Phospholipid-subclass-specific partitioning of lipophilic ions in membrane–water systems

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Herein, we systematically investigate phospholipid-subclass-specific alterations in the partitioning of both cationic and anionic amphiphiles to identify the importance of ester, ether and vinyl ether linkages at the *sn*-1 position of phospholipids in the partitioning of charged amphiphiles. The results demonstrated that the membrane–water partition coefficient of a prototypic cationic amphiphile (i.e. 3,3'-dipropylthiadicarbocyanine iodide) was approximately 2.5 times higher in membranes comprised of plasmenylcholine in comparison with membranes comprised of either phosphatidylcholine or plasmalogen. In striking contrast, the membrane–water partition coefficient of a prototypic anionic amphiphile [i.e. bis-(1,3-dibutylbarbituric acid)trimethine oxonol] in membranes comprised of plasmenylcholine was $\approx$ 2.5 times lower than that manifest in membranes comprised of phosphatidylcholine or plasmalogen. Utilizing these experimentally determined partition coefficients, the relative membrane dipole potential of membranes comprised of

plasmenylcholine was calculated and found to be $\approx$ 25 mV lower than in membranes comprised of phosphatidylcholine or plasmalogen. This lower membrane dipole potential in membranes comprised of plasmenylcholine is equivalent to the membrane potential induced by incorporation of $\approx$ 25 mol % of anionic phospholipids in membranes comprised of phosphatidylcholine. Collectively, these results demonstrate that phospholipid-subclass-specific differences in the membrane dipole potential contribute to alterations in the partitioning of lipophilic ions in membrane bilayers comprised of distinct phospholipid subclasses. Moreover, they suggest that these physicochemical differences can be exploited to facilitate the targeting of charged lipophilic drugs to specific cells and sub-cellular membrane compartments.

Key words: dipole potential, drug partitioning, ion–membrane interaction, membrane thermodynamics, plasmalogen.

INTRODUCTION

Many drugs are lipophilic or amphiphilic and hence readily partition into cellular membranes. The interaction of drugs with biological membranes has important implications regarding the type, magnitude and duration of the biological effects elicited by pharmaceutical agents [1–4]. A long-sought-after goal of modern pharmaceutical research is to selectively target biologically active agents to specific cells and/or cellular compartments. Despite the intense efforts directed towards this goal, only modest success has been achieved. Moreover, the biochemical mechanisms mediating the partitioning of drugs into specific cellular membranes in complex biological systems are just beginning to be understood.

In most cell types, biological membranes are comprised predominantly of diacyl phospholipid molecular species. However, in many specialized cell membranes (e.g. electrically active membranes), such as sarcolemma and sarcoplasmic reticulum, plasmalogen molecular species are the predominant phospholipid subclass [5–7]. Prior studies have demonstrated substantial differences in the dipole potential present in membranes comprised of plasmalogen compared with diacyl phospholipid molecular species [8,9]. Since alterations in membrane dipole potential change the free-energy profile of interactions between charged moieties and the host membranes, we anticipated that

substantial differences in partition coefficients could be achieved in membranes comprised of different phospholipid subclasses. Moreover, since alterations in partitioning of pharmacological agents could be exploited to target therapeutic agents selectively, we examined the partitioning of moieties containing a charged and a hydrophobic domain in model membranes comprised of plasmalogen, alkyl ether or diacyl phospholipid molecular species. We now report that the substantial differences in the membrane dipole potential between choline glycerophospholipid subclasses exist and can be exploited to affect the selective partitioning of amphiphilic moieties in membranes comprised of distinct phospholipid subclasses. These results provide a new approach to selectively target therapeutic agents to specialized membranes (i.e. electrically active membranes such as those present in heart and brain) to enhance the therapeutic efficiency of pharmaceutical agents containing charged moieties.

MATERIALS AND METHODS

Materials

3,3'-Dipropylthiadicarbocyanine iodide [diSC₃(5)] and bis-(1,3-dibutylbarbituric acid)trimethine oxonol [DiBAC₄(3)] were supplied by Molecular Probes (Eugene, OR, U.S.A.). Plasmenylcholine (PlasCho) was synthesized from bovine heart lecithin as described previously [10]. Other phospholipids, including phos-

