Electric field strength of membrane lipids from vertebrate species: Membrane lipid composition and Na\(^+\),K\(^+\)-ATPase molecular activity

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ABSTRACT

Intramembrane electric field strength is a very likely determinant of the activity of ion-transporting membrane proteins in living cells. In the absence of any transmembrane electrical potential or surface potential, its magnitude is determined by the dipole potential of the membrane’s lipid components and their associated water of hydration. Here we have used a fluorometric method to quantify the dipole potential of vesicles formed from lipids extracted from kidney and brain of eleven different animal species from four different vertebrate classes. The dipole potential was compared with the fatty acid composition and with the Na\(^+\),K\(^+\)-ATPase molecular activity of each preparation. The magnitude of the dipole potential was found to be relatively constant across all animal species, i.e. 236-334 mV for vesicles prepared from the total membrane lipids and 223-256 mV for phospholipids alone. The significantly lower value for phospholipids alone is potentially related to the removal of cholesterol and/or other common soluble lipid molecules from the membrane. Surprisingly, no significant dependence of the dipole potential on fatty acid composition was found. This may, however, be due to concomitant compensatory variations in lipid head group composition. The molecular activity of the Na\(^+\),K\(^+\)-ATPase was found to increase with increasing dipole potential. The fact that the dipole potential is maintained at a relatively constant value over a wide range of animal species suggests that it may play a fundamental role in ensuring correct ion pump conformation and function within the membrane.

Keywords: cholesterol, dipole potential, fatty acids, lipid, phospholipids, sodium pump
INTRODUCTION

Electric fields occur within membranes (23). Such electrical fields are known to be capable of influencing the function of numerous ion-transporting membrane proteins. Most notable is the case of voltage-sensitive ion channels, e.g. Na⁺, K⁺ and Ca²⁺ channels (21) as clearly demonstrated using the electrophysiological patch clamp technique of Neher and Sakmann (37). Here the relative open and closed times of voltage-sensitive ion channels can be measured as a function of an applied external voltage across the membrane. Less well recognized is that the site of action of any applied or naturally occurring transmembrane potential difference is actually within the membrane where the conformation of membrane proteins are likely influenced.

Factors that change the intramembrane electric field strength are likely to influence the function of ion-transporting membrane proteins. Such factors could include the binding of any charged or dipolar species to the membrane, or changes in the charged or dipolar components of the membrane associated with variations in membrane lipid composition. One of the most commonly occurring membrane phospholipids, phosphatidylcholine, for example contains a zwitterionic headgroup at physiological pH as well as dipolar carbonyl groups within the ester linkages to its hydrocarbon chains. Furthermore, the surfaces of lipid membranes are thought to be hydrated by oriented dipolar water molecules (33). Even in the absence of any transmembrane potential difference, these effects may contribute to a high electric field strength close to the membrane-water interface. In synthetic lipid membranes, the electric field strength is in the range 10⁸-10⁹ V m⁻¹ (8). The potential difference within the membrane, caused by the preferential orientation of lipid dipolar residues and water molecules, is often termed the dipole potential. Although its exact origin is still under discussion, theoretical studies (33) seem to suggest that the major contribution is the polarisation of surface water molecules, which overcompensates for a dipolar field of opposite polarity arising from the
lipid headgroup, e.g. phosphatidylcholine. The magnitude of the dipole potential cannot be measured by direct electrical means but can be determined indirectly from the relative conductance of lipid bilayers to hydrophobic anions and cations and from surface potential measurements on monolayers. In the case of asymmetric lipid bilayers, the dipole potential can also be estimated from the voltage dependence of the membrane capacitance (39, 41).

Based on the substantial variation in membrane phospholipid composition displayed by membranes of different species and between organs within a species (24, 25) it might be expected that different dipole potentials would exist and differentially influence the activities of membrane based proteins. Measurements of the dipole potential for membranes composed of phosphatidylcholine, of varying fatty acid composition, have yielded dipole potentials varying between 200-400 mV (1, 2, 19, 22, 40, 43, 45). The aim of this paper was firstly to investigate the magnitude of the dipole potential in membrane lipid vesicles prepared from lipids of natural membranes from a broad spectrum of species (4 avian, 6 mammalian, 1 reptilian and 1 amphibian species) and from distinctly different organs (brain and kidney). The second aim was to investigate if changes in the dipole potential correlated with the fatty acid composition of the membrane. The final aim was to investigate if there was a correlation between the activity (i.e. the molecular activity or turnover of substrate per enzyme) of a major energy-consuming membrane bound active transporter, namely the Na⁺,K⁺-ATPase (an enzyme with kinetics known to be sensitive to its local electric field; 3, 18, 29) and dipole potential. This paper reports on the results of these experiments and provides some of the first measurements of membrane dipole potentials conducted on natural lipid mixtures.
MATERIALS AND METHODS

Animals

Animals examined in the present study were adults of either sex. Tissues from sheep (Ovis aries), pigs (Sus scrofa) and cattle (Bos taurus) were obtained from a local abattoir (Wollondilly, N.S.W., Australia). Tissues were placed on ice immediately following the death of each animal and transported back to the University of Wollongong. Emus (Dromaius novaehollandiae) were purchased from Marayong Park Emu Farm (Falls Creek, NSW, Australia) and transported to the University of Wollongong and used immediately.

