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Molecular activity of Na^+/K^+-ATPase from different sources is related to the packing of membrane lipids

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Summary

The activity of the ubiquitous Na^+/K^+-ATPase represents a substantial portion of the resting metabolic activity of cells, and the molecular activity of this enzyme from tissues of different vertebrates can vary several-fold. Microsomes were prepared from the kidney and brain of the rat (*Rattus norvegicus*) and the cane toad (*Bufo marinus*), and Na^+/K^+-ATPase molecular activity was determined. The membrane lipids surrounding this enzyme were isolated and phospholipids prepared. 'Surface pressure/area' isotherms were measured in monolayers for both membrane lipids and phospholipids using classic Langmuir trough techniques. Microsomal lipid composition was also measured. Whilst significant correlations were observed between membrane composition and Na^+/K^+-ATPase molecular activity, the strongest correlations were found between the molecular activity and parameters describing the packing of the surrounding membrane lipids and phospholipids. The influence of membrane lipid composition, especially membrane acyl composition, on the activity of a membrane protein mediated by physical properties of the lipids may represent a fundamental principle applicable to other membrane proteins.

Key words: Na^+/K^+-ATPase, kidney, brain, membrane, lipid, sodium pump, phospholipid, monolayer, polyunsaturate, mono-unsaturate, metabolism, *Rattus norvegicus*, *Bufo marinus.*

Introduction

The energetic cost of living varies amongst vertebrate species in a predictable manner. For example, it is several-fold greater in endothermic than in ectothermic vertebrate species when measured at the same body temperature. This difference in metabolic activity appears to be primarily a cellular phenomenon, and it has recently been proposed that it is related to differences in membrane composition (Hulbert and Else, 1999, 2000). A substantial proportion of this cost of living is the cost of maintaining a very different intracellular composition compared with the extracellular compartment, and many aspects of this difference are maintained by the trans-plasmalemmal Na^+ concentration gradient. The membrane-bound Na^+/K^+-ATPase, a ubiquitous enzyme of animal cells, is known as the sodium pump and is responsible for maintenance of this Na^+ gradient. The *in vivo* activity of the sodium pump is estimated to be responsible for approximately 20 % of the resting metabolic rate of humans and rats, with its importance varying amongst tissues and being as high as 50–60 % in brain and kidney (Clausen et al., 1991; Rolfe and Brown, 1997). The activity of the sodium pump appears to be responsible for a similar proportion of metabolic rate in ectothermic vertebrates as it is in endothermic vertebrates. Although similar calculations have not been performed for the cane toad, a comparison of the resting rates of oxygen consumption of tissue slices from the rat and cane toad at the same temperature gave a similar percentage inhibition by ouabain in both species (Hulbert and Else, 1990).

In vertebrates, the concentration of this enzyme varies amongst tissues, being high in tissues such as the kidney, where it is involved in transcellular ion transport, and in the brain, where the Na^+/K^+ gradient is the immediate energy source for action potentials. Although tissue concentrations of the enzyme are relatively consistent amongst different vertebrate species, its *in vitro* activity can vary considerably. For example, the *in vitro* Na^+/K^+-ATPase activity is considerably greater in tissues from the endothermic laboratory rat (*Rattus norvegicus*) than in those from the ectothermic cane toad (*Bufo marinus*) when both are measured at 37 °C. Enzyme activity divided by enzyme number yields an average molecular activity of Na^+/K^+-ATPase of approximately 6000–9000 ATP min^{-1} in rat tissues but only approximately 1000–3000 ATP min^{-1} in toad tissues (Else et al., 1996; Else and Wu, 1999).

The lipid composition of the membrane surrounding the Na^+/K^+-ATPase varies between the rat and the cane toad, with delipidation producing the well-known phenomenon of a

decrease in molecular activity of this membrane-bound enzyme (Jorgensen, 1974; Cornelius, 1991). When relipidated with microsomal lipid from the same species, the molecular activity of the Na^+/K^+-ATPase returns to normal values for that species; however, when relipidated with microsomes from a second species, Na^+/K^+-ATPase molecular activity reflects that of the second species. Both brain and kidney microsomes show the same results (Else and Wu, 1999). A similar 'species-crossover' comparison between cattle and crocodiles has given identical results, with a double-relipidation technique resulting in an enhanced effect (B. J. Wu and P. L. Else, unpublished results). These 'species-crossover' experiments suggest that it is the membrane environment, rather than the pump itself, that primarily determines the enzymatic molecular activity of the sodium pump.

To examine this hypothesis further, we have measured the molecular activity of Na^+/K^+-ATPase in brain and kidney microsomal preparations from the laboratory rat (*Rattus norvegicus*) and the cane toad (*Bufo marinus*) and isolated membrane lipids from these microsomal preparations. We have measured the composition of membrane lipids and isolated the phospholipids from the membrane lipids, measuring their acyl composition. Using the classic Langmuir trough technique, pressure/area isotherms of monolayers of both microsomal membrane lipids and microsomal phospholipids from these preparations have been measured at 37 °C. Here, we report striking relationships between the molecular activity of Na^+/K^+-ATPase and parameters describing the molecular packing of the surrounding membrane lipids.

Materials and methods

The animals used in this study were male Sprague-Dawley laboratory rats (*Rattus norvegicus*), aged 12–14 weeks, and adult cane toads (*Bufo marinus*) of both sexes. Both species were maintained on 12 h:12 h photoperiod with free access to food and water. Rats were kept at 22±2 °C and toads at 28±2 °C. All experiments were approved by the University of Wollongong Animal Experimentation Ethics Committee.