Abbreviations used: DiBAC₄(3), bis-(1,3-dibutylbarbituric acid)trimethine oxonol; diSC₃(5), 3,3'-dipropylthiadicarbocyanine iodide; PG, phosphatidylglycerol; PS, phosphatidylserine; AlkCho, plasmalogen; PlasCho, plasmenylcholine; PtdCho, phosphatidylcholine; PA PlasCho, 1-O-(Z)-hexadec-1'-enyl-2-eicosa-5',8',11',14'-tetraenoyl-*sn*-glycero-3-phosphocholine; PO AlkCho, 1-O-hexadecyl-2-octadec-9'-enoyl-*sn*-glycero-3-phosphocholine; PO PhosCho, 1-hexadecanoyl-2-octadec-9'-enoyl-*sn*-glycero-3-phosphocholine; PO PlasCho, 1-O-(Z)-hexadec-1'-enyl-2-octadec-9'-enoyl-*sn*-glycero-3-phosphocholine; PO PS, 1-hexadecanoyl-2-octadec-9'-enoyl-*sn*-glycero-3-phosphoserine; PO PG, 1-hexadecanoyl-2-octadec-9'-enoyl-*sn*-glycero-3-phosphoglycerol; PA PhosCho, 1-hexadecanoyl-2-eicosa-5',8',11',14'-tetraenoyl-*sn*-glycero-3-phosphocholine; PA AlkCho, 1-O-hexadecyl-2-eicosa-5',8',11',14'-tetraenoyl-*sn*-glycero-3-phosphocholine.

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phatidylcholine (PtdCho) and plasmanylcholine (AlkCho, alkylacyl choline glycerophospholipid), were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). All choline glycerophospholipids were further purified by reversed-phase HPLC and extracted twice by the Bligh and Dyer procedure [11]. The concentration of phospholipids was determined by capillary GLC after acid methanolysis utilizing arachidic acid (20:0) as an internal standard [12]. Most other chemicals were obtained from Sigma (St. Louis, MO, U.S.A.).

Preparation of small unilamellar vesicles

Phospholipids (2 μ mol) were dissolved in chloroform, dried under a nitrogen stream, and subsequently subjected to high vacuum [less than 6.67 Pa (50 mTorr)] for at least 2 h. The dried lipid film was suspended in 0.6 ml of medium A (0.29 M K_2SO_4 , pH 6.3) by vigorous vortexing for 2 min and subsequent sonication twice (4 min, 40% duty cycle) utilizing a Vibra Cell Model VC 600 sonicator (Sonics Material, Danbury, CT, U.S.A.) under a nitrogen atmosphere. The average diameter of the vesicles, as measured by the light-scattering method [13] with a submicron particle analyser (Coulter, Hialeah, FL, U.S.A.), was about 30 nm, which was consistent with our previous results utilizing inulin trapping [14]. The average diameters of vesicles comprised of each choline glycerophospholipid subclass are almost identical within the limits of experimental error.

Preparation of large unilamellar vesicles

Phospholipids (2 μ mol) were dissolved in chloroform, dried under a nitrogen stream, and subsequently subjected to high vacuum (less than 6.67 Pa) for at least 2 h. The dried lipid film was suspended in 0.6 ml of medium A by vigorous vortex mixing for 2 min to form multilamellar vesicles. The freeze-thawed vesicles were obtained by freezing the vesicles in liquid nitrogen for 3 min and thawing in a 37 °C water bath (repeated five times). The resulting multilamellar vesicle dispersion was then transferred to an extrusion device (Lipex Biomembranes, Vancouver, Canada) to form large unilamellar vesicles. Extrusion of multilamellar vesicle dispersion was performed through polycarbonate filters (100 or 200 nm pore size) (Nuclepore Corp., Pleasanton, CA, U.S.A.). Iterative passage of vesicles (10 times) resulted in the formation of large unilamellar vesicles of selected size [15–17].

Measurement of partitioning of lipophilic ions by spin-column and UV methods

The concentration of vesicles in medium A was diluted 100-fold by addition of 20 μ l of vesicles to 2.0 ml of medium A while stirring. Next, 10 μ l of the lipophilic moieties [diSC₃(5) or DiBAC₄(3)] in ethanol were added into the diluted vesicle solution (final concentration 1.5 μ M). After the above solution had been gently stirred for selected time intervals to reach an equilibrium, uncaptured lipophilic moieties (free in water phase) were removed by a Sephadex G-25 spin column as described previously [18]. The vesicle solution passed through the spin column was extracted using the Bligh and Dyer procedure [11]. The amounts of the lipophilic moieties partitioning in the membrane phase were quantified by UV spectrophotometry at 660 nm for diSC₃(5) or 490 nm for DiBAC₄(3) (Beckman DU Series 70, Fullerton, CA, U.S.A.) by comparison with a standard curve. The overall spin-column yield determined by recovery experiments using radio-labelled lipids was 80 ± 5 mol% for all phospholipid subclasses. Control experiments demonstrated that more than 98% of the lipophilic ions were removed by the Sephadex G-25 spin column. Analysis of vesicle phospholipids by either HPLC or TLC showed

that phospholipids were stable during the time course of the experiment.

