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PHOSPHOLIPID REQUIREMENTS FOR ($Na^+ + K^+$)-ATPase ACTIVITY: HEAD-GROUP SPECIFICITY AND FATTY ACID FLUIDITY*

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SUMMARY

We have re-examined the question of phospholipid activation of latent ($Na^+ + K^+$)-ATPase activity in delipidized preparations. The influence of both polar head-groups and fatty acyl chain fluidity have been examined. The results indicate the following:

1. The enzyme can be reactivated not only by phosphatidylserine, but also equally well by phosphatidylglycerol. A large variety of other purified phospholipids were found to have little or no effect.

2. This specificity for ($Na^+ + K^+$)-ATPase reactivation correlates with the observation that only phosphatidylserine and phosphatidylglycerol vesicles show substantial discrimination for K^+ over Na^+ in terms of permeability.

3. Maximal reactivation is obtained only when the fatty acyl chains are fluid. Activation by dipalmitoyl phosphatidylglycerol is inhibited below its transition temperature, or in the presence of cholesterol. Arrhenius plots for the activation by dipalmitoyl phosphatidylglycerol show a break for ($Na^+ + K^+$)-ATPase activity corresponding to the presumed transition temperature for the gel to liquid–crystalline transition for this phospholipid. We suggest the term "viscotropic" to describe such effects of membrane fluidity on enzyme activity.

4. The ATPase preparations used in this paper were obtained by deoxycholate treatment of microsomal fractions of rabbit kidney and beef brain. This treatment results in the removal of more than 90 % of the phospholipid and inhibition of ($Na^+ + K^+$)-ATPase activity. It was noted that exclusive specificity for reactivation by phosphatidylserine and phosphatidylglycerol was obtained only with those preparations where the inhibition of ($Na^+ + K^+$)-ATPase activity was essentially complete.

5. These results are discussed in relation to previous work on the phospholipid requirement for ($Na^+ + K^+$)-ATPase activity, the ability of phospholipids to exhibit K^+/Na^+ discrimination and current concepts of protein–lipid interactions in biological membranes.

INTRODUCTION

Attempts to purify and solubilize the membrane-bound ($Na^+ + K^+$)-dependent ATPase using detergents, have generally resulted in a partially purified preparation

* A preliminary report of some of this work was included in ref. 17 as unpublished observations.

which exhibits a requirement for added phospholipid to show maximal activity[1-3]. A number of studies in which phospholipases and/or organic solvents have been used to remove phospholipids from membrane fractions containing ($Na^+ + K^+$)-ATPase, have also indicated the involvement of phospholipids[4-10]. Thus an absolute requirement for phospholipid by the ($Na^+ + K^+$)-ATPase seems firmly established. Still unclear, however, is the specificity of this requirement. An early study[5] indicated that phosphatidylserine, but not phosphatidylethanolamine or phosphatidylinositol, would restore activity to phospholipase-treated erythrocytes. Subsequently, phosphatidylcholine was found to be effective in reactivating a soluble ($Na^+ + K^+$)-ATPase obtained by deoxycholate treatment of brain microsomes[1]. More recent work, however[3,11-13] seems to indicate that the requirement may indeed be specific for phosphatidylserine. In contrast, it has been reported recently that phosphatidic acid and lysolecithin[15] is nearly as effective as phosphatidylserine, and that even cholesterol can be effective under particular conditions[16].

In view of these current conflicts on the specificity of the lipid requirement for ($Na^+ + K^+$)-ATPase activation we decided to examine this question using a wide range of purified phospholipids. Recent work from this laboratory[17] indicated that phosphatidylserine and phosphatidylglycerol exhibit a substantial discrimination between K^+ and Na^+ in terms of permeability rates. In this report we present evidence demonstrating that the activation of ($Na^+ + K^+$)-ATPase correlates with the ability of various phospholipids to discriminate for K^+ over Na^+. Furthermore we will present data indicating that the fluidity of the fatty acyl chains of the phospholipids may also be involved in their ability to activate.

METHODS AND MATERIALS

Preparation of ($Na^+ + K^+$)-dependent ATPase

The procedure was based on Towle and Copenhaver's[2] modification of Tanaka and Strickland's[1] method, using frozen rabbit kidneys. Frozen rabbit kidney cortex was obtained from Pel-Freez Biologicals Inc. and thawed in 0.25 M sucrose (200 g tissue per 800 ml). This was then homogenized in a "Willems" Polytron (Bronwill Scientific) for 6 times 30-s intervals, using a combination of the high setting on the polytron and an appropriate setting on an external powerstat to minimize splashing. The temperature was maintained at approximately 5 °C. The homogenate was then centrifuged at 9000 × *g* for 20 min and the supernatant poured through two layers of cheesecloth. This was then centrifuged at 13500 rev./min for 2 h at 5 °C in the 15 rotor of the Beckman L3-50. The precipitate was then taken up in 200 ml of 50 mM Tris acetate, pH 7.2–7.4. This was treated with deoxycholate and fractionated with $(NH_4)_2SO_4$ exactly according to the procedure of Tanaka and Strickland[1] and Towle and Copenhaver[2]. The final precipitate was taken up in 5 ml of 50 mM Tris acetate, pH 7.2, 1 mM dithiothreitol, 1 mM EDTA, 20 % glycerol (v/v). This was then dialysed *versus* 200 ml of the same medium for 18–24 h at 4 °C.