Microsomes were prepared from brain and kidney from both rats and toads. Sodium pump density was measured using [^{3}H]ouabain binding and Na^+/K^+-ATPase activity by methods described previously (Else et al., 1996; Else and Wu, 1999), both at 37 °C. In rats, microsomes were prepared from the cortex of the kidney. Microsomes were prepared from 10 % tissue homogenates that had been centrifuged at 12 500 ***g*** in a Beckman Ultracentrifuge to remove nuclei and mitochondria. The supernatants were centrifuged at 98 000 ***g***, and the resultant microsomal pellet was resuspended in 25 mmol l^{-1} Tris, 3 mmol l^{-1} ATP, 2 mmol l^{-1} EDTA (pH 7.6). Sodium pump concentration was determined by measuring [^{3}H]ouabain binding of microsomal membranes with a phosphate-facilitated method at 37 °C. Specific binding was measured at 10^{-6} mol l^{-1} ouabain, with a non-specific binding correction determined using 10^{-2} mol l^{-1} ouabain, as described previously (Else and Wu, 1999). Na^+/K^+-ATPase activity was measured at 37 °C as the difference in the liberation of phosphate from ATP in the absence and presence of 10^{-3} mol l^{-1} ouabain and K^+, as extensively described by Else et al. (1996).

All measurements were made on six microsomal preparations from each source, except phospholipid headgroup composition where N=3 for microsomes from each source. Because of the amount of material required, each microsomal preparation was a pooled preparation from 10 rats and 20 toads for kidney preparations and from five rats and 50 toads for brain preparations. Tritiated ouabain was from Dupont NEN, Hionic-Fluor scintillation cocktail was from Packard and ATP was from Boehringer Mannheim. All chemicals were of analytical grade and, except where specified, were from Sigma. Solvents used in lipid extraction were of ultrapure grade.

The cholesterol content of microsomal preparations was determined by enzymatic assay (Sigma Chemicals), and protein content was determined by the Lowry (1951) method using bovine serum albumin (Sigma Chemicals) as standard. The phospholipid content of each microsomal preparation was determined using the method of Chen et al. (1956). Lipids were extracted from microsomal preparations by standard methods (Folch et al., 1957) using ultrapure-grade chloroform and methanol (2:1, v/v) containing butylated hydroxytoluene (0.01 % w/v) as an antioxidant. In this study, we have called these extracts 'membrane lipids' because they include both phospholipids and other membrane lipids (primarily cholesterol). Phospholipids were separated from samples of each preparation of microsomal membrane lipids using Sep-Pak silica cartridges (Waters, Milford, MA, USA). The acyl composition of each microsomal phospholipid fraction was determined by methods described in detail elsewhere (Pan and Storlien, 1993).

To assess the physical properties of these microsomal lipids, the molecular packing of monolayers was measured as 'surface pressure/average molecular area' isotherms using classic Langmuir trough techniques (Adamson and Gast, 1997). Membrane lipids and phospholipids from each microsomal preparation were dried under nitrogen at 40 °C and resuspended in chloroform:methanol (2:1 v/v) at 1 mg ml^{-1}. The dissolved lipids were spread onto the surface of 8 mmol l^{-1} H_2SO_4 in a Langmuir trough (1.2 l capacity), and 10 min was allowed for evaporation of the solvent. The entire Langmuir trough was in a cabinet maintained thermostatically at 37 °C. Following evaporation of solvent, the monolayer film was compressed with a movable Teflon barrier in 1 cm steps, with 15–30 s intervals between compressions, until the monolayer collapsed. Surface pressure was measured with a calibrated mica float, and the entire apparatus was meticuluously cleaned with petroleum/ether and the fluid replaced after each pressure/area isotherm measurement. The average surface area per lipid molecule was calculated as the area of the monolayer film divided by the number of lipid molecules in the monolayer. The number of lipid molecules was calculated using Avogadro's number, the mass of lipid added to the fluid surface and an average phospholipid molecular mass calculated for each microsomal preparation from its known

composition. These values were 789, 781, 745 and 741 Da for rat kidney, rat brain, toad kidney and toad brain, respectively.

The relative compositions of the phospholipid classes of the microsomal fractions were determined using thin-layer chromatography on Silica Gel 60 plates using a two-solvent system (Aloia and Mlekusch, 1988). Solvent I was a mixture of chloroform:methanol:28 % ammonia (13:5:1 by volume), whilst solvent II was a mixture of chloroform:acetone:methanol:acetic acid:water (6:8:2:2:1 by volume). Because of a lack of material, two microsomal phospholipid preparations were combined for each determination of phospholipid headgroup composition.

All data are expressed as means ± S.E.M. Figures (including curve fits and correlation coefficients) were produced using the KaleidaGraph 3.0 software package (Synergy Software). Linear curve fits are plotted in Fig. 2 and Fig. 3, whilst in Fig. 1 the lines are polynomial curve fits. Statistical analyses were performed using Statview II (Abacus Concepts Inc.). The relationships in this study were analysed by analysis of variance (ANOVA), and significance was determined by Scheff's *S*-test or unpaired *t*-test, as appropriate.

Results

As can be seen from Table 1, in both species, the density of sodium pumps in brain microsomes is approximately twice that measured in kidney microsomes. Similarly, for both tissues, the microsomes prepared from cane toads had more than twice the pump density measured for microsomes from rats. However, although there were substantial differences observed in the density of sodium pumps in microsomes from the four different sources, these differences were not manifest when the Na^+/K^+-ATPase activity was measured. Knowing the number of individual enzyme molecules in a preparation as well as the total enzymic activity allows the average activity of the individual enzyme molecules to be calculated. The molecular activities of the Na^+/K^+-ATPase ranged from 1420 ATP min^{-1} for microsomes prepared from toad brain to 7194 ATP min^{-1} for preparations from rat kidney (see Table 1). This represents an approximately fivefold range in molecular activity of the sodium pump. These values are similar to those reported previously for microsomal preparations from the same sources (Else and Wu, 1999) and to those calculated for whole-tissue homogenates from the same sources (Else et al., 1996).