Measurement of partitioning of lipophilic ions by a fluorescence method

Alternatively, the amounts of the lipophilic moieties partitioning in the membrane phase were quantified by using a fluorescence method which was based on the self quenching of dye in a membrane environment [18,19]. Vesicles in medium A (prepared as described above) were diluted 100-fold by addition of 20 μ l of vesicles to 2.0 ml of medium A while stirring. Fluorescence intensities were recorded immediately after the addition of the lipophilic cation diSC₃(5) by a SLM 4800C spectrofluorimeter (SLM Instruments, Urbana, IL, U.S.A.) employing an excitation wavelength of 618 nm and an emission wavelength of 690 nm.

Data analysis

We have defined the membrane–water partition coefficient (K) as the molar fraction of the lipophilic ions in the membrane phase divided by their molar fraction in the water phase [20–22]. The molar fraction of the lipophilic ions was calculated from the UV absorbance of the ions from the membrane phase (considering the overall spin-column yield of 80%), utilizing an experimentally-derived calibration curve of UV absorbance and lipophilic ion concentration. The standard free energy change (ΔG°) in moving 1 mol of the lipophilic ions from the water to membrane phase can be computed from the membrane–water partition coefficient (K) by:

$$\Delta G^\circ = -RT \cdot \ln K \quad (1)$$

and ΔG° can be expressed as the sum of Born (ΔG_{Bo}°), image (ΔG_{Im}°), neutral (ΔG_{Ne}°) and dipole (ΔG_{Di}°) energies according to the model proposed by Flewelling and Hubbell [22]:

$$\Delta G^\circ = \Delta G_{Bo}^\circ + \Delta G_{Im}^\circ + \Delta G_{Ne}^\circ + \Delta G_{Di}^\circ \quad (2)$$

Born energy is the free energy of transfer in moving 1 mol of the lipophilic ions from a region of dielectric strength ϵ_1 to a region of dielectric strength ϵ_2 . Image energy represents the interactions of the ions with membrane interfaces (excluding the dipole energy). Neutral energy is the free energy change in moving 1 mol of lipophilic ions from the water to membrane phase, excluding the electrical contribution. Dipole energy results from the selective orientation of phospholipid head groups, phospholipid carbonyls and surface-water molecules with the charged amphiphile [22–24].

For a first-order approximation we neglected the differences in the other energy terms (such as Born, image, neutral energy) between membranes comprised of different subclasses of choline glycerophospholipids or choline glycerophospholipids containing substitutional impurities such as anionic phospholipids. Therefore, the relative membrane dipole potential (ΔE) can be calculated from the alteration in the membrane–water partition coefficient:

$$\Delta(\Delta G^\circ) \approx \Delta(\Delta G_{Di}^\circ) = -nF(\Delta E) \quad (3)$$

and thus:

$$\Delta E = \Delta[RT \cdot \ln K / (nF)] \quad (4)$$

where n is the number of the charge of each lipophilic ion, and F is Faraday's constant (96 500 C · mol⁻¹).

Membrane potentials can be calculated from the partition coefficients by Gouy–Chapman theory [25–27]:

$$C_M = C_{aq} \cdot e^{-nFE/RT} \quad (5)$$

where C_M and C_{aq} are probe concentrations in membrane and water phases, respectively. Previous studies have demonstrated that the partitioning of amphiphilic molecules was enthalpy-driven in small unilamellar vesicles, and was entropy-driven in large unilamellar vesicles [28–31]. The molar enthalpy change (ΔH) and molar heat capacity (ΔC_p) can be calculated from the temperature dependence of partition coefficients by assuming that the partitioning was enthalpy-driven in small unilamellar vesicles:

$$\Delta G \approx \Delta H = \Delta H(T_1) + \Delta C_p(T_2 - T_1) \quad (6)$$

where ΔG is the molar free energy change and T is temperature. Similarly, the molar entropy change (ΔS) can be calculated by assuming that the partitioning was entropy-driven in large unilamellar vesicles (i.e. $\Delta S \approx -\Delta G/T$).