Preparation of lipids

Most phospholipids used in this study were prepared and characterized in this laboratory. The isolation procedures were based on a combination of several methods and described in detail elsewhere[18]. All lipids were chromatographically pure and the

fatty acid ester content was essentially similar to earlier studies[18]. Phosphatidylserine, phosphatidylinositol and sulfatide were isolated from beef brain. Phosphatidylcholine and phosphatidylethanolamine were isolated from egg yolk. Phosphatidic acid was prepared by enzymatic hydrolysis of phosphatidylcholine by phospholipase D. Egg phosphatidylglycerol and dipalmitoyl phosphatidylglycerol were prepared enzymatically from egg phosphatidylcholine and dipalmitoyl phosphatidylcholine respectively by the method of Dawson[19]. Dipalmitoyl phosphatidylcholine was prepared from egg phosphatidylcholine by the re-acylation procedure of Robles *et al.*[20]. In addition, phosphatidylethanolamine from pig erythrocytes was a generous gift of Dr D. O. Tinker. Sulfatide from beef brain, and diphosphatidylglycerol (cardiolipin) from beef heart was purchased from Supelco Inc., Bellefonte, Pa. Cholesterol (99 %+) was obtained from Sigma Chemical Co. (St. Louis, Mo.) and recrystallized twice from methanol. The later stages of the purification of phospholipids were performed under nitrogen, in order to avoid air oxidation. The purified samples were stored under nitrogen in sealed ampoules at −50 °C. Each ampoule contained approximately 10–20 μmoles of phosphate (determined as inorganic P after $HClO_4$ digestion) in 1 ml of chloroform solution and was newly opened for each experiment.

Preparation of phospholipid vesicles

Dispersions of the various phospholipids were sonicated for 1 h under nitrogen in a bath type sonicator as previously described[21,22] Sonication was carried out in the same medium as used in the measurement of $(Na^+ + K^+)$-ATPase activity (see below), but omitting Mg^{2+}. The sonicated dispersions were not passed through a Sephadex column. The phospholipid concentration was usually 10 μmoles/ml. When permeability measurements were made, the dispersion was sonicated in the presence of $^{22}Na^+$ and $^{42}K^+$, passed through a Sephadex G-50 column and treated as previously described[17,21,22].

Other materials

Crystalline ATP disodium salt (grade 1) was obtained from Sigma and neutralized to pH 7.4 with NaOH. Ouabain was also obtained from Sigma. $(NH_4)_2SO_4$ and Tris were special enzyme grade from Mann Research Laboratories. All other chemicals were reagent grade.

Measurements of ATPase activity

ATPase activity was measured in the following medium: 100 mM NaCl, 10 mM KCl, 50 mM Tris acetate, 0.1 mM Na_2EDTA and 3 mM $MgCl_2$, pH 7.2, at 36 °C. Aliquots containing 0.5–1.0 mg enzyme protein ± 1.5 μmoles ouabain were added to the above medium to a final volume of 1.5 ml, and equilibrated at 36 °C for 15 min. The reaction was then initiated by adding neutralized ATP, usually 5 μmoles. After 20 min the reaction tubes were placed on ice, and the reaction terminated by adding 0.3 ml, 40 % (w/v) trichloroacetic acid. After a 10-min centrifugation, 1.0 ml of the supernatant was removed and assayed for inorganic phosphorus content. A zero time enzyme *plus* ATP blank was always run, in which trichloroacetic acid was added before ATP. Inorganic phosphorus was measured in a mixture containing 1.5 % (v/v) H_2SO_4, 1 % (w/v) NH_4MbO_4 and a 1:10 dilution of Elon (30 g $NaHSO_3$ + 10 g *p*-dimethylaminophenol sulfate per l). The final volume was 5 ml. The mixture was

left to stand for 15 min and then read at 660 nm. The assay was linear over the range of 0–1.0 μmole P_i.

Phospholipid concentration was assayed by measuring inorganic phosphorus after $HClO_4$ digestion. Protein was measured by the biuret reaction[23].