In the present study, enzyme activity was measured at 37 °C in all preparations. Whilst this is the normal body temperature of the rat, it is higher than the preferred body temperature of the cane toad. The critical thermal maximum for *Bufo marinus* is reported to be 41–42 °C (Brattstrom, 1963). A comparison of the Na^+/K^+-ATPase activity of kidney from the rat and cane toad at 25 and 37 °C gave very similar Q_{10} values for both species (1.74 for rat and 1.95 for cane toad) (see table 2 in Else et al., 1996). We conclude from this that no significant thermal denaturation occurs in the cane toad preparations relative to the rat preparations and, thus, that the measured activity differences are real rather than an artefact due to the measurement temperature.

Assuming a molecular mass of approximately 147 kDa for Na^+/K^+-ATPase, we calculate that Na^+/K^+-ATPase protein represents approximately 2.7 % of the total protein content of rat kidney microsomes. The respective values for microsomal preparations from rat brain, toad kidney and toad brain are 4.7 %, 5.7 % and 13.7 % of the total microsomal protein.

For the microsomal preparations from toad kidney, the mass of phospholipid was approximately 48 % of the mass of microsomal protein, whilst it ranged between 62 and 69 % for the microsomes prepared from the other three sources (see Table 2). The cholesterol content (per milligram protein) of brain microsomes was approximately twice that of kidney microsomes for both species, and for both tissues cholesterol content was approximately 20 % higher in the toad microsomes that in rat microsomes. On a molar basis, the ratio of cholesterol to phospholipid ranges from 0.18 in rat kidney microsomes to 0.41 in toad brain microsomes (Table 2). If membrane lipids were to consist solely of phospholipids and cholesterol, cholesterol molecules would represent 15 % and 24 % of membrane lipid molecules in rat and toad kidney microsomes, respectively, whilst they would represent 25 % and 29 % of membrane lipid molecules in rat brain and toad brain microsomes, respectively.

The relative compositions of the different phospholipid headgroups were similar in microsomes prepared from all four sources (Table 3). The only statistically significant differences observed were that the relative amount of phosphatidylcholine (PC) was greater in rat than in toad microsomes for both tissues and that the relative amount of phosphatidylethanolamine (PE) was greater in rat kidney than in toad kidney microsomes. In

Table 1. *Sodium pump density and enzymic activity of Na^+/K^+-ATPase in microsomes from kidney and brain of rats (*Rattus norvegicus*) and toads (*Bufo marinus*)*

	Kidney microsomes		Brain microsomes	
	Rat	Toad	Rat	Toad
Sodium pump density (pmol mg^{-1} protein)	182±26	389±42*	318±37	934±94*
Na^+/K^+-ATPase activity (μmol P_i mg^{-1} protein h^{-1})	78.6±2.2	68.5±4.4	89.7±5.4	79.6±3.3
Sodium pump molecular activity (ATP min^{-1})	7194±197	2943±190*	4699±283	1420±60*

Values are means ± S.E.M. (N=6).
* indicates a value significantly different from the respective rat value (P<0.01).
P_i, inorganic phosphate.

Table 2. *Cholesterol and phospholipid content of microsomes from kidney and brain of rats (*Rattus norvegicus*) and toads (*Bufo marinus*)*

	Kidney microsomes		Brain microsomes	
	Rat	Toad	Rat	Toad
Cholesterol content (μg mg^{-1} protein)	56.2±0.5	69.9±2.2*	116.0±1.1	136.3±1.0*
Phospholipid content (μg mg^{-1} protein)	616.6±9.0	476.5±20.4*	692.4±33.2	676.3±27.7
Cholesterol:phospholipid (mol:mol)	0.18±0.00	0.30±0.01*	0.34±0.02	0.41±0.02*

Values are means ± S.E.M. (*N*=6).
* indicates a value significantly different from the respective rat value (*P*<0.05).

both tissues, the molar ratio of PE to PC was significantly lower in the rat than in toad microsomes (Table 3).

The fatty acyl compositions of microsomal phospholipids from the four different sources are presented in Table 4. The average acyl chain length was similar in all microsomal preparations. A consistent finding is that microsomes prepared from the rat tissues had a greater unsaturation index (average number of double bonds per 100 fatty acyl chains) than those from the toad tissues. However, this is not due to the presence of more unsaturated acyl chains in the rat microsomes, but to the greater average number of double bonds per unsaturated acyl chain. The mono-unsaturates were present in equal amounts in brain microsomes from both species, but were more prevalent in toad kidney microsomes than those from the rat. In both species, polyunsaturates comprised approximately half of all acyl chains in brain microsomes and approximately one-third of all acyl chains in kidney microsomes. A notable difference between tissues was the predominance of *n*-6 polyunsaturates in kidney microsomes compared with the brain microsomes, where there is a more even balance between *n*-6 and *n*-3 polyunsaturates. This difference is also manifest when the acyl composition of whole-tissue phospholipids are compared (Couture and Hulbert, 1995).

Brain microsomes from both species have approximately the same relative amount of total unsaturates, as well as total mono-unsaturates and total polyunsaturates. In the brain microsomes, *n*-3 polyunsaturates predominate in the rat, whilst *n*-6 polyunsaturates are more common in the toad. Kidney microsomes from the rat have a lower level of total unsaturates than toad kidney microsomes, and this difference is almost solely due to a lower content of mono-unsaturates. Both species have the same total polyunsaturate content in their kidney microsomes, although rat kidney microsomes have approximately twice the *n*-3 polyunsaturate content of the toad kidney microsomes. This difference is also manifest in the sum of 20- and 22-carbon acyl chains and probably indicates a greater activity of the desaturase and elongase enzyme systems in the rat. Such a conclusion regarding the elongase system is supported by the higher ratio of 18:0/16:0 in the rat compared with the toad.