Mice (Mus musculus) and rats (Rattus norvegicus) were purchased from Gore Hill Research Laboratories (Sydney, NSW, Australia). Ducks (Anas platyrhynchos) and geese (Anser anser) were purchased from the Narellan Aviary Bird Auction (NSW, Australia). Currawongs (Strepera graculina) were free-living animals caught locally in the Wollongong area. Shingleback lizards (Trachydosaurus rugosus) were collected from areas in the North-Western parts of NSW. Cane toads (Bufo marinus) from Queensland where obtained from a commercial supplier. Mice were killed by cervical dislocation whereas lizards, rats and birds were killed by Nembutal® or Lethabarb® overdose (pentobarbitone sodium, 100mg.kg\(^{-1}\) body mass; intraperitoneal, except in the case of the emus where the injection was intrajugular). Cane toads were killed under tricaine methanesulfonate (MS222, pH adjusted to 7.4) anaesthesia.

Animals housed in the animal house at the University of Wollongong were maintained individually (with the exception of the toads that where maintained together in large plastic containers) at between 22-28 ± 2°C under a 12h:12h light dark photoperiod. Lizards had access to heating lamps for behavioral thermoregulation. All animals had free access to food
appropriate to the species and fresh water. Animals were maintained and procedures performed in accordance with the National Health and Medical Research Council Guidelines for Animal Research in Australia. Experiments were approved by the University of Wollongong Animal Experimentation Ethics Committee. Collection of wild species was conducted under license from the NSW National Parks and Wildlife Service.

**Na⁺,K⁺-ATPase molecular activity**

Kidneys and brains were removed following the death of each animal and Na⁺,K⁺-ATPase concentration was determined by [³H]ouabain binding as described in detail elsewhere (48). Na⁺,K⁺-ATPase activity was measured as the ouabain-inhibitable ATPase activity in detergent treated (sodium deoxycholate 1mg.ml⁻¹) homogenates (10%, w/v). Molecular activity, which is defined as the maximal rate of substrate turnover by a protein, was derived by dividing maximal Na⁺,K⁺-ATPase activity (expressed as pmol Pi.mg wet weight⁻¹.min⁻¹) by the Na⁺,K⁺-ATPase concentration (in pmol.mg wet weight⁻¹) for the same preparation. The net result was expressed as the number of ATP molecules hydrolysed by each Na⁺,K⁺-ATPase per minute. Some of the molecular activity data has been reported elsewhere in investigations of its correlation with body mass and lipid composition (48, 49). Samples of each tissue were kept at -80°C until required for the preparation of microsomal membranes and subsequent extraction of lipids and measurements of dipole potential following vesicle formation.

**Preparation of microsomal membranes**

Microsomal membrane fragments were prepared according to the procedure of Jørgensen (28). Tissue homogenates (10% in 250mM sucrose, 30mM histidine; pH 7.4), were centrifuged at 6,000g for 15 minutes with the pellet resuspended and centrifuged for a further 15 minutes at 6,000g. The supernatants from both spins were combined and centrifuged at
48,000g for 35 minutes. The resultant pellet, designated microsomal membranes, was resuspended in 250mM sucrose, 30mM histidine (pH 7.2).

**Extraction of total membrane lipids and phospholipid separation**

All solvents used in the lipid extractions were of ultra-pure grade and were from Merck Pty Ltd (Kilsyth, VIC, Australia). Analytical grade butylated hydroxytoluene was from Sigma Aldrich (Castle Hill, NSW, Australia). Lipids were extracted from microsomal membrane fractions by a standard method (17) using chloroform:methanol (2:1, v/v) containing butylated hydroxytoluene (0.01% w/v) as an antioxidant. In this study we have termed these extracts ‘total membrane lipids’ because they include both phospholipids and other membrane lipids (primarily cholesterol). Phospholipids were separated by solid phase extraction on Phenomenex SPE® silica cartridges (Pennant Hills, NSW, Australia). Phospholipid concentration of total membrane lipids and phospholipids was determined using a phosphorus assay as described by Mills et al (34).

**Preparation of vesicles from synthetic and natural lipids**

Synthetic phospholipids used for calibration purposes were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Unilamellar vesicles were prepared by the ethanol injection method described in detail elsewhere (52, 53). The final vesicle suspension (in 30 mM Tris, 1 mM EDTA, 150 mM NaCl; pH 7.2) contained no detectable trace of ethanol, i.e. [ethanol] ≤ 10 μM, according to a nicotinamide adenine dinucleotide/alcohol dehydrogenase enzymatic assay (Boehringer, Mannheim). The phospholipid content of the synthetic vesicle suspension was determined by the phospholipids-B test from Wako (Wako Pure Chemical Industries, Osaka, Japan). The final phosphatidylcholine concentration following dialysis (from Medicell International London, UK) was in the range 1.5-3.2 mM. The origins of the various reagents used were as follows: Tris-[(hydroxymethyl)amino)methane (99.9%, Sigma
Chemical Co., St Louis, MO, USA), EDTA (99%, Sigma Chemical Co.), NaCl (analytical grade, Merck, Darmstadt, Germany), HCl (1.0M Titrisol solution, Merck) and ethanol (analytical grade, Merck).