RESULTS

Partitioning of the lipophilic cation diSC₃(5) in membranes comprised of distinct choline glycerophospholipid subclasses

Incubation of the lipophilic cation diSC₃(5) with vesicles comprised of each choline glycerophospholipid subclass resulted in a linear increase in incorporated diSC₃(5) in each subclass as a function of concentration (Figure 1A). Remarkably, membranes comprised of PO PlasCho [1-*O*-(*Z*)-hexadec-1'-enyl-2-octadec-9'-enoyl-*sn*-glycero-3-phosphocholine] incorporated approximately 2.5 times as much diSC₃(5) at each concentration in comparison with membranes comprised of PO PhosCho (1-hexadecanoyl-2-octadec-9'-enoyl-*sn*-glycero-3-phosphocholine) or PO AlkCho (1-*O*-hexadecyl-2-octadec-9'-enoyl-*sn*-glycero-3-phosphocholine) (Figure 1A). In all three cases, the amount of incorporated amphiphile was linear with concentrations under the experimental conditions employed. Moreover, in timed incubations of 1.5 μ M diSC₃(5) with membrane vesicles, the partitioning occurred rapidly (i.e. was complete before the first measurement at 5 min) and was stable over the entire experimental interval (60 min; Figure 1B). These results demonstrated that PO PlasCho possessed a partition coefficient for the lipophilic cation diSC₃(5) which was approximately 2.5-fold higher than that manifested by PO PhosCho or PO AlkCho (2.4×10^5 compared with 0.9×10^5) (Figure 1B). To determine if lipophilic cation partitioning was substantially affected by alterations in the individual molecular species present in the membrane bilayer, additional experiments were performed utilizing each of the three choline glycerophospholipid subclasses, containing palmitate at the *sn*-1 position and arachidonate at the *sn*-2 position. Incubation of 1.5 μ M diSC₃(5) with vesicles comprised of PA PlasCho [1-*O*-(*Z*)-hexadec-1'-enyl-2-eicosa-5',8',11',14'-tetraenoyl-*sn*-glycero-3-phosphocholine] demonstrated that diSC₃(5) rapidly entered the membrane bilayer and was stably associated with vesicles over the entire 60 min experimental time course. The partition coefficient of diSC₃(5) in PA PlasCho was approximately 2.5-fold higher than that manifest in PA PhosCho (1-hexadecanoyl-2-eicosa-5',8',11',14'-tetraenoyl-*sn*-glycero-3-phosphocholine) or PA AlkCho (1-*O*-hexadecyl-2-eicosa-5',8',11',14'-tetraenoyl-*sn*-glycero-3-phosphocholine), similar to the results utilizing phospholipid subclasses containing oleic acid at the *sn*-2 position (compare Figures 1B and 1C). To demonstrate that the results were independent of the separation method employed, we utilized the fluorescence self quenching of diSC₃(5) to document the increase in partitioning of diSC₃(5) in membranes comprised of PlasCho. Examination of the fluorescence profiles of diSC₃(5) demonstrated substantially less fluorescence in membranes comprised of PlasCho in comparison with membranes comprised of either

Figure 1 Partitioning of diSC₃(5) in membranes comprised of distinct choline glycerophospholipid subclasses

Small unilamellar vesicles comprised of PlasCho (Δ), AlkCho ($\circ$) or PhosCho ($\square$; 2 μ mol of each) were prepared in K^+ medium by high-power sonication. The prepared vesicles were diluted 100-fold prior to the addition of diSC₃(5). Uncaptured diSC₃(5) was removed by a Sephadex G-25 spin column and the amount of diSC₃(5) incorporated into the membrane was quantified as described in the Materials and methods section. (A) Concentration dependence of amphiphile partitioning in each phospholipid subclass. (B, C) Time-course partitioning of diSC₃(5) in membranes comprised of all three PO phosphocholine subclasses or in membranes comprised of all three PA phosphocholine subclasses respectively. Data represent the means $\pm$ S.D. for at least three independent experiments.

PhosCho or AlkCho. Collectively, these results demonstrated that the lipophilic cation diSC₃(5) rapidly and stably associates with phospholipid vesicles in a concentration-dependent manner and manifests substantially different partition coefficients in vesicles comprised of PlasCho in comparison with vesicles comprised of PhosCho or AlkCho.

Partitioning of the lipophilic anion DiBAC₃(3) into membranes comprised of distinct choline glycerophospholipid subclasses

We hypothesized that the differential partitioning of the lipophilic cation into membranes comprised of each choline glycerophospholipid subclass was due mainly to the substantially different dipole potentials present in PlasCho in comparison with PhosCho or AlkCho. If this was the case, utilization of a lipophilic anion should result in a reverse rank order of partition coefficients. Accordingly, we incubated the negatively charged amphiphile

Figure 2 Partitioning of DiBAC₄(3) in membranes comprised of distinct choline glycerophospholipid subclasses

Small unilamellar vesicles comprised of Plascho (Δ), AlkCho (○) or PhosCho (□; 2 μmol of each) were prepared in 0.6 ml of K⁺ medium by high-power sonication. The amount of DiBAC₄(3) incorporated into the membrane was quantified after Bligh and Dyer extraction of the 'pass-through' volume of the spin column. The membrane–water partition coefficients (*K*) were calculated. (A) Concentration dependence of amphiphile partitioning in each phospholipid subclass. (B, C) Time-course partitioning of DiBAC₄(3) in membranes comprised of all three PO phosphocholine subclasses or in membranes comprised of all three PA phosphocholine subclasses, respectively. Data represent the means ± S.D. for at least three independent experiments.

