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Na^+–K^+ discrimination by "pure" phospholipid membranes

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SUMMARY

Phospholipid vesicles composed of phosphatidylserine and phosphatidylglycerol exhibit considerable discrimination between univalent cations. Under optimal conditions such membranes show a K^+/Na^+ diffusion rate ratio of approx. 10. Other phospholipids tested under the same conditions do not exhibit any appreciable discrimination. The influence of pH, cholesterol, Ca^{2+} and proteins was found to be significant in determining the degree of discrimination.

Model membrane systems, such as phospholipid bilayer films and vesicles, exhibit some characteristics remarkably similar to those of biological membranes[1-4]. They differ, however, in that model membranes exhibit a low permeability to ions in general, and an apparent lack of discrimination between Na^+ and K^+ found in many biological membranes[4,5]. The use of various "ionophoric" compounds such as valinomycin, *etc.*, has been valuable in establishing that phospholipid membranes can be induced to exhibit a low electrical resistance and a high discrimination between various monovalent cations[6]. Heptanol has also been reported to increase the K^+ selectivity of phospholipid bilayer membranes[7]. Nevertheless these compounds are not normally components of biological membranes, and it still seems relevant to question whether any of the naturally occurring membrane phospholipids exhibit Na^+–K^+ specificity.

Evidence from binding studies[8-10] indicated that phosphatidylserine could be exhibiting Na^+–K^+ discrimination. Early work with phosphatidylserine vesicles revealed that these membranes were discriminating to a small degree between monovalent cations, with K^+ diffusing faster than Na^+ at high temperatures (45–60°) and also in the presence of Ca^{2+} (ref.11).

We have recently repeated and expanded these experiments, studying several phospholipids and forming vesicles under conditions minimizing air oxidation and thus reducing non-specific leakage. The results indicate that purified phosphatidylserine as

well as phosphatidylglycerol in the absence of any "ionophoric" antibiotics, Ca^{2+}, or proteins are capable of considerable discrimination among monovalent cations. Other phospholipids such as phosphatidylcholine, phosphatidylethanolamine, and phosphatidic acid do not show any such specificity under the same experimental conditions.

Most of the phospholipids used in this study were prepared and characterized in this laboratory. Methods for the preparation of chromatographically pure phosphatidylserine, phosphatidylcholine, phosphatidic acid, and phosphatidylethanolamine have been described previously[12]. Phosphatidylglycerol was prepared enzymatically from egg phosphatidylcholine by the method of Dawson[13]. Phosphatidylethanolamine was obtained from pig erythrocytes and was kindly supplied by Dr. D.O. Tinker. All lipid samples were kept in chloroform solution in sealed ampules under nitrogen at $-50°$ to avoid oxidation. Ion fluxes were measured with sonicated, single-compartment vesicles, under conditions avoiding oxidative and thermal breakdown[14]. Self-diffusion rates were determined by dialysis and estimation of the amount of isotope ($^{22}Na^+$ or $^{42}K^+$ or $^{86}Rb^+$) appearing outside the vesicles per unit time[15,16]. The salt solutions were usually 100 mM in NaCl or KCl or RbCl and 50 mM for each Na^+ or K^+ when both were present. Solutions also contained 2 mM histidine, 2 mM *N*-tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid (TES) and 0.1 mM EDTA, adjusted to the appropriate pH by HCl, NaOH, or KOH. Salt concentrations and pH of solutions were the same throughout each experiment unless otherwise indicated.

The self-diffusion rates of Na^+, K^+, Rb^+ measured separately through phosphatidylserine vesicles are shown in Table I, line 1. The rates differ considerably following the series $K^+ > Rb^+ > Na^+$, and the K^+/Na^+ ratio is 9.0. In each of the above cases the diffusion rate of each ion was also determined at different temperatures. The Arrhenius plots indicate a linear relationship between 10 and 52°, with an activation energy of approx. 29 kcal/mole. The presence of equimolar amounts of cholesterol reduces the activation energy to approx. 14 kcal/mole and also substantially reduces the ion self-diffusion rates[17].