To prepare vesicles from natural lipids, total membrane lipid and phospholipid extracts (approx. 2mM) from microsomal preparations were dried under nitrogen and resuspended in a buffer containing 30 mM Tris, 1 mM EDTA, and 150 mM NaCl in de-ionised water (pH 7.2). The resuspension process involved 20-30 min of sonication under nitrogen to prevent any oxidation. Lipid and phospholipid solutions were then given 11 passes through an Avanti Mini-Extruder (Avanti Polar Lipids, Alabaster, AL, USA), equipped with a 100nm pore-size Nucleopore (Whatman, Maidstone, UK) polycarbonate membrane, to produce a unimodal, normal distribution of unilamellar lipid vesicles of 100nm size.

**Fluorescence measurements**

Di-8-ANEPPS was obtained from Molecular Probes (Eugene, OR). In vesicles and in aqueous solution, the dye showed a single long wavelength fluorescence emission band. For spectral measurements in the presence of lipid vesicles, 5 µl of an ethanolic dye solution (1.00 mM) was added to 1 ml of the vesicle-containing aqueous solution, thus giving a final di-8-ANEPPS concentration of 5 µM. After addition of the dye, the solutions were left overnight in a nitrogen environment to allow for dye disaggregation and incorporation into the membrane. Due to the addition of the ethanolic dye solution, the final solution contained a small percentage of ethanol (0.5%). The effect of the small volume of ethanol added on the fluorescence spectra of membrane-bound dye was checked in separate control experiments and found to be negligible.
Steady-state fluorescence measurements were recorded with a Shimadzu RF-5301PC spectrofluorophotometer. To minimise contributions from scattering of the excited light and higher order wavelengths, a glass cut-off filter was used in front of the emission monochromator. The fluorescence emission of di-8-ANEPPS was measured at an emission wavelength of 670 nm (+RG645 glass cut-off filter, Schott, Mainz, Germany). The fluorescence ratio, $R$, is defined as the ratio of the fluorescence intensity at an excitation wavelength of 420 nm divided by that at 520 nm. The wavelengths were chosen based on a previous study (9) to avoid any effects of membrane fluidity on the measured fluorescence ratios. The use of the ratiometric method avoids any effects of small variations in the dye concentration on the fluorescence results. Control experiments showed that as long as the lipid concentration is approximately a factor of 300 in excess of the dye concentration, no effects of variations of the dye concentration on the measured $R$ value are observed (9). Measurement temperature was thermostatically controlled to at least 2°C above the main phase transition temperature of each synthetic lipid, so that each lipid was in its liquid crystalline state. For the natural lipid extracts, measurements were conducted at both 37°C and 25°C.

*Phospholipid fatty acid composition*

Portions of the fatty acid composition of the mammalian and avian species have previously been reported elsewhere (48, 49). Fatty acid analysis of the phospholipid fractions of natural membrane lipid extracts from the shingleback and cane toad were determined using the same method as for the other species as described in detail in Pan and Storlien (38). Briefly, phospholipid fractions were transmethylated with 14% (w/v) boron trifluoride in methanol and fatty acid methyl esters were separated by gas-liquid chromatography on a Hewlett-Packard 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) with a fused silica capillary column. Individual fatty acids were identified by comparing each
peak’s retention time to those of external standards. The relative amount of each fatty acid (% of total fatty acid) was determined by integrating the area under the peak and dividing the result by the total area for all fatty acids.

Statistical analyses

Values are presented as means ± standard deviation. Linear and logarithmic analyses were conducted using either JMP® 4.0.1 software (SAS Institute Inc., NC, USA) or Origin 6.0 (Microcal Software Inc., MA, USA). Figures were produced using KaleidaGraph™ 3.51 software (Synergy Software, PA, USA) or Origin 6.0 (Microcal Software Inc., MA, USA).
RESULTS

Calibration of di-8-ANEPPS

For the measurement of the dipole potential a recently developed spectroscopic technique (9, 20) involving the fluorescence intensity ratio of a voltage-sensitive styrylpyridinium dye, di-8-ANEPPS, was used. This method has the advantage that it is applicable directly to membrane vesicles and, in principle, even living cells, and does not require the formation of planar black lipid membranes or monolayers. In order to convert the fluorescence ratio $R$ of di-8-ANEPPS to a dipole potential value in mV a calibration of the dye in pure synthetic lipid was required. This new calibration was performed using dipole potential values from phosphatidylcholine bilayers corrected for the differences in hydration energies of the hydrophobic ions used in the experimental determination (43) as well as values determined from monolayer data (see Table 1). The calibration is shown in Figure 1. The monolayer dipole potentials have been calculated for the area per lipid molecule expected from X-ray crystallographic data according to the Helmholtz equation as given by Smaby and Brockman (45; see Eq. (2)). The values thus calculated include an area-independent contribution to the dipole potential.

As shown in Figure 1, the $R$ values obtained using di-8-ANEPPS, plotted against the literature values of $\psi_d$ show an approximately linear relationship (correlation coefficient, $r = 0.79$, $P < 0.01$). Considering the very different electrical methods used and the difficulties involved in the determination of $\psi_d$, a certain degree of scattering of the values of $\psi_d$ is to be expected. Nevertheless, a significant correlation between the value of $R$ and $\psi_d$ is present. If the data in Table 1 are fitted to a straight line as defined by the equation (1):

$$R = m\psi_d + d$$  \hspace{1cm} (1)
the slope, $m$, and the intercept, $d$, are calculated to be $4.3 \pm 1.2 \cdot 10^{-3} \text{mV}^{-1}$ and $−0.3 \pm 0.4$, respectively. Rearranging Eq. (1) results in the equation (2),

$$
\psi_d = \frac{R + 0.3}{4.3 \cdot 10^{-3}}
$$

which allows the dipole potential of a given membrane to be calculated from the measured $R$ value.