DiBAC₄(3) (0.5–3 μM) with vesicles comprised of each choline glycerophospholipid subclass and demonstrated that DiBAC₄(3) preferentially bound to vesicles comprised of PO PhosCho and PO AlkCho approximately 2.5-fold more effectively than it bound to vesicles comprised of PO Plascho (Figure 2A). Timed incubations of DiBAC₄(3) (1.5 μM) with vesicles comprised of each choline glycerophospholipid subclass demonstrated that DiBAC₄(3) was rapidly incorporated into the vesicles (within 5 min) and that the observed incorporation was stable (> 60 min). Calculation of the partition coefficient of DiBAC₄(3) in vesicles comprised of PO PhosCho and PO AlkCho demonstrated that it was about 2.5-fold higher than that manifest in vesicles comprised of PO Plascho (Figure 2B). To determine if these alterations were related to the individual molecular species of choline glycerophospholipid subclass present, vesicles containing arachidonic acid at the *sn*-2 position of each choline glycerophospholipid subclass were employed. Similar to the results

Figure 3 Effects of membrane surface charge on apparent anionic or cationic amphiphile partitioning in vesicles comprised of PO PhosCho or PO Plascho

Vesicles comprised of binary mixtures of either PO PhosCho (A–D) or PO Plascho (E–H) and indicated amounts of either 1-hexadecanoyl-2-octadec-9'-enoyl-*sn*-glycero-3-phosphoserine (PO PS) or 1-hexadecanoyl-2-octadec-9'-enoyl-*sn*-glycero-3-phosphoglycerol (PO PG) were prepared and diluted, and the apparent partitioning of either anionic or cationic amphiphiles in these vesicles was determined as described in the Materials and methods section. The apparent membrane–water partition coefficients of either cationic DiSC₃(5) (A, C, E, G) or anionic DiBAC₄(3) (B, D, F, H) were calculated as described in the Materials and methods section. Data represent the means ± S.D. for at least three independent experiments.

found in Figure 1, alterations of the molecular species of the choline glycerophospholipid subclasses did not result in any demonstrable change in the partitioning of the lipophilic anion (compare Figures 2B and 2C). Collectively, these results demonstrate that lipophilic anions and cations partition differentially into choline glycerophospholipid subclasses and that the ratios of the partition coefficients are equal and opposite for anionic and cationic lipophilic moieties.

Alterations in anionic and cationic lipophile binding to vesicles which have systematic alterations in membrane-surface charge

Incorporation of negatively charged phospholipids into membrane bilayers will alter the membrane potential and result in an

Figure 4 Correlation of anionic phospholipid induced membrane potentials with the molar fraction concentrations of anionic phospholipids incorporated as substitutional impurities in vesicles comprised of distinct choline glycerophospholipid subclasses

The membrane potentials induced by the incorporation of either PO PS or PO PG in membranes comprised of either PO PhosCho (A) or PO PlasCho (B) were calculated from the partition coefficients of diSC₃(5) or DiBAC₄(3) in these systems, as shown in Figure 3, after assuming that the differences in the other energy terms (e.g. Born, image and neutral energies) between membranes comprised of different subclasses of choline glycerophospholipids containing substitutional impurity amounts of anionic phospholipids can be neglected. The effects of PS on the induced membrane potentials were determined with diSC₃(5) (□) or DiBAC₄(3) (○), and the effects of PG on the induced membrane potentials were determined with diSC₃(5) (◇) or DiBAC₄(3) (△), utilizing results from Figure 3.

increase in lipophilic cation binding and a decrease in a lipophilic anion binding. As anticipated, incorporation of either phosphatidylserine (PS) or phosphatidylglycerol (PG, 0–20 mol %) into membrane bilayers comprised of PO PhosCho resulted in an increase in lipophilic cation binding and a decrease in lipophilic anion binding, as expressed by their apparent partitioning (Figures 3A–3D). Similar results were obtained utilizing vesicles comprised of PO PlasCho containing PS or PG as substitutional impurities (Figures 3E–3H). The induced membrane potential, calculated as described in the Materials and methods section, was identical utilizing either lipophilic cations or lipophilic anions, whether in the presence of PS or PG. Moreover, the induced potential in each combination was identical when utilizing vesicles comprised of either diacyl or vinyl ether choline glycerophospholipid subclasses (Figure 4). To document that