Lipid extraction of microsomes and enzymes

The microsome preparation (22 mg protein per ml) or the $(NH_4)_2SO_4$ enzyme (9.2 mg protein per ml), were mixed with 5 vol. of a chloroform–methanol (2:1, v/v) mixture at room temperature[24]. The lower organic phase was saved as the first extraction. A mixture of 50 ml chloroform, 25 ml methanol and 15 ml 0.1 M NaCl was then allowed to settle out. The lower, organic phase from this was used to wash the extracted microsomes a second time, and the lower phase collected again and combined with the first extract. The upper phase was then acidified with HCl to 0.1 M final concentration, washed in the same way as above, and the lower phase taken as the third extraction.

Preparation of dipalmitoyl phosphatidylglycerol vesicles

This phospholipid was suspended and sonicated as previously described[21,22] except that the temperature was kept at 42 °C throughout the procedure. The final suspension (usually 10 μmoles/ml) was left at 42 °C for an additional 30 min and then equilibrated at the temperature required for the ATPase assay. The above procedure produces a clear suspension which does not aggregate visibly following a drop in temperature below the region of the phase-transition (30–40 °C). It is stable even at 0 °C for several hours.

RESULTS

Specificity of different phospholipids for the reactivation of $(Na^+ + K^+)$-ATPase

Rabbit kidney microsomes usually show very low $(Na^+ + K^+)$-ATPase activity after treatment with sodium deoxycholate and subsequent $(NH_4)_2SO_4$ fractionations. The activity of the original microsomes averaged 102.7 ± 23.7 nmoles P_i per mg protein per minute for $(Na^+ + K^+)$-ATPase activity and 137.1 ± 25.3 nmoles P_i per mg protein per min for Mg^{2+}-ATPase activity in the presence of 1 mM ouabain. Table I summarizes a large amount of data from different preparations with and without added phospholipids. In cases where means ± S.E. are given, the number of observations ranged from a minimum of three up to eight. It can be seen that phosphatidylserine and phosphatidylglycerol are always most effective in activating $(Na^+ + K^+)$-ATPase. However, the degree of stimulation of $(Na^+ + K^+)$-ATPase activity by phosphatidylserine can vary greatly since when the residual activity is very low, as in Prepn 1, 500-fold stimulation can be obtained, whereas in Prepn 3 there is only 10-fold stimulation and in Prepn 2, 5-fold. Prepn 4 is anomalous since it showed relatively high $(Na^+ + K^+)$-ATPase activity even in the absence of added phospholipids. In this case both phosphatidic acid and phosphatidylcholine gave considerable stimulation of activity. In the other preparations (1, 2 and 3) phosphatidic acid and phosphatidylcholine were ineffective, as were PE, diphosphatidylglycerol, and sulfatide. Phosphatidylinositol showed a moderate stimulation. In most cases the Mg^{2+}-ATPase activity was not stimulated, but a stimulation of up to 1.8-fold was sometimes found.

TABLE I

ACTIVATION OF $(Na^+ + K^+)$-ATPase ENZYME BY DIFFERENT PHOSPHOLIPIDS

ATPase activity measurements and preparation of sonicated phospholipid vesicles, as described in Methods and Materials. The phospholipid suspension (0.4–0.6 μmole) and the protein (0.5 mg) were incubated together for 15 min at 36 °C. The reaction was then initiated by adding 5 μmoles ATP and run for 20 min. Final reaction volume, 1.5 ml. Prepns 1 through 4 were from rabbit kidney. Prepn 5 was from beef cerebral cortex grey matter. The results are expressed ± S.E., $n = 3$–8.

Prepn	*ATPase activity (nmoles P_i per mg protein per min) Phospholipid added:*								
	—	*Phosphatidyl-serine*	*Phosphatidyl-glycerol*	*Phosphatidyl-choline*	*Phosphatidic acid*	*Phosphatidyl-ethanolamine*	*Di-phosphatidyl-glycerol*	*Phosphatidyl-inositol*	*20% sulfatide in phosphatidyl-choline*
	$(Na^+ + K^+)$-ATPase								
1	0.2	100.4 ± 11.5	120.3 ± 14.8	0	51.0 ± 3.1	5	16	—	—
2	13.5 ± 3.5	131.8 ± 5.7	119.7 ± 3.8	14.5 ± 1.5	19.0 ± 1.5	12	—	44.7 ± 0.3	30
3	6.3 ± 0.6	58.0 ± 3.7	—	—	5.5 ± 1.5	4	—	48.0 ± 10.5	—
4	41.3 ± 9.1	179.0 ± 27.6	269.5 ± 41.5	131	219	—	—	—	—
5	12	75	—	13	—	—	—	—	—
	Mg^{2+}-ATPase								
1	34.2 ± 4.1	38.6 ± 5.2	39.0 ± 3.4	38.7 ± 6.0	35.0 ± 9.6	52	26	—	—
2	43.7 ± 2.4	76.0 ± 4.6	69.3 ± 1.8	51.5 ± 0.5	30.0 ± 0.6	79	—	57.3 ± 0.9	65
3	25.0 ± 1.7	46.8 ± 4.4	—	—	27.0 ± 2.0	23	—	44.3 ± 1.9	—
4	36.0 ± 1.2	46.3 ± 2.5	48.5 ± 4.5	18	44	—	—	—	—
5	48	72	—	47	—	—	—	—	—

