayden, D. A., and J. E. Kimura. Some effects of *n*-pentane on the sodium and potassium currents of the squid giant axon. *J. Physiol. (Lond.)* **312**:57–70 (1981).
6. Bangham, A. D., M. W. Hill, and W. T. Mason. A biochemical and biophysical view of anesthesia. *Prog. Anesthesiol.* **2**:69–77 (1980).
7. Lee, A. G. Model for action of local anesthetics. *Nature (Lond.)* **262**:545–548 (1976).
8. Frenzel, J., K. Arnold, and P. Nuhn. Calorimetric, ^{13}C NMR, and ^{31}P NMR studies on the interaction of some phenothiazine derivatives with dipalmitoyl phosphatidylcholine model membranes. *Biochim. Biophys. Acta* **507**:185–197 (1978).
9. Forrest, B. J., P. T. Linehan, and J. Mattai. Interactions of a phenothiazine tranquilizer with phosphatidylcholine and phosphatidylcholine/cholesterol membranes. *Biochemistry* **23**:2288–2293 (1984).
10. Yasuhara, H., C. A. Dujovne, and I. Ueda. Relationship between surface

activity and toxicity to Chang liver cultures of tricyclic antidepressants. *Pharmacology* **18**:95–102 (1979).
11. Herbette, L., A. M. Katz, and J. M. Sturtevant. Comparisons of the interaction of propranolol and timolol with model and biological membrane systems. *Mol. Pharmacol.* **24**:259–269 (1983).
12. Kuroda, Y., and Y. Fujiwara. Locations and dynamical perturbations for lipids of cationic forms of procaine, tetracaine, and dibucaine in small unilamellar phosphatidylcholine vesicles as studied by nuclear Overhauser effects in ^1H nuclear magnetic resonance spectroscopy. *Biochim. Biophys. Acta* **903**:395–410 (1987).
13. Reasor, M. J. A review of the biology and toxicological implications of the induction of lysosomal lamellar bodies by drugs. *Toxicol. Appl. Pharmacol.* **97**:47–56 (1989).
14. Baldessarini, R. J. Drugs and the treatment of psychiatric disorders, in *The Pharmacological Basis of Therapeutics* (A. G. Gilman, L. S. Goodman, T. W. Rall, and F. Murad, eds.). MacMillan Publishing Company, New York, 387–445 (1985).
15. Douglas, W. W. Histamine and 5-hydroxytryptamine (serotonin) and their antagonists, in *The Pharmacological Basis of Therapeutics* (A. G. Gilman, L. S. Goodman, T. W. Rall, and F. Murad, eds.). MacMillan Publishing Company, New York, 605–638 (1985).
16. Henry, J. A., and S. L. Cassidy. Membrane stabilising activity: a major cause of fatal poisoning. *Lancet* **1**:1414–1417 (1986).
17. Voet, D., and J. G. Voet. *Biochemistry*. John Wiley and Sons, New York (1990).
18. Cantley, L. C. Structure and mechanism of the (Na,K)-ATPase. *Curr. Top. Bioenerg.* 201–237 (1981).
19. Zemkova, H., J. Teisinger, and F. Vyskocil. Inhibition of the electrogenic Na,K pump and Na,K-ATPase activity by tetraethylammonium, tetrabutylammonium, and apamin. *J. Neurosci. Res.* **19**:497–503 (1988).
20. Forbush, B. The interaction of amines with the occluded state of the Na,K-pump. *J. Biol. Chem.* **263**:7979–7988 (1988).
21. Post, R. L., C. Hegyvary, and S. Kume. Activation of adenosine triphosphate in the phosphorylation kinetics of sodium and potassium ion transport adenosine triphosphatase. *J. Biol. Chem.* **247**:6530–6540 (1972).
22. Kimmelberg, H., and D. Papahadjopoulos. Effects of phospholipid acyl chain fluidity, phase transitions, and cholesterol on (Na^++K^+)-stimulated adenosine triphosphatase. *J. Biol. Chem.* **249**:1071–1080 (1974).
23. Abeywardena, M. Y., T. M. Allen, and J. S. Charnock. Lipid-protein interactions of reconstituted membrane-associated adenosinetriphosphatases. *Biochim. Biophys. Acta* **729**:62–74 (1983).