Figure 5 Independence of partition coefficients of lipophilic ions on the vesicle size

Vesicles with diameters of 100 (◇) and 200 nm (□) were prepared utilizing an extrusion apparatus, and vesicles with diameters of 30 nm (○) were prepared by high-energy sonication as described in the Materials and methods section. The partition coefficients of diSC₃(5) in different-sized vesicles comprised of binary mixtures of PO PhosCho and indicated amounts of PO PS were determined and calculated as described in the legend of Figure 1. Data represent the means $\pm$ S.D. of at least three independent experiments.

these apparent partitioning coefficients were independent of alterations in vesicle size, we prepared unilamellar liposomes possessing diameters of 100 and 200 nm utilizing an extrusion apparatus. There were no demonstrable differences in amphiphile apparent partitioning utilizing either small or large unilamellar vesicles (Figure 5).

Temperature dependence

To determine the relative effects of changes in enthalpy and entropy in the differential partitioning of charged amphiphiles into choline glycerophospholipid subclasses, the temperature dependence of the partition coefficients of lipophilic anions and cations was investigated. Incubation of PO PhosCho or PO PlasCho with DiBAC₄(3) resulted in the differential partitioning of charged amphiphiles into each phospholipid subclass (e.g. y intercept = 14.0 for PO PhosCho and 13.2 for PO PlasCho at 37 °C; Figure 6). Increasing the temperature resulted in a similar decrease in the membrane–water partitioning of DiBAC₄(3) for each phospholipid subclass (slope = 3.1×10^3 for PO PhosCho and 2.8×10^3 for PO PlasCho; Figure 6). Moreover, incorporation of the positively charged amphiphile diSC₃(5) into membrane vesicles comprised of either PO PhosCho or PO PlasCho resulted in the differential, but opposite, partitioning of the positively charged amphiphile (e.g. y intercept = 11.0 for PO

Figure 6 Temperature dependence of the membrane–water partition coefficients (K) of either DiBAC₄(3) or diSC₃(5) in vesicles comprised of either PO PhosCho or PO PlasCho

Small unilamellar vesicles comprised of either PO PhosCho (□, ■) or PO PlasCho (○, ●) were prepared and the membrane–water partition coefficients (K) of either DiBAC₄(3) (□, ○) or diSC₃(5) (■, ●) in these vesicles were determined, as described in the Materials and methods section, at the indicated temperatures. Data are the means $\pm$ S.D. for three independent experiments.

PhosCho and 12.2 for PO PlasCho at 37 °C). Increases in the temperature also resulted in a decrease in the membrane–water partitioning of the positively charged amphiphile diSC₃(5) which possessed similar slopes for both subclasses (e.g. slope = 1.8×10^3 for PO PhosCho and 2.1×10^3 for PO PlasCho).

At 22 °C, the free energy change (ΔG°) in moving the lipophilic cation diSC₃(5) from water into membranes comprised of PhosCho was -6.6 kcal/mol ($1 \text{ cal}_{\text{m}} = 4.184 \text{ J}$) (Table 1). In

contrast, ΔG° for moving diSC₃(5) into membranes comprised of PlasCho was -7.3 kcal/mol. This result suggests that the partitioning of lipophilic cations in membranes comprised of PlasCho is thermodynamically more favourable than in membranes comprised of PhosCho. The ΔG° for moving 1 mol of the lipophilic anion DiBAC₄(3) from water to membranes comprised of PhosCho was -8.6 kcal/mol. The ΔG° for partitioning of DiBAC₄(3) into membranes comprised of PlasCho was -8.0 kcal/mol. This result suggests that the partitioning of lipophilic anions in the membrane comprised of PlasCho is thermodynamically less favourable than in partitioning in membranes comprised of PhosCho. Previous studies have demonstrated that the partitioning of amphiphilic molecules was enthalpy-driven in small unilamellar vesicles, but was mainly entropy-driven in large unilamellar vesicles [25–31]. The molar enthalpy change (ΔH) and molar heat capacity (ΔC_p) can thus be calculated from the temperature dependence of partition coefficients, utilizing the assumption that partitioning was enthalpy-driven in these small unilamellar vesicles. Calculation of ΔC_p in membranes comprised of PhosCho or PlasCho resulted in values of 11.1 and 14.2 cal/mol per K for diSC₃(5) and 21.4 and 19.2 cal/mol per K for DiBAC₄(3), respectively (Table 1). Similarly, the molar entropy change (ΔS) for this process can be calculated by assuming that the partitioning was entropy-driven in large unilamellar vesicles as described in the Materials and methods section. The calculated ΔS values in membranes comprised of PhosCho or PlasCho were 102 and 112 cal/mol per K for diSC₃(5) and 122 and 114 cal/mol per K for DiBAC₄(3), respectively (Table 1).