Also presented in Table 4 are the linear correlation coefficients obtained when the acyl composition parameters were plotted against the molecular activity of the sodium pump for each preparation. Each correlation coefficient was determined from 24 data points (six microsomal preparations from each of four sources) and, as can be seen from these results, several of these correlations were statistically significant. There were significant negative correlations between sodium pump molecular activity and 16:0, 16:1(*n*-7), 18:1(*n*-9) and 22:5(*n*-6) as well as with the composite parameters of percentage mono-unsaturates and percentage *n*-9 acyl chains. There were significant positive correlations

Table 3. *Phospholipid headgroup composition of microsomes from kidney and brain of rats (*Rattus norvegicus*) and toads (*Bufo marinus*)*

	Phospholipid content (% total phospholipids by mass)			
	Kidney microsomes		Brain microsomes	
Phospholipid class	Rat	Toad	Rat	Toad
Phosphatidylcholine (PC)	36.1±1.2	27.5±0.8*	36.4±1.0	30.0±0.9*
Phosphatidylethanolamine (PE)	23.8±0.9	19.5±0.4*	22.4±0.8	24.3±0.7
Sphingomyelin	13.8±2.8	18.5±0.7	17.5±0.6	16.0±0.8
Phosphatidylserine (PS)	11.9±0.3	13.1±0.6	9.7±0.8	10.6±0.5
Phosphatidylinositol (PI)	10.5±0.8	11.6±0.5	8.1±0.3	8.3±0.3
Diphosphatidylglycerol	1.9±0.4	2.1±0.3	2.7±0.4	2.7±0.3
PE:PC	0.66±0.00	0.71±0.01*	0.62±0.04	0.81±0.04*
Negatively charged phospholipid (PS+PI)	22.3±0.8	24.7±1.0	17.9±0.9	18.9±0.3

Values are means ± S.E.M. (*N*=3).
* indicates a value significantly different from the respective rat value (*P*<0.05).

Table 4. *Fatty acyl composition of microsomal phospholipids from kidney and brain of rats (*Rattus norvegicus*) and toads (*Bufo marinus*)*

	Kidney microsomes		Brain microsomes		Correlation with molecular activity
	Rat	Toad	Rat	Toad	
Acyl chain (% total)					
14:0	0.9±0.2	0.4±0.1	1.0±0.2	1.2±0.1	–0.01
16:0	16.9±0.6	17.1±0.2	20.8±0.9	23.8±0.9	–0.58**
17:0	0.4±0	0.4±0	0.2±0	0.2±0	0.23
18:0	16.8±0.7	8.9±0.3	18.1±0.5	16.0±0.2	0.38
16:1(*n*-7)	0.4±0	1.7±0	0.3±0.1	2.1±0.2	–0.83***
17:1(*n*-7)	0.4±0	0.3±0	0.7±0.1	0.3±0	0.35
18:1(*n*-9)	9.5±0.4	19.0±0.1	15.5±0.3	16.8±0.4	–0.82***
18:1(*n*-7)	2.7±0.4	1.7±0.1	4.9±0.6	3.0±0.1	0.08
18:2(*n*-6)	8.4±0.6	27.8±0.3	0.6±0.1	1.7±0	–0.09
18:3(*n*-3)	0±0	0.8±0	0±0	0±0	–0.28
20:3(*n*-9)	0.4±0	0.8±0	0.1±0	0.6±0	–0.25
20:3(*n*-6)	0±0	0.8±0	0.2±0	0.5±0	0.38
20:3(*n*-3)	2.4±0.9	0.3±0.1	0.1±0.1	0.3±0	0.52**
20:4(*n*-6)	33.6±0.5	15.5±0.2	11.9±0.4	16.9±0.4	0.69***
20:5(*n*-3)	0.4±0.1	0.7±0	0.2±0.1	0.1±0	0.10
22:4(*n*-6)	0.2±0.1	0.5±0	2.9±0.2	2.1±0.1	–0.35
22:5(*n*-6)	0±0	0.3±0	0.6±0.1	1.6±0.1	–0.76***
22:5(*n*-3)	1.1±0.2	0.7±0	0.6±0.1	0.6±0	0.48*
22:6(*n*-3)	2.9±0.1	0.9±0	19.7±0.4	11.4±0.1	–0.15
24:1(*n*-9)	0.3±0.1	0.7±0	0.3±0.1	0.4±0	–0.37
% Saturates	35.0±1.1	27.0±0.3	40.1±1.3	41.2±0.8	–0.06
% Mono-unsaturates	13.7±0.7	23.6±0.3	22.2±1.0	22.9±0.4	–0.82***
% Polyunsaturates	50.1±0.5	48.6±0.2	36.4±0.5	35.2±0.4	0.46*
% *n*-9	10.1±0.3	20.4±0.2	15.9±0.4	17.9±0.3	–0.85***
% *n*-7	4.0±0.5	4.0±0.2	6.4±0.7	5.6±0.2	–0.25
% *n*-6	43.4±0.2	45.1±0.2	16.2±0.3	22.7±0.4	0.32
% *n*-3	6.7±0.6	3.5±0.1	20.2±0.5	12.5±0.1	–0.06
Unsaturation index	200±3	166±1	207±3	187±2	0.56**
18:0/16:0	1.0± 0.1	0.5±0	0.9±0.1	0.7±0	0.71***
n-6/*n*-3	6.8±0.7	12.9±0.3	0.8±0	1.8±0	0.06
20+22C polyunsaturates	42.0±1.0	20.7±0.3	35.8±0.5	34.2±0.4	0.58**
Polyunsaturates/saturates	1.2±0.1	0.8±0	0.9±0	0.8±0	0.76***
Average chain length	18.4±0.1	18.1±0	18.5±0.2	18.4±0	0.11

Values are means ± S.E.M. (N=6).
Unsaturation index is the average number of double bonds per 100 fatty acyl chains.
Linear correlation coefficients were determined using individual data points (i.e. N=24).
Asterisks represent statistical significance; *P<0.05, **P<0.01, ***P<0.001.

between sodium pump molecular activity and 20:3(*n*-3), 20:4(*n*-6), 22:5(*n*-3) as well as the composite parameters of percentage polyunsaturates, unsaturation index, percentage total 20- to 22-carbon acyl chains and the ratios of 18:0/16:0 and polyunsaturates/saturates.