24. Schachter, D. Fluidity and function of hepatocyte plasma membranes. *Hepatology* **4**:140–151 (1984).
25. Tsakiris, S., and G. Deliconstantinos. Influence of phosphatidylserine on (Na^+-K^+)-stimulated ATPase and acetylcholinesterase activities of dog brain synaptosomal plasma membranes. *Biochem. J.* **220**:301–307 (1984).
26. Chatelain, P., R. Laruel, and M. Gillard. Effect of amiodarone on membrane fluidity and Na^+/K^+ ATPase activity in rat-brain synaptic membranes. *Biochem. Biophys. Res. Commun.* **129**:148–154 (1985).
27. Prasada Rao, K. S., S. B. Rao, P. H. Camus, and H. M. Mehendale. Effect of amiodarone on Na^+-K^+-ATPase and Mg^{2+}-ATPase activities in rat brain synaptosomes. *Cell Biochem. Funct.* **4**:143–151 (1986).
28. Shacoori, V., G. Leray, L. Guenet, F. Gueble-Val, A. Le Treut, and J. Y. Le Gall. Inhibition of (Na^+,K^+)-ATPase and Mg^{++}-ATPase by a lysosomotropic drug: perhexiline maleate. *Res. Commun. Chem. Pathol. Pharmacol.* **59**:161–172 (1988).
29. Deliconstantinos, G., L. Kopeikina-Tsiboukidou, and V. Villiotou. Evaluation of membrane fluidity effects and enzyme activity alterations in Adriamycin neurotoxicity. *Biochem. Pharmacol.* **36**:1153–1161 (1987).
30. Okuda, C. The effects of volatile anesthetics on the binding of 1-anilino-8-naphthalene sulphonate to biological membranes and lipid vesicles: the role of cholesterol. *J. Biochem. (Tokyo)* **92**:357–363 (1982).
31. Peura, P., P. Mackenzie, U. Koivusaari, and M. Lang. Increased fluidity of a model membrane caused by tetrahydro-β-carbolines. *Mol. Pharmacol.* **22**:721–724 (1982).
32. Haque, S. J., and M. K. Poddar. Effect of tetracaine on membrane-bound acetylcholinesterase activity and anilinonaphthalene sulphonate-induced membrane fluorescence. *Biochem. Pharmacol.* **34**:2599–2603 (1985).
33. Hillard, C. J., R. A. Harris, and A. S. Bloom. Effects of cannabinoids on physical properties of brain membranes and phospholipid vesicles: fluorescence studies. *J. Pharmacol. Exp. Ther.* **232**:579–588 (1985).
34. Slavik, J. Anilinonaphthalene sulfonate as a probe of membrane composition and function. *Biochim. Biophys. Acta* **694**:1–25 (1982).
35. Shinitzky, M., and Y. Barenholz. Fluidity parameters of lipid regions determined by fluorescence polarization. *Biochim. Biophys. Acta* **515**:367–394 (1978).
36. Brocklehurst, J. R., R. B. Freedman, D. J. Hancock, and G. K. Radda. Membrane studies with polarity dependent and eximer-forming fluorescent probes. *Biochem. J.* **116**:721–734 (1970).
37. Gitler, C., B. Rubalcava, and A. H. Caswell. Fluorescence changes of ethidium bromide on binding to erythrocyte and mitochondrial membranes. *Biochim. Biophys. Acta* **193**:479–491 (1969).
38. Gomperts, B., F. Lantelme, and R. Stock. Ion association reactions with biological membranes, studied with the fluorescent dye 1-anilino-8-naphthalenesulfonate. *J. Membr. Biol.* **3**:241–266 (1970).
39. Radda, G. K. The design and use of fluorescence probes for membrane studies, in *Current Topics in Bioenergetics* (D. Rao Sanadi, ed.). Academic Press, New York, 81–126 (1971).
40. Van Blitterswijk, W. J., R. P. VanHoeven, and B. W. VanDerMeer. Lipid structural order parameters (reciprocal of fluidity) in biomembranes derived from steady-state fluorescence polarization measurements. *Biochim. Biophys. Acta* **644**:323–332 (1981).