DISCUSSION

The experimental results presented herein demonstrate large differences in the partitioning of lipophilic cations and anions in membranes comprised of distinct choline glycerophospholipid subclasses. The data suggest that these differences in partitioning of lipophilic ions are mainly attributable to alterations in the membrane dipole potential of vesicles comprised of each choline glycerophospholipid subclass (see Scheme 1). Specifically, for the cation diSC₃(5) the membrane–water partition coefficient in membranes comprised of PlasCho was approximately 2.5-fold higher than in membranes comprised of PhosCho or AlkCho. In contrast, in the case of the anion DiBAC₄(3), the membrane–water partition coefficient in membranes comprised of PlasCho was only approximately one-third of those in membranes comprised of PhosCho or AlkCho. The incorporation of anionic phospholipids (PS or PG) decreases the membrane potential and increases the membrane–water partition coefficient of the cation diSC₃(5), whereas it decreases that of the anion DiBAC₄(3). The magnitude of the effect of phospholipid-subclass partitioning is

Table 1 Thermodynamic parameters for the partitioning of lipophilic ions in phospholipid bilayers at 22 °C

ΔG is the average value calculated from the partition coefficients for both small and large unilamellar vesicles. ΔH and ΔC_p were computed from the temperature dependence of the partition coefficients by assuming the partition process is enthalpy driven in small unilamellar vesicles. ΔS was calculated by assuming the process is entropy-driven in large unilamellar vesicles [28–31].

Probe	Phospholipid	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔC_p (cal/mol per K)	ΔS (cal/mol per K)
diSC ₃ (5)	PO PhosCho	-6.6 ± 0.3	-6.6 ± 0.2	11.1 ± 0.4	102 ± 4
diSC ₃ (5)	PO PlasCho	-7.3 ± 0.3	-7.2 ± 0.3	14.2 ± 0.5	112 ± 5
DiBAC ₄ (3)	PO PhosCho	-8.6 ± 0.5	-8.5 ± 0.4	21.4 ± 0.9	122 ± 8
DiBAC ₄ (3)	PO PlasCho	-8.0 ± 0.4	-7.9 ± 0.3	19.2 ± 0.8	114 ± 7

Scheme 1 Simplified schematic profile of the dipole and free energies of univalent lipophilic cations (A, C) or anions (B, D) in membrane bilayers comprised of PhosCho (A, B) or PlasCho (C, D)

In each panel, the dipole energy (ΔG_{Di}), other energy terms ($\Delta G - \Delta G_{Di}$) and total free energy (ΔG) were defined as in the Materials and methods section. Lines are not to scale.

Table 2 Determination of the relative dipole potential in membranes comprised of distinct subclasses of choline glycerophospholipids at 22 °C

Membrane potentials were calculated from the Gouy–Chapman equation [25–27]. The dipole potential relative to phosphatidylcholine membranes was computed by assuming that other energy items were identical [22].

Probe	Phospholipid	ΔG (kcal/mol)	Membrane potential (mV)	Relative dipole (mV)
diSC ₃ (5)	PhosCho	−6.6	−286	—
diSC ₃ (5)	PlasCho	−7.3	−315	−29
diSC ₃ (5)	AlkCho	−6.7	−290	−4
diBAC ₄ (3)	PhosCho	−8.6	−371	—
diBAC ₄ (3)	PlasCho	−8.0	−347	−24
diBAC ₄ (3)	AlkCho	−8.6	−371	0

equivalent to having ≈ 25 mol % anionic phospholipids in the membrane.