The relationships between average molecular area and surface pressure for lipids from each of the four sources of microsomes, using Langmuir trough techniques, are presented in Fig. 1. Each relationship is the average curve for six separate preparations. Fig. 1A shows the relationships for membrane lipids, which includes both phospholipids and cholesterol (as well as any unidentified lipids), whilst Fig. 1B shows the relationships for phospholipids only. As can be seen from this figure, during the early stages of monolayer compression (i.e. at very low surface pressures), the average area per lipid molecule is large. Initially, there is a substantial reduction in average molecular area with little increase in surface pressure. This is followed by smaller reductions in average molecular area and larger increases in surface pressure during the later stages of monolayer compression. This sort of relationship is observed whether it is membrane lipids (phospholipids plus cholesterol) or only phospholipids being measured. For both the membrane lipid study and the phospholipid study, the brain lipids showed a steeper relationship between surface pressure

and average molecular area (above pressures of 10 mN m^{-1}) than that observed for the kidney lipids. The slopes of these relationships were similar for both species, although the relationship for the toad was always substantially left-shifted compared with the respective curve for the rat lipids (see Fig. 1). Although there are a large number of studies using Langmuir trough techniques reported in the literature, most have been very mechanistically oriented. They generally examine a single molecular species of phospholipid, and there is a remarkable scarcity of studies that have examined complex mixtures of lipids sourced directly from natural membranes.

That the calculated average molecular area of membrane lipids is much smaller than the average area of phospholipids is partly because cholesterol molecules occupy much smaller surface areas than do phospholipid molecules. However, this is only part of the explanation. For example, when first placed in a monolayer (i.e. at zero surface pressure), phospholipids from rat kidney microsomes occupy 1.14 nm^2 molecule^{-1}, whilst membrane lipids from the same source average 0.79 nm^2 molecule^{-1}. Cholesterol molecules under the same conditions occupy 0.48 nm^2 molecule^{-1} (data not shown). Because cholesterol molecules represent only 15 % of membrane lipid molecules in rat kidney microsomes, we can calculate that the arithmetical average molecular area of membrane lipids from the rat kidney microsomes should be only 1.04 nm^2 molecule^{-1} rather than the measured 0.79 nm^2 molecule^{-1}. This effect is described as the 'condensation effect' of cholesterol and is described in some detail in the literature on the physical chemistry of membrane lipids (Chapman et al., 1969). It is largely due to the predominant influence of the acyl chains in the determination of the average surface area occupied by phospholipid molecules in monolayers. For example, the surface area occupied by phospholipid headgroups varies between phospholipid types (and headgroup orientation) but can be estimated to be generally approximately 0.30–0.35 nm^2 molecule^{-1}. This value is approximately 26–30 % of the area occupied by the average phospholipid from rat kidney microsomes before monolayer compression and approximately 45–50 % of the corresponding value for phospholipids from toad brain microsomes. Not coincidentally, it is also the approximate molecular surface area at which phospholipid monolayers collapse during compression (see Fig. 1).