In order to substantiate the above results, the diffusion rates for Na^+ and K^+ were also measured concomitantly in experiments where both metals were present inside and outside of the vesicles in equimolar amounts. As indicated in Table I, line 2, the diffusion rate for K^+ was still higher than that of Na^+, the ratio being 5.1. Similar experiments were also performed at pH 4.5 in which case the ratio was found to be higher ($K^+/Na^+ = 7.2$, line 3) compared to pH 7.4. This change is of interest since pH 4.5 is close to the p*K* of the carboxyl group of phosphatidylserine[18]. An increase in the K^+/Na^+ permeability ratio at lower pH has also been observed with yeast cells[19],

Results obtained with phosphatidylserine vesicles at lower ionic strength (10 mM) or higher pH (9.0) do not differ substantially from the results shown in Table I, line 2. However, the inclusion of equimolar amounts of cholesterol to phosphatidylserine resulted in vesicles with diminished K^+/Na^+ diffusion ratio (line 4). Similarly, mixtures of 15% phosphatidylserine in phosphatidylcholine were also found to exhibit a lower discrimination between these cations (line 5). These results with mixtures are of interest since they corroborate earlier evidence[5] indicating no appreciable Na^+–K^+ discrimination with membranes composed of total lipid extracts, which are usually equimolar mixtures of phospholipids and cholesterol, containing 10–20% phosphatidylserine. It should be noted here that

TABLE I

EFFLUX OF Na^+, K^+ AND Rb^+ THROUGH PHOSPHOLIPID VESICLES

The phospholipid vesicles were sonicated and passed through a Sephadex column at room temperature[14]. They were then dialysed overnight at 0° under nitrogen atmosphere and finally equilibrated at the desired temperature against 10 ml of buffer which was changed every 2 h. The self-diffusion rates given here were determined at 36° after 4 h of equilibration at this temperature and were expressed as: percent of total ion captured per h. The amount of ions captured per μmole of phosphatidylserine is usually 0.2 μmole. Taking this figure into account, the diffusion rates of K^+ through phosphatidylserine vesicles can be calculated as $1.86 \cdot 10^{-7}$ equiv per mole of phosphatidylserine per sec. Assuming unilamellar vesicles and a packing of 67 $Å^2$ per phospholipid molecule, the area of a phospholipid bilayer is approx. 2000 cm^2 per μmole. The K^+ diffusion rate can thus be calculated as $9.3 \circ 10^{-17}$ equiv/cm^2 per sec. Taking into account a concentration difference of $1 \cdot 10^{-4}$ mole/cm^3 the diffusion coefficient for K^+ through phosphatidylserine can be calculated as $9.3 \cdot 10^{-13}$ cm/sec. The results included in this table are averages of at least two separate experiments not varying more than 20% from each other. Within each experiment the reproducibility was usually better than 5% of the mean value.

Phospholipid	*Self-diffusion rate (% total per h)*			K^+
	Na^+	K^+	Rb^+	Na^+
(1) Phosphatidylserine at pH 7.4*	0.038	0.340	0.285	9.0
(2) Phosphatidylserine at pH 7.4**	0.092	0.466	–	5.1
(3) Phosphatidylserine at pH 4.5**	0.065	0.467	–	7.2
(4) Phosphatidylserine/cholesterol at pH 7.4*	0.015	0.045	–	3.0
(5) 15% phosphatidylserine in phosphatidylcholine at pH 7.4**	0.107	0.260	–	2.4
(6) Phosphatidic acid at pH 7.4**	0.128	0.144	–	1.1
(7) Phosphatidylethanolamine at pH 8.0	0.658	0.696	–	1.0
(8) Phosphatidylglycerol at pH 7.4**	1.29	7.03	–	5.4
(9) Phosphatidic acid at pH 4.5*	1.39	2.98	–	2.1
(10) Phosphatidylserine at pH 7.4 (outside pH 4.5)**	0.072	0.642	–	8.9
(11) Phosphatidylserine at pH 7.4 (outside pH 3.5)**	1.55	1.93	–	1.2

*Self-diffusion rates for each cation were determined independently with sonicated phosphatidylserine vesicles in solutions containing 100 mM salt, either NaCl or KCl or RbCl, buffered with 4 mM histidine–TES at the indicated pH.