Although not used for calibration purposes, because no electrical data is available, fluorescence ratio measurements were also carried out on synthetic vesicles composed of the lipid docosahexaenoylphosphatidylcholine (22:6 (n-3)). This lipid was measured in addition to those listed in Table 1, because 22:6(n-3) is both the longest and most polyunsaturated fatty acid chain commonly found in natural membranes and is a particularly important constituent in the membranes of brain (see Table 3). The fluorescence ratio measured was 0.359 ($\pm 0.001$). Applying equation (2), this corresponds to a dipole potential value of 153 ($\pm 102$) mV. This value represents the lowest dipole potential of any lipid studied so far. The particularly low value could be explained by the high degree of unsaturation of its hydrocarbon chains which cause it to occupy a relatively large surface area in the membrane and hence lead to a relatively low density of molecular dipoles within the membrane surface (7).

*Dipole potential of total membrane lipids and phospholipids*

The dipole potential of the natural total membrane lipid and phospholipid extracts at 37°C are shown in Table 2. In the phospholipids, there was little variation in dipole potential between species and across tissues, with values ranging from 223mV to 256mV. In the measurements of dipole potential in total membrane lipids a modest level of variation was
found in the kidney. Here values ranged between 236mV in the duck to 334mV in the mouse. In the brain all total membrane lipids displayed a fairly similar dipole potential of approximately 300mV.

Dipole potential was also determined at 25°C in all samples with very little difference observed to those measured at 37°C. Dipole potentials at 25°C were only approximately 2 - 4% greater than the value at 37°C for most samples, with a maximum difference of 7%. This relative independence of temperature is to be expected because the magnitude of the dipole potential depends on the average orientation of the dipoles from which it is formed and their surface density, but not on their speed of movement. If the dipoles move more rapidly but maintain the same average orientation in the membrane surface, no change in dipole potential would be predicted. This is supported by previous work (9) that has shown that the fluorescence ratio of di-8-ANEPPS is independent of membrane fluidity as long as the membrane does not undergo a phase transition. A more significant effect is the fact that the magnitude of the dipole potential in phospholipid extracts was on average 22% less than that of the total membrane lipid extracts. The possible origin of this effect is considered in the discussion.

**Na⁺,K⁺-ATPase molecular activity and phospholipid fatty acid composition**

Some of the Na⁺,K⁺-ATPase molecular activity data and phospholipid fatty acid composition of the species used (except for those of the toad and lizard species) have previously been reported (48, 49). In the present study however, they are used to examine any potential link with the dipole potential of the phospholipids and lipids extracted from the same membranes. Both Na⁺,K⁺-ATPase molecular activity and phospholipid fatty acid composition showed substantial variation between and within tissues (Table 3) which contrasts sharply against the relatively consistent values observed for dipole potential. Molecular activity
values were generally higher in the brain than in the kidney and varied approximately 7-fold between species. The highest Na\(^{+}\),K\(^{+}\)-ATPase molecular activity value determined was for the mouse brain at 29,000 ATP.min\(^{-1}\), while duck kidney displayed the lowest molecular activity at 4,500 ATP.min\(^{-1}\).

The kidney and brain also showed a number of differences in fatty acid profile (Table 3). The kidney of most species contained a relatively greater proportion of n-6 polyunsaturated fatty acids (PUFA) including 18:2(n-6) and 20:4(n-6), while the brain was characterized by high levels of n-3 PUFA, primarily 22:6(n-3). The content of a number of individual fatty acids showed dramatic variation between species, with the percentage of 18:2(n-6) and 22:6(n-3) varying approximately 120-fold and 60-fold respectively.

Comparing the fatty acid compositions of each tissue with its corresponding dipole potential value, there were no obvious determinants of the dipole potential. When plotted against Na\(^{+}\),K\(^{+}\)-ATPase molecular activity, the dipole potential of membrane lipids showed a positive correlation (\(r=0.66\), n=15, \(P=0.008\)) but no similar relationship with the dipole potential of membrane phospholipids was observed. An examination of the residual plots of these findings suggested that a non-linear curve might better describe the relationship between Na\(^{+}\),K\(^{+}\)-ATPase molecular activity and the dipole potential of membrane lipids. A logarithmic equation \([y = -22 + 76\ln (x)]\) was a better fit for describing this relationship (\(r=0.76\), \(P<0.0001\)) (Fig. 2).

**DISCUSSION**

The intramembrane electric field strength of phosphatidylcholine bilayers is known to vary depending on the fatty acid composition (1, 2, 19, 22, 40, 43, 45). In general it has been
found that there is an inverse relationship between the dipole potential and the level of membrane unsaturation (7). Using the same spectroscopic technique, we have utilized the large natural variation in fatty acid composition of membranes that occur between vertebrate species (including mammals, birds and ectothermic vertebrates), to investigate the dipole potential in natural lipid extracts.