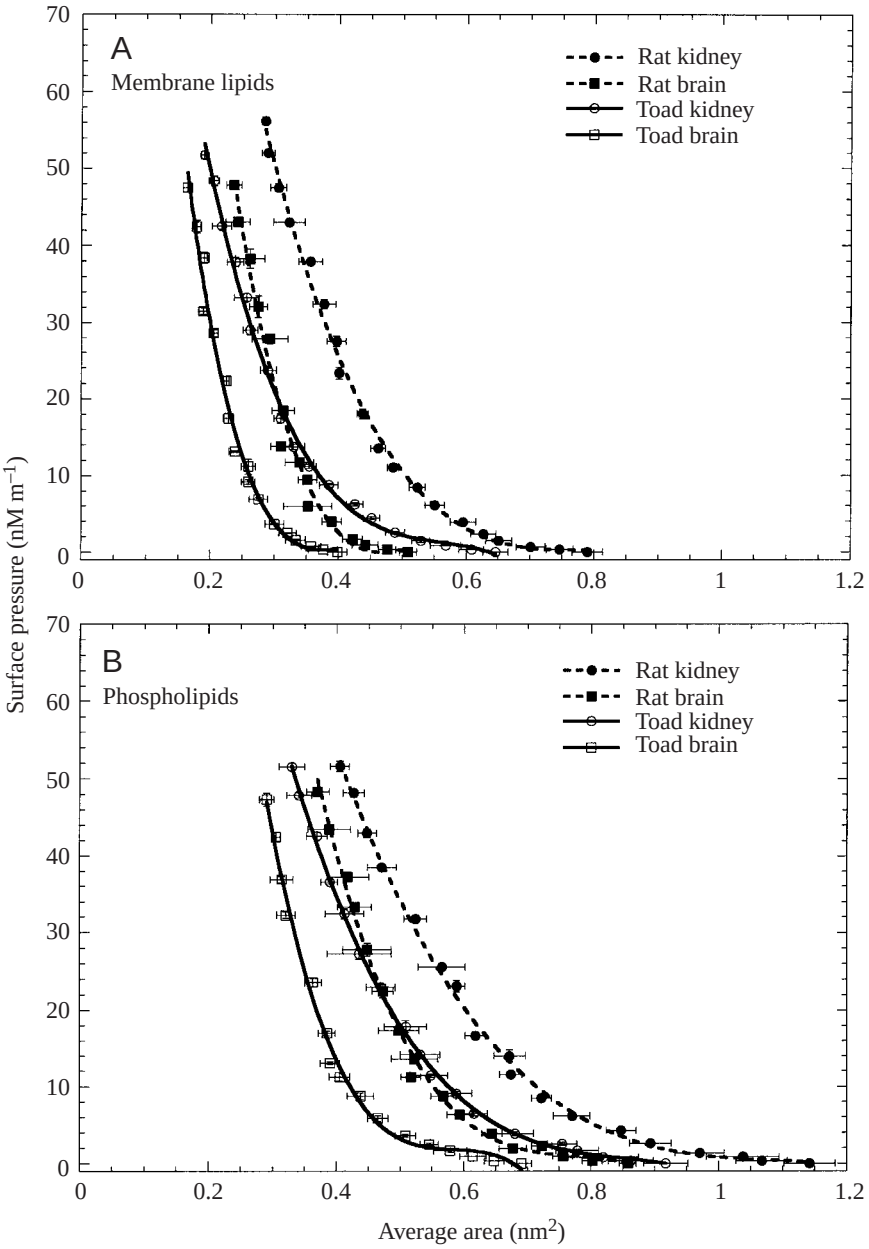

Fig. 1. The relationships between average molecular area (per lipid molecule) and surface pressure at 37 °C of monolayers of (A) membrane lipids and (B) phospholipids from kidney and brain microsomes of rats (*Rattus norvegicus*) and cane toads (*Bufo marinus*). Each symbol represents the mean ± S.E.M. of six determinations.

Discussion

We have examined whether there is any correlation between the physical behaviour of membrane lipids and phospholipids measured in monolayers and the molecular activity of sodium pumps measured in microsomes (i.e. in bilayers). Fig. 2 shows that there are striking correlations (P<0.001) between Na^+/K^+-ATPase molecular activity and the average molecular surface area of both membrane lipids and phospholipids measured in monolayers. Because the average surface area per lipid molecule varies with the lateral pressure within the monolayer we have used, the individual values at a surface pressure of

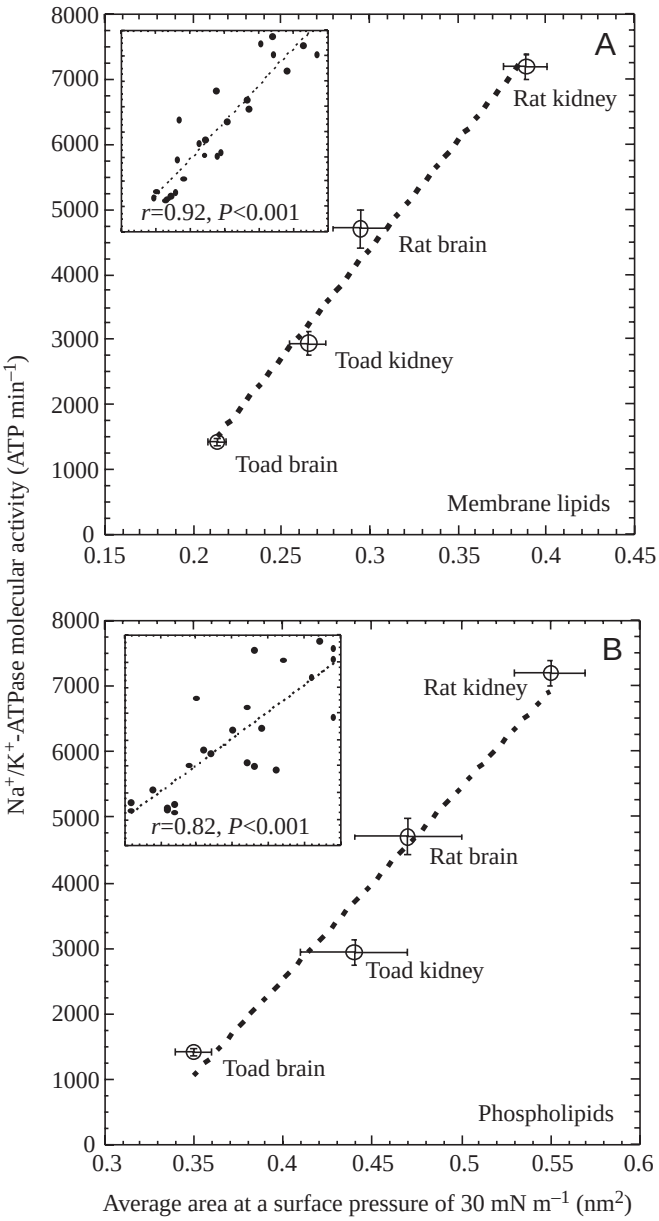

Fig. 2. The relationship between the molecular activity of the sodium pump (Na^+/K^+-ATPase) and the average molecular area per lipid molecule (at a surface pressure of 30 mN m^{-1}) of (A) membrane lipids and (B) phospholipids from kidney and brain microsomes of rats (*Rattus norvegicus*) and cane toads (*Bufo marinus*). Each symbol represents the mean ± S.E.M. of six determinations. Each inset graph show the relationship for the 24 individual data points.

30 mN m^{-1} are shown in Fig. 2. Although the lateral surface pressure in microsomal bilayers is not known, several lines of evidence suggest that the packing of lipids in a biological membrane is comparable with that observed in phospholipid monolayer (Rebecchi et al., 1992). Almost identical correlation coefficients were obtained when surface area values at monolayer pressures of 20 or 10 mN m^{-1} were plotted against the respective Na^+/K^+-ATPase molecular activity (data not shown).