**Self-diffusion rates for Na^+ and K^+ were determined concomitantly, with sonicated phosphatidylserine vesicles in solutions containing NaCl (50 mM) and KCl (50 mM) and buffered as above at the indicated pH.

Na^+–K^+ discrimination was also observed with non-sonicated, multilamellar vesicles composed of purified phosphatidylserine. However, the K^+/Na^+ ratio was lower (approx.2) compared to the ratio obtained with sonicated vesicles under the same conditions (Table I, line 2). A low ratio ($K^+/Na^+ = 2.2$) was also observed when phosphatidylserine was sonicated for 5 min in the presence of air, under conditions identical to those of the early experiments with phosphatidylserine vesicles[11].

In order to investigate the effect of the different polar groups on Na^+ and K^+ permeability, several phospholipids were studied under similar conditions. As lines 6 and 7 of Table I indicate, no appreciable discrimination was observed with phosphatidic acid at pH 7.4, nor with phosphatidylethanolamine at pH 8.0. However, considerable discrimination was observed with vesicles formed from phosphatidylglycerol ($K^+/Na^+ = 5.4$, line 8), the only other phospholipid tested, except for phosphatidylserine, to show such discrimination. Phosphatidic acid showed a small degree of discrimination only at low pH (line 9). This result is of considerable interest because: first, it indicates the importance of subtle differences within the environment of the ionic head-groups of the phospholipids in determining the

ability for Na^+–K^+ discrimination; second, it eliminates the possibility that the carboxyl is the only group responsible for the discriminatory ability of phosphatidylserine; third, it can be concluded that at least in this case the observed discrimination could not be attributed to unknown "ionophoric" compounds extracted from the original source and present as impurities. This follows from the fact that both phosphatidylglycerol and phosphatidic acid were prepared enzymatically from the same parent compound, phosphatidylcholine, under the same conditions. The mere addition of glycerol *via* transesterification produces considerable Na^+–K^+ discrimination in phosphatidylglycerol which is not apparent in phosphatidylcholine or phosphatidic acid.

The influence of the ionization of the carboxyl group is further described by the experiments shown on lines 10 and 11. In this case the phosphatidylserine vesicles were formed at pH 7.4 with both Na^+ and K^+ present, as in line 2, and the outside pH was subsequently decreased. As the outside pH reached 4.5, the K^+ diffusion rate increased more than the Na^+ rate, and the K^+/Na^+ ratio increased to 8.9. Yet when the pH was further decreased to 3.5, the diffusion rate of both ions increased considerably, with a lower K^+/Na^+ ratio of 1.2. This latter increase in diffusion rate and loss of Na^+–K^+ discrimination seems to be related to the asymmetric distribution of the negative charges across the bilayer membrane which has been observed to induce instability[20].

Further evidence of discrimination between monovalent cations by phosphatidylserine was obtained in experiments where K^+ was added initially inside the vesicles and the outside K^+ was replaced by Na^+ or Li^+ at the same ionic strength. The results showed that the diffusion rate of K^+ was inhibited 50% by Na^+ and 60% by Li^+. When Rb^+ was added initially inside the vesicles in a similar experiment, the Rb^+ diffusion rate was inhibited 4% by K^+, 29% by Na^+ and 59% by Li^+. This inhibition by external ions could be related to the creation of a trans-membrane potential arising from the diffusion potential of the most permeable cation, K^+ or Rb^+. Such bi-ionic potentials have been observed independently by Dr. S. Ohki with black lipid films composed of phosphatidylserine (personal communication).