41. Conrad, M. J., and S. J. Singer. The solubility of amphipathic molecules in biological membranes and lipid bilayers and its implications for membrane structure. *Biochemistry* **20**:808–818 (1981).
42. Chatelain, P., N. Reckinger, and R. Roncucci. Effect of suloctidil on Na^+/K^+-ATPase activity and on membrane fluidity in rat brain synaptosomes. *Biochem. Pharmacol.* **28**:3677–3680 (1979).
43. Keeffe, E. B., N. M. Blankenship, and B. F. Scharschmidt. Alteration of rat liver plasma membrane fluidity and ATPase activity by chlorpromazine hydrochloride and its metabolites. *Gastroenterology* **79**:222–231 (1980).
44. Deliconstantinos, G. Phenobarbital modulates the (Na^+,K^+)-stimulated ATPase and Ca^{2+} stimulated ATPase activities by increasing the bilayer fluidity of dog brain synaptosomal plasma membranes. *Neurochem. Res.* **8**:1143–1152 (1983).
45. East, J. M., O. T. Jones, A. C. Simmonds, and A. G. Lee. Membrane fluidity is not an important physiological regulator of the (Ca^{2+}-Mg^{2+})-dependent ATPase of sarcoplasmic reticulum. *J. Biol. Chem.* **259**:8070–8071 (1984).
46. Ma, J. Y., J. K. Ma, and K. C. Weber. Fluorescence studies of the binding of amphiphilic amines with phospholipids. *J. Lipid Res.* **26**:735–744 (1985).
47. Joshi, U. M., P. R. Kodavanti, B. Coudert, T. M. Dwyer, and H. M. Mehendale. Types of interaction of amphiphilic drugs with phospholipid vesicles. *J. Pharmacol. Exp. Ther.* **246**:150–157 (1988).
48. Jones, D. H., and A. I. Matus. Isolation of synaptic plasma membrane from brain by combined flotation-sedimentation density gradient centrifugation. *Biochim. Biophys. Acta* **356**:276–287 (1974).
49. Lowry, O. H., N. J., Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
50. Forbush, B. Assay of Na,K-ATPase in plasma membrane preparations: increasing the permeability of membrane vesicles using sodium dodecyl sulfate buffered with bovine serum albumin. *Anal. Biochem.* **128**:159–163 (1983).
51. Ahmed, K., and J. D. Judah. Preparation of lipoproteins containing cation-dependent ATPase. *Biochim. Biophys. Acta* **93**:603–613 (1964).
52. Segel, I. H. *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*. John Wiley and Sons, New York (1975).
53. Azzi, A. The use of fluorescent probes for the study of membranes. *Methods Enzymol.* **32**:234–246 (1974).
54. Christian, S. T. The interaction of phenothiazine derivatives with synaptosomal membranes: the use of fluorescent probes as an indicator of drug-induced conformational perturbations. *Int. J. Neurosci.* **6**:57–65 (1973).
55. Schroeder, F. Use of fluorescence spectroscopy in the assessment of biological membrane properties, in *Advances in Membrane Fluidity: Methods for Studying Membrane Fluidity* (R. C. Aloia, C. C. Curtain, and L. M. Gordon, eds.). Alan R. Liss, New York, 193–217 (1988).
56. Brooks, B. R., R. E. Bruccoleri, B. D. Olafson, D. J. States, S. Swaminathan, and M. Karplus. CHARMm: a program for macromolecular energy minimization and dynamics calculations. *J. Comput. Chem.* **4**:187–217 (1983).
57. Davis, P. W., and T. M. Brody. Inhibition of Na^+K^+-activated adenosine triphosphatase activity in rat brain by substituted phenothiazines. *Biochem. Pharmacol.* **15**:703–710 (1966).
58. Roufogalis, B. D. Comparative studies on the membrane actions of depressant drugs: the role of lipophilicity in inhibition of brain sodium and potassium-stimulated ATPase. *J. Neurochem.* **24**:51–61 (1974).
59. Hansch, C., and W. J. Dunn. Linear relationships between lipophilic character and biological activity of drugs. *J. Pharm. Sci.* **61**:1–19 (1972).