Why should the molecular activity of a membrane-bound enzyme be related to the average surface area occupied by its surrounding lipids? What does 'average molecular area' represent? The calculated average area per molecule presumably represents a composite of both the size of the phospholipid molecule and its relative movement. Membrane lipids are reported to have rates of lateral diffusion within membranes that are approximately two orders of magnitude greater than those typical of membrane proteins (Storch and Kleinfeld, 1985). This implies that membrane proteins (and specifically that part of the protein located within the membrane bilayer) are in a state of constant collision with their surrounding membrane lipids. As described in the Results section, the average area occupied by a membrane phospholipid is a reflection more of the membrane area occupied by the acyl chains of phospholipids than of their headgroups. This can also be deduced from the fact that, whilst the membrane lipids from the four different sources exhibited different pressure/area isotherms in the Langmuir trough experiments (Fig. 1), they did not differ greatly in their phospholipid headgroup composition (Table 3) but showed substantial differences in their phospholipid acyl composition (Table 4). Generally, the lipids that showed the greatest degree of compressibility in monolayers were those that had the highest relative polyunsaturate content. As discussed elsewhere (Hulbert and Else, 1999), since lipids are not compressible in volume, reductions in the average area occupied must be accompanied by increases in the average height of these molecules. In addition, since these are calculated average areas, there will be a range of surface areas occupied per molecule. Assuming that, within each particular molecular species of phospholipid, no individual molecule is different from another, it implies that each acyl chain, and thus each phospholipid molecule, will oscillate between being 'tall and thin' and 'short and wide'.

Another way of relating the pressure/area isotherm of monolayers to the activity of a membrane enzyme is to compare enzyme molecular activity with the lateral pressure exerted by phospholipids when they are confined to specific average surface area. This has been done in Fig. 3, in which Na^+/K^+-ATPase molecular activity is plotted against the lateral pressure in monolayers of the same preparations when membrane lipids and phospholipids are confined to an average surface area of, respectively, 0.30 nm^2 and 0.45 nm^2 (these represent arbitary choices as standard molecular areas in the present study). As can be seen from Fig. 3, there are strong relationships (P<0.001) between the molecular activity of the sodium pump and the surface pressure exerted by both membrane lipids and phospholipids measured in monolayers. It is conceptually easy to appreciate that the lateral pressure within a membrane (which is presumably related to the number of collisions between the membrane molecules) will probably affect the behaviour of a protein located within the membrane.

The correlation coefficients between the physical properties exhibited by these lipids in monolayers (which indicate the degree of molecular packing in membranes) and the molecular

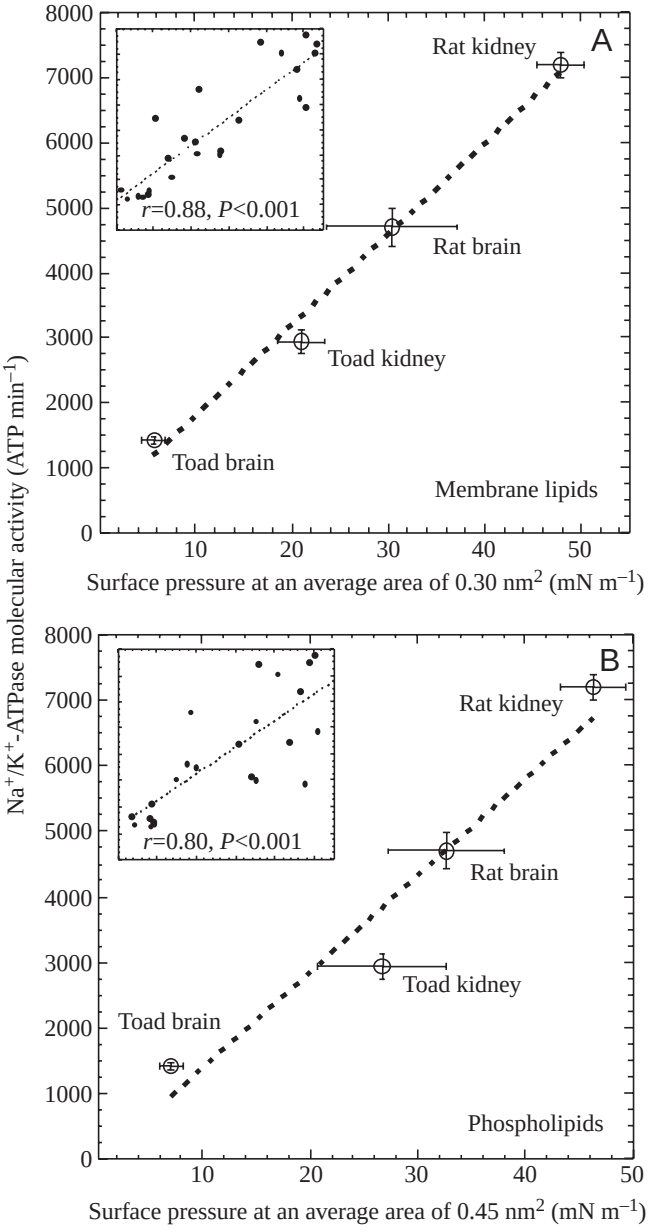

Fig. 3. The relationship between the molecular activity of the sodium pump (Na^+/K^+-ATPase) and the surface pressure of (A) membrane lipids and (B) phospholipids from kidney and brain microsomes of rats (*Rattus norvegicus*) and cane toads (*Bufo marinus*). Surface pressures were calculated at an average molecular area (per lipid molecule) of 0.30 nm^2 and 0.45 nm^2, respectively, for membrane lipids and phospholipids. Each symbol represents the mean ± S.E.M. of six determinations. Each inset graph show the relationship for the 24 individual data points.

activity of the membrane-bound sodium pump are stronger than those generally observed between individual lipid composition parameters and sodium pump molecular activity.