The ability of phosphatidylserine membranes to discriminate between the cations is retained to a certain degree even under conditions of increased permeability induced by the addition of proteins. Previous studies have indicated[21] that the addition of cytochrome *c* or lysozyme to phosphatidylserine vesicles increases the Na^+ permeability by 100 to 1000 fold. It is therefore pertinent to report here on experiments with phosphatidylserine vesicles containing both Na^+ and K^+ in the presence of cytochrome *c*. These lipoprotein membranes show a relatively high permeability (close to the range of biological membranes) and still discriminate between monovalent cations with a K^+/Na^+ ratio of approx. 2.

Differential binding of Na^+ and K^+ to phosphatidylserine vesicles was also studied but the observed difference was rather small and could not by itself account for the discrimination expressed in the diffusion rates. Equilibrium dialysis of phosphatidylserine vesicles in an equimolar K^+–Na^+ salt solution indicated a higher affinity for K^+, the K^+/Na^+ ratio being 1.29 at pH 7.4. In experiments where only the ions captured inside the phosphatidylserine vesicles were measured, the ratio was found to be low ($K^+/Na^+ = 0.84$) Differences in binding dependent on ionic strength and local pH may be of physiological significance and the subject is presently under further investigation.

The observations reported here on Na^+–K^+ discrimination by phosphatidylserine

and phosphatidylglycerol membranes would seem to be highly relevant to possible physiological mechanisms of trans-membrane ion movement, both active and passive. Phosphatidylserine is usually found in most cell membranes in amounts varying from 10 to 20% of total phospholipids[22]. Furthermore, membranes lacking phosphatidylserine, such as those of numerous microorganisms, mitochondria and chloroplasts, usually contain considerable amounts of phosphatidylglycerol or other glycerol containing phospholipids[22]. The results presented in Table I with vesicles composed of mixtures of phosphatidylserine and phosphatidylcholine or cholesterol indicate that such mixtures exhibit diminished Na^+–K^+ discrimination compared to vesicles of pure phosphatidylserine. However, it is conceivable that in biological membranes, phosphatidylserine or other specific phospholipids are segregated topologically. The areas of membranes containing high percentages of these phospholipids could represent "sites" concerned with specific permeability functions. Their function and permeability properties would probably be controlled and modulated by other membrane components such as proteins and bivalent metals.

In terms of the participation of phospholipids in the active transport of Na^+ and K^+, it is important to note that phosphatidylserine has been shown to be most effective in activating partially de-lipidized ($Na^+ + K^+$)-ATPase[23–25]. Moreover, recent experiments in our laboratory (Kimelberg and Papahadjopoulos, unpublished observations) provide further evidence for a correlation between ATPase activation and Na^+–K^+ discrimination by phospholipid vesicles. Thus it has been observed that phosphatidylserine and phosphatidylglycerol are the most effective phospholipids in reactivating an ATPase preparation[25] from rabbit kidney cortex. Of the other phospholipids tested phosphatidylcholine and phosphatidylethanolamine are completely inactive, while phosphatidic acid produces only partial activation. This correlation seems to be relevant to the mechanism of active transport of Na^+ and K^+ across biological membranes. The interaction of the ($Na^+ + K^+$)-activated ATPase with specific phospholipids is presently under further investigation.

The mechanism for monovalent cation discrimination by specific phospholipids reported here is not clear at present. It seems possible that the observed differences in diffusion rates can be rationalized in terms of Eisenman's[26] theory of anionic site field strength. Although this report appears to be the first experimental observation describing a substantial Na^+–K^+ discrimination by "pure" phospholipid membranes, it has already been predicted on the basis of carboxyl and phosphate group field strength[27], that Na^+–K^+ discrimination in biological membranes could depend on such groups. Earlier evidence indicating lack of monovalent cation discrimination by phospholipid membranes could be explained as the result of oxidative degradation of the phospholipid samples or use of mixtures containing high amounts of non-discriminatory switter-ionic phospholipids.

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