60. Moffat, A. C. *Clarkes's Isolation and Identification of Drugs*. The Pharmaceutical Press, London (1986).
61. Schuurmans Stekhoven, F. M. A. H., H. G. P. Swarts, G. K. 't Lam, Y. S. Zou, and J. J. H. H. M. De Pont. Phosphorylation of (Na^++K^+)-ATPase; stimulation and inhibition by substituted and unsubstituted amines. *Biochim. Biophys. Acta* **937**:161–176 (1988).
62. Wallace, R. A., L. Wallace, M. Harrold, D. Miller, and J. Uretsky. Interaction of permanently charged chlorpromazine and dopamine analogs with the striatal D-1 dopaminergic receptor. *Biochem. Pharmacol.* **38**:2019–2025 (1989).
63. Abernathy, C. O., L. Lukacs, and H. J. Zimmerman. Toxicity of tricyclic antidepressants to isolated rat hepatocytes. *Biochem. Pharmacol.* **24**:347–350 (1975).
64. Jarvik, M. E. Therapeutic uses of psychotropic drugs, in *The Pharmacological Basis of Therapeutics* (L. S. Goodman and A. Gilman, eds.). MacMillan, New York, 151–203 (1970).
65. Auger, M., H. C. Jarrell, and I. C. P. Smith. Interactions of the local anesthetic tetracaine with membranes containing phosphatidylcholine and cholesterol: a ^2H NMR study. *Biochemistry* **27**:4660–4667 (1988).
66. Haynes, D. H., and H. Staerk. 1-Anilino-8-naphthalenesulfonate: a fluores-

cent probe of membrane surface structure, composition and mobility. *J. Membr. Biol.* **17**:313–340 (1974).
67. Podo, F., and J. K. Blasie. Nuclear magnetic resonance studies of lecithin bimolecular leaflets with incorporated fluorescent probes. *Proc. Natl. Acad. Sci. USA* **74**:1032–1036 (1977).
68. Kelusky, E. C., Y. Boulanger, S. Schreier, and I. C. P. Smith. A ^{2}H-NMR study on the interactions of the local anesthetic tetracaine with membranes containing phosphatidylserine. *Biochim. Biophys. Acta* **856**:85–90 (1986).
69. Lullmann, H., and M. Wehling. The binding of drugs to different polar lipids *in vitro*. *Biochem. Pharmacol.* **28**:3409–3415 (1979).
70. Kursch, B., H. Lullmann, and K. Mohr. Influence of various cationic amphiphilic drugs on the phase-transition temperature of phosphatidylcholine liposomes. *Biochem. Pharmacol.* **32**:2589–2594 (1983).
71. Arguello, J. M., and J. H. Kaplan. Evidence for essential carboxyls in the cation-binding domain of the Na,K-ATPase. *J. Biol. Chem.* **266**:14627–14635 (1991).
72. Sachs, J. R., and M. E. Conrad. Effect of tetraethylammonium on the active cation transport system of the red blood cell. *Am. J. Physiol.* **215**:795–798 (1968).
73. Bazzi, M. D., and G. L. Nelsestuen. Extensive segregation of acidic phospholipids in membranes induced by protein kinase C and related proteins. *Biochemistry* **30**:7961–7969 (1991).
74. Kelusky, E. C., and I. C. P. Smith. The influence of local anesthetics on molecular organization in phosphatidylethanolamine membranes. *Mol. Pharmacol.* **26**:314–321 (1984).
75. Houslay, M. D., and K. K. Stanley. *Dynamics of Biological Membranes*. John Wiley and Sons, New York (1982).
76. Gordon, L. M., R. D. Sauerheber, J. A. Esgate, I. Dipple, R. J. Marchmont, and M. D. Houslay. The increase in bilayer fluidity of rat liver plasma membranes achieved by the local anesthetic benzyl alcohol affects the activity of intrinsic membrane enzymes. *J. Biol. Chem.* **255**:4519–4527 (1980).
77. Stryer, L. *Biochemistry*. Freeman and Company, New York (1988).
78. Hille, B., A. M. Woodhull, and B. I. Shapiro. Negative surface charge near sodium channels of nerve: divalent ions, monovalent ions, and pH. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **270**:301–318 (1975).

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