Examination of the dipole potentials of the natural lipid mixtures in the present investigation showed that they existed within the range i.e. 220 – 335mV, which is within the range (200-400 mV) of those previously reported for synthetic phospholipid bilayers (7). However, although the natural membrane lipid extracts display dipole potentials within the expected range, a number of distinct differences were observed compared to those measured on synthetic phospholipid bilayers. Firstly, the dipole potential remained relatively constant in the natural phospholipids despite very large variation in fatty acid composition. Secondly, the dipole potential showed no obvious dependence on the level of unsaturation (as measured by unsaturation index). This is in marked contrast to what is observed in bilayers composed of various phosphatidylcholine molecular species where increasing unsaturation of the fatty acid chains causes a significant reduction in the dipole potential (7).

From measurements on synthetic phosphatidylcholine vesicles it appears that the major effect of fatty acid chain unsaturation on membrane dipole potential is indirectly via its effect on lipid packing (7). The acyl chains themselves appear to make a minimal contribution to the dipole potential (39, 41). The effect of packing is explained in analogy to a parallel-plate capacitor by the Helmholtz equation,

$$\psi_d = \frac{\mu}{A\varepsilon_0\varepsilon}$$

(3)
where $\mu_{\perp}$ is the average component of the lipid dipole moment (including membrane-associated water molecules) perpendicular to the plane of the membrane, $\varepsilon_0$ is the permittivity of free space and $\varepsilon$ is the local dielectric constant. From equation (3) it can be seen that $\psi_d$ is expected to be directly proportional to $1/A$, the lipid packing density, as long as the local dielectric constant doesn't vary. For the lipid extracts studied here, the values of the unsaturation index (UI), given in Table 3, show that the average number of double bonds per fatty acid chain is in the range 1.82-3.58. Taking a $\psi_d$ value of approximately 400 mV (from the $R$ values of Table 1 and applying equation (2)) for a fully saturated phosphatidylcholine and its area per lipid molecule of 0.65 nm$^2$ (7) as reference values, one can estimate from equation (3) the approximate range of $\psi_d$ values expected for such a range of the unsaturation index. For this purpose we make the rather drastic approximation that the membrane is composed totally of phosphatidylcholine (PC). For the PC lipids 18:2 n-6, 18:3 n-3 and 20:4 n-6, $A$ values of 0.97, 1.08 and 1.11 nm$^2$, respectively, have previously been calculated (7). From these values equation (3) allows one to estimate an expected range of dipole potential values of 234-268 mV. In spite of the significant simplifications made in this calculation, it can be seen that this range agrees very well with the experimental phospholipid values of $\psi_d$ listed in Table 2. The fact that the dipole potential does not greatly vary between the different phospholipid extracts would, therefore, seem to be a reflection of the fact that the average number of double bonds per fatty acid chain is only varying between values of approximately 2 and 4 and each additional double bond has only a relatively small effect on lipid packing.

Natural membranes contain a mixture of hundreds of different phospholipid molecular species (11), and while the fatty acid moieties of the phospholipids are known to have an important influence on dipole potential (7, 39), it is unclear what effect the different phospholipid headgroups have on membrane dipole potential. Measurements using synthetic
lipids have primarily concentrated on phosphatidylcholine molecules of varying acyl composition, at least in part because of the relatively large region of their phase diagrams in which they form the biologically relevant L\text{\textsubscript{\alpha}} lamellar phase (6, 32). In contrast, the natural lipid extracts used in the current study would have been composed of a variety of phospholipids with different headgroups. Although the zwitterionic headgroups of phosphatidylcholine and phosphatidylethanolamine are not thought to be a major contributor to the magnitude of the dipole potential (4, 5), this may not be true for all classes of phospholipid, e.g. phosphatidylserine, phosphatidylglycerol. One reason why the dipole potential was found to be relatively consistent in the natural membrane lipids, despite large changes in fatty acid composition, could have been that the effects of different fatty acid chain unsaturation were compensated for by the effects of differences in phospholipid headgroup composition.

While our measurements were conducted on natural membrane lipid extracts, and are therefore far more representative of cellular membranes than synthetic lipid vesicles composed of a single lipid species, they may still not represent the natural condition in cells. In the natural condition, bilayer lipids are regulated in an asymmetric distribution such that all naturally-occurring membranes exhibit a distinct sidedness, i.e. the exofacial and cytofacial leaflets of the membrane bilayer are different (42). This relationship has been most extensively studied in human erythrocytes where it has been shown that phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol are primarily located in the cytoplasmic leaflet of the bilayer, while phosphatidylcholine and sphingomyelin are located predominantly in the exofacial leaflet (42). In the present study, the preparation of vesicles via extrusion is likely to have disrupted the normal lipid asymmetry of the membrane. Considering that di-8-ANEPPS distributes itself in the exofacial leaflet of the
bilayer, disruption to the distribution of lipids needs to be kept in mind when interpreting the current data.