Membrane potentials can be calculated from the membrane–water partition coefficients utilizing Gouy–Chapman theory [25–27]. Our data (Table 2) for diacyl phospholipids are similar to values described by Flewelling and Hubbell for lipophilic ion partitioning [22]. For a first-order approximation of membranes comprised of different choline glycerophospholipid subclasses [22], the relative dipole potentials were compared neglecting the differences in the other energy terms (i.e. Born, image and neutral

energies). The membrane dipole potential calculated from the membrane–water partition coefficients was approximately 25 mV lower in membranes comprised of PlasCho than in membranes comprised of PhosCho or AlkCho (Table 2). This substantial difference in dipole potential in membrane bilayers comprised of different phospholipid subclasses can be explained by both the differential membrane conformation and the covalent structure of each phospholipid subclass near the membrane interface including: (i) the presence of an induced dipole in the double bond of the vinyl ether linkage of plasmalogens [8,9]; (ii) the differences in both the conformation and dynamics of the phospholipid head groups in choline glycerophospholipid subclasses [32,33]; and (iii) the alterations in the orientation of the carbonyl group at the *sn*-2 fatty acid chains [22–24]. It should be noted that previous workers have assumed that the *sn*-1 acyl chain in diacyl phospholipids does not contribute substantially to the membrane dipole potential since the dipole of carbonyl is oriented perpendicularly to the membrane director [24,34,35]. Our results support this assumption since both diacyl and alkyl ether choline glycerophospholipids have similar membrane potentials whereas vinyl ether choline glycerophospholipids have a different dipole potential.

The partitioning of many amphiphilic molecules (including natural metabolites, small peptides, potential-sensitive dyes and drugs) in biological membranes has been studied extensively [25–28]. Traditionally, partitioning has been considered to be driven primarily by hydrophobic interactions between the metabolite or drug of interest and the recipient membrane. There are conflicting results in the literature on the relative contributions of enthalpic and entropic forces to the free energy of transfer of amphiphile to its host membrane. Recently, Beschiaschvili and Seelig [26] investigated the partitioning of small peptides and potential-sensitive dyes in phospholipid membranes with high-sensitivity titration calorimetry, and found that for highly curved small vesicles with a diameter of 30 nm, the partitioning process was enthalpy-driven with ΔH of approximately -7 kcal/mol. In contrast, for large vesicles with more tightly packed lipids, the partitioning was mainly entropy-driven [26]. Our experimental results showed that the membrane–water partitioning coefficients were independent of vesicle size, similar to the results of Beschiaschvili and Seelig [26]. Accordingly, based on their studies and conclusions, we calculated the molar enthalpy change (ΔH) and the molar heat capacity (ΔC_p) from the temperature dependence of partition coefficients, by assuming that the partitioning was enthalpy-driven in small unilamellar vesicles. Similarly, we computed the molar entropy change (ΔS) by assuming that the partitioning was entropy-driven in large unilamellar vesicles. We specifically point out that it is likely, in most cases, that neither the enthalpy effect nor the entropy effect is responsible solely for the free energy of transfer of the partitioning process and that these conclusions represent the predominating interactions in each case.

The demonstration of differences in membrane dipole potential in phospholipid subclasses probably has profound influence on the interactions of proteins, charged lipids and ions with cellular membranes comprised of each phospholipid subclass. For example, a difference in dipole potential affects the conformation and kinetics of conformational alterations (i.e. dynamics) of intrinsic (i.e. transmembrane) and extrinsic (i.e. surface-bound) proteins in the membrane bilayers. The results suggest that the conformation of the protein near the hydrophobic–hydrophilic interface is substantially different in membranes comprised of PlasCho compared with PhosCho. Moreover, it seems likely that the observed differences in membrane potential modulate the activation-energy barriers for protein conformational alterations

near the membrane interface. In this regard, the salutary effect of plasmalogens on the conformation of ion channels or on the energy barriers determining channel conformational changes could be one reason underlying the predominance of plasmalogens in electrically active membranes. Similarly, the altered dipole potential could facilitate altered distributions of both ions and peptides at the membrane interface. We point out that prior studies have identified phospholipid-subclass-specific alterations in the kinetics of ion channels (e.g. gramicidin) and ion carriers (e.g. valinomycin) across biological membranes [18]. Recent studies have demonstrated > 10-fold differences in passive-ion permeability properties of vesicles comprised of different choline glycerophospholipid subclasses [36,37]. Thus, differences in membrane dipole potential could modulate ion flux either directly by modulating passive-ion flux or indirectly by modulating the conformation and dynamic kinetic properties of ion channels.

Many drugs are lipophilic ions under physiological conditions. Based on the present results, it seems likely that the partitioning of these charged drugs in biological membrane systems is phospholipid-subclass specific. The majority of mammalian phospholipids in most cell types are comprised of diacyl phospholipids. However, plasmalogens are the predominant phospholipid subclass present in many electrically active membranes. Indeed, in two specialized electrically active membrane systems in muscle, sarcolemma and sarcoplasmic reticulum, plasmalogen molecular species actually constitute the major phospholipid subclass [5,6]. We suggest that these differences in membrane dipole potential can be exploited to target drugs selectively to subcellular membrane compartments containing plasmalogen molecular species.

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