The sodium pump occurs in a number of different isoforms that have been extensively studied in tissues of the rat, especially in kidney and brain (Sweadner, 1989), but there is no specific information of their relative tissue distribution (apart from the urinary bladder) in the cane toad. The ancestral catalytic subunit of Na^+/K^+-ATPase is thought to be similar to the α-1 isoform, which is common to all vertebrates (Horisberger, 1994) and is the isoform of Na^+/K^+-ATPase found in the kidneys of vertebrates (Sweadner, 1989). If, as is most likely, it is also the isoform present in the toad kidney, isoform differences are unlikely to explain the substantial differences in sodium pump molecular activity between rat and toad kidney (see Table 1). Most studied in the rat, the adult brain has three isoforms (α1, α2 and α3), with different abundances in different types of brain cell (Sweadner, 1989). The distribution of these isoforms in the cane toad brain is unknown, but it is probably similar to that in the rat brain. Whether different isoforms exhibit different molecular activities is not known.

Such isoform differences may have a role in the species difference in the enzyme molecular activities observed in the present study, although this is probably a very small influence for a number of reasons. There are differences in ouabain sensitivities between isoforms of the sodium pump. Such differences are probably not important because the ouabain concentration used in the present study (1 mmol l^{-1}) to determine Na^+/K^+-ATPase activity is several-fold greater than affinity differences reported for different isoforms, which are generally in the micromolar concentration range. Similarly, the results of the 'species-crossover' experiments (Else and Wu, 1999), in which sodium pump isoform remains constant but molecular activity is altered by the change in membrane lipid, rule out different isoforms being the predominant cause of different molecular activity. Membrane 'crossover' studies between different tissues within a single species will also probably illuminate the relative importance of isoform differences in explaining the substantial differences in the molecular activity of the sodium pump from various sources.

The membrane-crossover results (Else and Wu, 1999) provide experimental evidence that the membrane lipids are important determinants of Na^+/K^+-ATPase molecular activity. The present results suggest that, as well as their chemical effects (such as being eicosanoid precursors), the physical properties of membrane acyl chains, in providing an appropriate environment, will influence membrane protein function.

The maintenance of the trans-plasmalemmal Na^+ gradient is a significant component of the cost of living in animals, as is the maintenance of other transmembrane gradients (Rolfe and Brown, 1997; Brand et al., 1994). Recently, it has been suggested that both the amount of membrane and the relative abundance of long-chain polyunsaturates in membrane bilayers may be important determinants of cellular metabolic activity in animals (Hulbert and Else, 1999, 2000). Specific effects have sometimes been ascribed to fatty acid subclasses (e.g. *n*-3 fatty acids) and individual fatty acyl chains among them (e.g. docosahexaenoic acid or 22:6). However, what is of particular interest here is that, although there were significant correlations between Na^+/K^+-ATPase molecular activity and the prevalence of some individual fatty acyl chains and various composite

parameters, the highest correlation coefficients were generally associated with average membrane packing parameters.

In both kidney and brain, the cane toad had a greater density of sodium pumps than the same tissues from the rats (see Table 1). Whether the maintenance of the higher density of pumps in the toad tissues results in a larger energetic cost is not known. The cost of maintenance will depend on the rate of degradation/synthesis of pumps and, in view of the relatively low proportion of the resting metabolic rate of mammals that is associated with protein synthesis (Rolfe and Brown, 1997), this is not likely to be a large part of the resting metabolic rate of toads.

The two tissues chosen for the present study, kidney and brain, are the same as used in previous studies from this laboratory and were initially chosen because of their relatively high tissue density of sodium pumps. The unplanned, but very different, relative abundance of *n*-3 and *n*-6 polyunsaturates in their membrane lipids (and in this respect they are at extreme ends of the spectrum of tissue phospholipid acyl composition) has allowed the physical properties of membrane lipid packing to be highlighted, more than the presence of specific acyl chains, in its potential influence on the molecular activity of the sodium pump.

The importance of the physical properties of membrane lipids on the functional activities of membrane-bound proteins has also been demonstrated in other systems. For example, the mechanical interactions between membrane lipid bilayers and gramicidin monomers are important functional determinants of these ion channels (Lundbaek et al., 1997), and the relative importance of physical properties for these interactions, compared with chemical effects, is demonstrated by the inability of phospholipid chirality to influence the function of these channels (Providence et al., 1995). Similarly, Ca^{2+} channel function can be influenced by manipulation of the surrounding membrane stiffness through application of exogenous membrane-active compounds (Lundbaek et al., 1996).

The idea that physical effects are likely to be a general phenomenon operating on membrane proteins (as well as chemical effects) opens up new ways of understanding and approaching some modern medical problems. For example, many prevalent disease conditions in western societies (e.g. obesity, hypertension, blood dyslipidaemias, type II diabetes, atherosclerosis) are associated and represent a ‘metabolic syndrome’ centred around tissue insulin-resistance (Reaven, 1993). Insulin-resistance has been shown to be related to a relative deficiency of polyunsaturated fatty acids in both rat (Storlien et al., 1991) and human (Borkman et al., 1993; Vessby et al., 1994) tissue. When rats are fed a diet high in saturated fat but deficient in dietary-essential *n*-3 polyunsaturates, they first develop insulin-resistance, followed by hyperinsulinaemia, hypertension and obesity (Barnard et al., 1998). Changes in membrane acyl composition, achieved by dietary and possibly pharmacological means, may thus offer a potential therapy to improve insulin action. Similarly, changes in the acyl composition of phospholipids, now being considered as a potential biochemical basis for the development of schizophrenia (Horrobin, 1998), supported by *post-mortem* brain analysis (Yao et al., 2000), suggest that consideration should be given to the physical as well as the chemical effects of changes in membrane bilayer composition.

The insights generated by this study are largely due to expressing enzyme activity in units of molecular activity; i.e. expressing enzyme activity relative to the number of individual enzyme molecules present. This approach has distinct advantages in that it allows comparison both amongst tissues within a single species and amongst species. Such comparisons, by using the natural laboratory of evolution, can provide considerable understanding and insight into how living systems operate. We believe this approach will also be beneficial when applied to other enzymes.

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