One of the most consistent findings in the current investigation was a clear difference in dipole potential between the total membrane lipid and phospholipid extracts. This difference may partially be due to the influence of membrane cholesterol, which was only present in the total membrane lipids. Cholesterol is an important constituent of animal membranes and is primarily located in the plasma membrane (10, 16). In monolayers, cholesterol interacts with phospholipids to reduce the mean area per molecule (46, 50, 51). The higher dipole potential observed in the membrane lipids in the present investigation, may partially be due to cholesterol reducing the mean area per molecule and thereby effectively ‘concentrating’ the molecular dipoles and increasing the local electric field strength. Such an effect is consistent with the results of Clarke (7), where dipole potential was shown to be higher in phosphatidylcholine vesicles that occupied a reduced mean surface area. The effect could also be partially explained by the electric field induced by the cholesterol molecule itself and its polarisation of surrounding lipid headgroups and water of hydration. It should be noted however that the magnitude of the difference in dipole potential between the total membrane lipid and phospholipid extracts was not correlated simply to the amount of cholesterol present in the membrane.

The lack of direct correlation with cholesterol content may partially be explained by the fact that the condensation effect of cholesterol and therefore its potential influence on dipole strength is itself nonlinear as we have measured in purified phospholipid monolayers of varying cholesterol molar ratio (Wu and Else, unpublished results). Other factors that could explain the dipole potential difference could include other lipid soluble molecules that may be present in the lipid but not transfer into the phospholipid extract during separation. These
molecules might include some vitamins and hormones (e.g. thyroid hormones) that may possess dipoles themselves or polarise others within the membrane.

A final interesting observation was that, in total membrane lipids, the molecular activity of the Na⁺,K⁺-ATPase increased with increasing dipole potential (see Fig. 2). This is consistent with the results of Ganea et al. (18), who showed that a reduction of the dipole potential induced by the adsorption of lyotropic anions to the membrane caused a significant decrease in the rate constant for the enzyme’s conformational change in its phosphorylated state (E₁P → E₂P). Effects of the dipole potential on the kinetics of reaction steps of the enzyme would be expected if they involve charge displacement (either ions or charged protein residues) and are hence sensitive to their local electric field. It appears, therefore, that modulation of dipole potential via membrane composition could be a possible fine-tuning mechanism for optimizing ion pump function.

At this stage it cannot be excluded that some of the variation in Na⁺,K⁺-ATPase activity observed here could be related to differences in the protein between the different species rather than their lipids. Different isoforms of the enzyme do exist, which are expressed to different degrees in different organs or tissues (47). In the kidney, which was one of the organs studied here, the α₁ isoform appears to be expressed almost exclusively. In the brain, on the other hand, the α₁, α₂ and α₃ isoforms all appear to be present, with the expression level changing with the degree of development. Convincing data supporting the role of lipids in determining Na⁺,K⁺-ATPase activity has, however, come from membrane crossover experiments performed by Else and Wu (15). By isolating both the enzyme and their surrounding lipids from rat and toad and then reconstituting the enzyme in the lipids of the other animal, they were able to provide direct experimental evidence that the activity of the Na⁺,K⁺-ATPase enzyme is largely determined by its surrounding lipid environment.
Significant effects of lipids on the activity of the Na\(^+\),K\(^+\)-ATPase have also been found in experiments in which the enzyme has been reconstituted into artificial vesicles consisting of synthetic lipids of defined composition (11, 12, 31). These effects have predominantly been discussed in terms of the lipid chain length and it has been proposed that there must be a hydrophobic match between the hydrophobic transmembrane segments of the protein and the hydrocarbon fatty acid chains of the lipids. The cholesterol content of the membrane has also been found to have a significant effect on Na\(^+\),K\(^+\)-ATPase activity. The effect of cholesterol has similarly been discussed in terms of the hydrophobic match between the membrane and the protein (11), since cholesterol induces an ordering of the lipid acyl chains and hence an increase in membrane thickness. Cornelius et al. (12) recently found, however, that hydrophobic matching could not explain all of the lipid effects on Na\(^+\),K\(^+\)-ATPase activity and he suggested additional mechanisms, including effects on intramembrane charge distribution (consistent with the findings reported here) or effects on the oligomeric state of the protein within the membrane. As a caveat it is important to point out, however, that the correlation found here between Na\(^+\),K\(^+\)-ATPase activity and the membrane dipole potential does not necessarily imply that the change in intramembrane electric field strength caused by differences in the dipole potential is the actual cause of the change in enzyme activity. It is still feasible that the underlying cause of the change in Na\(^+\),K\(^+\)-ATPase activity is another property of the lipid membrane which also incidentally affects the dipole potential. Further controls would still be necessary in order to make a definitive statement.

The relatively constant value of the dipole potential over the range of animals studied, i.e. 236-334 mV for total membrane lipids and 223-256 mV for phospholipids, may be a reflection of the importance of the dipole potential in determining proper ion pump conformation within the membrane and hence its correct function. Recently the lipid dipole potential was proposed as a possible cause of the different structures of anion and cation ion channels within cell membranes (14). It is worth making the point, however, that the dipole
potential values reported here represent average values for the entire membrane surface of each vesicle suspension measured. In membranes of living cells, on the other hand, it appears very likely that the membrane composition is not uniform, rather that local variations occur, e.g. the formation of so-called “lipid rafts” (44). This could lead to lateral variation in the magnitude of the dipole potential and localized regions of high or low dipole potential, particularly around membrane proteins such as the Na\textsuperscript{+},K\textsuperscript{+}-ATPase. Such variations could play a regulatory role in determining pump activity. Future investigations might benefit from the use of fluorescent probes in combination with fluorescence microscopy to examine for localized dipole potential variations within the membrane of living cells and their potential to influence ion pump activity and other membrane functions.

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Figure Legends

Figure 1: Correlation between the fluorescence excitation ratio, $R$, of di-8-ANEPPS and the membrane dipole potential, $\psi_d$. $R$ is defined as the fluorescence intensity at a $\lambda_{ex}$ of 420 nm divided by that at a $\lambda_{ex}$ of 520 nm. In each case the emission wavelength used was 670 nm. The values of $\psi_d$ have been taken from the literature (see Table 1). The line represents a fit of the data to a straight line ($r = 0.79$, $P < 0.01$).

Figure 2: Correlation between the Na$^+$,K$^+$-ATPase molecular activity of the kidney and brain of various animals and the magnitude of the dipole potential of vesicles formed from total membrane lipids extracted from the same tissues. Data are from Table 2 and Table 3. Logarithmic regression was used for the analysis ($r = 0.76$, $P < 0.0001$).
Figure 1

![Graph showing the relationship between $R$ and $\psi_d / mV$. The x-axis represents $\psi_d / mV$ ranging from 200 to 450, and the y-axis represents $R$ ranging from 0.6 to 1.6. The graph includes scattered data points and a trend line.]
Table 1. Comparison of the fluorescence ratio, $R$, of di-8-ANEPPS with electrical bilayer and monolayer measurements of the dipole potential, $\psi_d$, of various phosphatidylcholines

<table>
<thead>
<tr>
<th>Phosphatidylcholine</th>
<th>$R$</th>
<th>$T(°C)$</th>
<th>$\psi_d$(mV)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilauroyl (12:0)</td>
<td>1.419 (± 0.008)</td>
<td>20</td>
<td>335</td>
<td>30, 45</td>
</tr>
<tr>
<td>Dimyristoyl (14:0)</td>
<td>1.463 (± 0.010)</td>
<td>30</td>
<td>396</td>
<td>26, 27, 45</td>
</tr>
<tr>
<td>Dimyristoyl (14:0)</td>
<td>1.463 (± 0.010)</td>
<td>30</td>
<td>403</td>
<td>26, 27, 45</td>
</tr>
<tr>
<td>Dipalmitoyl (16:0)</td>
<td>1.437 (± 0.007)</td>
<td>45</td>
<td>400</td>
<td>35, 36, 45</td>
</tr>
<tr>
<td>Dipalmitoyl (16:0)</td>
<td>1.437 (± 0.007)</td>
<td>45</td>
<td>346</td>
<td>19, 43</td>
</tr>
<tr>
<td>Dioleoyl (18:1n-9)</td>
<td>0.937 (± 0.010)</td>
<td>20</td>
<td>320</td>
<td>30, 45</td>
</tr>
<tr>
<td>Dioleoyl (18:1n-9)</td>
<td>0.937 (± 0.010)</td>
<td>20</td>
<td>335</td>
<td>30, 45</td>
</tr>
<tr>
<td>Dioleoyl (18:1n-9)</td>
<td>0.937 (± 0.010)</td>
<td>20</td>
<td>343</td>
<td>40, 43</td>
</tr>
<tr>
<td>Stearoyl-Oleoyl (18:0/18:1n-9)</td>
<td>1.068 (± 0.004)</td>
<td>20</td>
<td>316</td>
<td>40, 43</td>
</tr>
<tr>
<td>Dihexadecyl</td>
<td>0.757 (± 0.036)</td>
<td>50</td>
<td>228</td>
<td>19, 43</td>
</tr>
</tbody>
</table>

*R* values are the average of 5 measurements with their standard deviations in brackets.
Table 2. Fluorescence ratio, $R$, and dipole potential, $\Psi_d$, of natural lipid extracts at 37°C

<table>
<thead>
<tr>
<th></th>
<th>Total membrane lipid</th>
<th>Phospholipid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R$</td>
<td>$\Psi_d$ (mV)</td>
</tr>
<tr>
<td><strong>Kidney</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>1.149 (± 0.060)</td>
<td>334 (± 132)</td>
</tr>
<tr>
<td>Rat</td>
<td>0.942 (± 0.020)</td>
<td>286 (± 123)</td>
</tr>
<tr>
<td>Pig</td>
<td>0.894 (± 0.001)</td>
<td>275 (± 120)</td>
</tr>
<tr>
<td>Cow</td>
<td>0.935 (± 0.026)</td>
<td>284 (± 123)</td>
</tr>
<tr>
<td>Duck</td>
<td>0.729 (± 0.006)</td>
<td>236 (± 115)</td>
</tr>
<tr>
<td>Emu</td>
<td>0.753 (± 0.003)</td>
<td>242 (± 115)</td>
</tr>
<tr>
<td>Shingleback</td>
<td>0.859 (± 0.003)</td>
<td>267 (± 119)</td>
</tr>
<tr>
<td><strong>Brain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>1.012 (± 0.071)</td>
<td>302 (± 126)</td>
</tr>
<tr>
<td>Rat</td>
<td>1.005 (± 0.025)</td>
<td>300 (± 126)</td>
</tr>
<tr>
<td>Sheep</td>
<td>0.974 (± 0.057)</td>
<td>293 (± 124)</td>
</tr>
<tr>
<td>Pig</td>
<td>1.029 (± 0.007)</td>
<td>306 (± 126)</td>
</tr>
<tr>
<td>Cow</td>
<td>1.022 (± 0.023)</td>
<td>304 (± 126)</td>
</tr>
<tr>
<td>Currawong</td>
<td>0.975 (± 0.015)</td>
<td>293 (± 124)</td>
</tr>
<tr>
<td>Duck</td>
<td>0.961 (± 0.015)</td>
<td>290 (± 123)</td>
</tr>
<tr>
<td>Goose</td>
<td>0.986 (± 0.007)</td>
<td>296 (± 125)</td>
</tr>
<tr>
<td>Emu</td>
<td>0.979 (± 0.072)</td>
<td>294 (± 125)</td>
</tr>
<tr>
<td>Toad</td>
<td>0.974 (± 0.011)</td>
<td>293 (± 124)</td>
</tr>
</tbody>
</table>

$R$ values are the average of 5 measurements with their standard deviations in brackets. The $\Psi_d$ values were calculated from the $R$ values according to eq. 2. Their errors quoted include significant contributions from the standard errors in the slope and intercept of the calibration (Fig. 1) and have been calculated using eq. 2 according to the laws of error propagation.
Table 3. Molecular activity and microsomal phospholipid fatty acid profile of vertebrate kidney and brain

<table>
<thead>
<tr>
<th>Species</th>
<th>Molecular Activity (ATP.min⁻¹)</th>
<th>16:0</th>
<th>18:0</th>
<th>18:1(n-9)</th>
<th>18:1(n-7)</th>
<th>18:2(n-6)</th>
<th>20:4(n-6)</th>
<th>22:4(n-6)</th>
<th>22:5(n-6)</th>
<th>22:5(n-3)</th>
<th>22:6(n-3)</th>
<th>UI Cholesterol: Phospholipid (mole:mole)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kidney</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Mouse</td>
<td>23,038</td>
<td>13.5</td>
<td>9.7</td>
<td>6.1</td>
<td>1.8</td>
<td>7.6</td>
<td>25.5</td>
<td>1.3</td>
<td>0.5</td>
<td>1.1</td>
<td>29.2</td>
<td>322</td>
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<tr>
<td>Rat</td>
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<td>12.3</td>
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<td>6.1</td>
<td>1.7</td>
<td>9.6</td>
<td>46.4</td>
<td>1.5</td>
<td>0.2</td>
<td>0.7</td>
<td>5.2</td>
<td>264</td>
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<tr>
<td>Pig</td>
<td>6,681</td>
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<td>11.4</td>
<td>11.1</td>
<td>2.2</td>
<td>18.7</td>
<td>28.4</td>
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<td>0.2</td>
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<td>21.3</td>
<td>0.7</td>
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<td>10.5</td>
<td>2.7</td>
<td>10.7</td>
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<td>0.1</td>
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<tr>
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<td>0.3</td>
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<td>1.5</td>
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<tr>
<td>Shingleback</td>
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<td>10.1</td>
<td>12.4</td>
<td>9.0</td>
<td>23.7</td>
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<td>2.1</td>
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<tr>
<td><strong>Brain</strong></td>
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<tr>
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<td>4.5</td>
<td>0.8</td>
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<tr>
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<td>15.1</td>
<td>14.6</td>
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<td>0.5</td>
<td>8.6</td>
<td>3.6</td>
<td>0.6</td>
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<td>16.5</td>
<td>19.1</td>
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<td>5.5</td>
<td>3.0</td>
<td>0.7</td>
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<tr>
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<td>3.9</td>
<td>0.8</td>
<td>1.8</td>
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<td>8.2</td>
<td>4.4</td>
<td>0.5</td>
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<td>3.5</td>
<td>2.7</td>
<td>0.4</td>
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<td>Duck</td>
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<td>13.5</td>
<td>10.5</td>
<td>7.7</td>
<td>3.5</td>
<td>0.2</td>
<td>14.0</td>
<td>5.7</td>
<td>8.2</td>
<td>0.6</td>
<td>34.0</td>
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<tr>
<td>Goose</td>
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<td>13.9</td>
<td>9.8</td>
<td>4.0</td>
<td>0.3</td>
<td>10.5</td>
<td>3.5</td>
<td>2.2</td>
<td>1.8</td>
<td>33.3</td>
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<td>8.5</td>
<td>6.5</td>
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<td>4.7</td>
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<td>0.6</td>
<td>40.5</td>
<td>358</td>
</tr>
<tr>
<td>Toad</td>
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<td>10.2</td>
<td>11.9</td>
<td>2.2</td>
<td>1.7</td>
<td>25.7</td>
<td>3.0</td>
<td>1.2</td>
<td>1.4</td>
<td>22.3</td>
<td>285</td>
</tr>
</tbody>
</table>

Mammalian and avian data are mean values from Turner et al. (48, 49). Molecular activity was determined at 37°C. Fatty acids are expressed as mole percentage of total fatty acids. Fatty acids making up <1.5% of total fatty acids in all species are not included. Unsaturation index (UI) is the number of double bonds per 100 fatty acid chains. * moles of phospholipid calculated assuming a molecular weight of 780. N=1 for the shingleback kidney and toad brain.