Table I also shows the effects of phosphatidylserine and phosphatidylcholine on the $(Na^+ + K^+)$-ATPase enzyme prepared from beef brain cerebral cortex, grey matter (Prepn 5). Again phosphatidylserine was effective while phosphatidylcholine was ineffective, in agreement with earlier findings[3,12,13]. The enzyme from brain, therefore, behaves in the same manner as that from rabbit kidney with regard to these two phospholipids.

Correlation of $(Na^+ + K^+)$-ATPase activation with K^+/Na^+ discrimination by phospholipids

As the results in Table I show, considerable variation can be obtained for phospholipid activation of $(Na^+ + K^+)$-ATPase activity, depending on the state of the delipidized enzyme. However, the present studies confirm that phosphatidylserine, and in addition phosphatidylglycerol, are most effective in stimulating the $(Na^+ + K^+)$-ATPase activity of the phospholipid-deficient enzyme. This fits the pattern of selectivity for K^+ permeability, recently described by Papahadjopoulos[17]. The K^+/Na^+ selectivity ratios for different phospholipids and also sulfatide, are compared in Fig. 1 with their ability to activate $(Na^+ + K^+)$-ATPase activity. The results from Prepn 2 have been used. The data for K^+ and Na^+ permeability previously described[17] are used, together with new data for phosphatidylinositol and sulfatide. It is interesting to note that sulfatide is unique in discriminating in favor of Na^+ permeability. Phosphatidylinositol shows a 1.6-fold discrimination in favor of K^+.

Phospholipid content of microsomes and enzyme

As can be seen from Table II, the enzyme contains less than 10 % of the phospholipid present in the original microsomes. The lipids present in the microsomes and

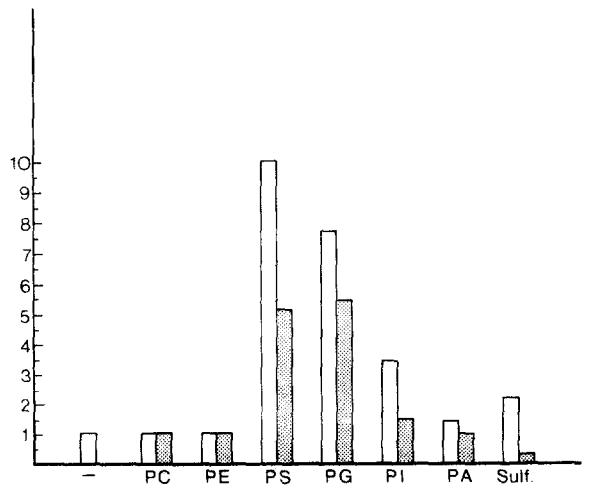

Fig. 1. Comparison of the K^+/Na^+ selectivity ratios for different lipids and their reactivation of $(Na^+ + K^+)$-ATPase. Permeability measurements were made as previously described[17,22,39]. See Table I and Materials and Methods for conditions for phospholipid-activated ATPase activity assays. Stippled column is K^+/Na^+ ratio of diffusion rates through vesicles. Open columns: activation of $(Na^+ + K^+)$-ATPase, expressed as the ratio of activity with lipid added over activity without lipid. No lipid control: 0.8 μmole P_i per mg protein per h. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PA, phosphatidic acid; Sulf., sulfatide.

$(NH_4)_2SO_4$ enzyme were extracted as described under Methods. Two-dimensional thin-layer chromatography (Fig. 2) of microsome Extracts 1 and 2 showed the main phospholipid components to be phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and phosphatidylserine in approximate ratios of 1:1:1:0.5. They were identified on the basis of purified standards, sprays for specific groups, and from comparison with the published chromatograms of Rouser *et al.*[25]. The two spots by the solvent front (to the left) are probably fatty acids (middle left side) and cholesterol

TABLE II

PHOSPHOLIPID CONTENT OF MICROSOMES AND $(NH_4)_2SO_4$ ENZYME

The extraction procedure is described in Methods and Materials.

Stage of extraction	*μmoles P_i per mg protein*	
	Microsomes	*Enzyme*
Original	0.51	0.035
After 1st and 2nd extraction	0.15	0.017
After 3rd extraction	0.11	—

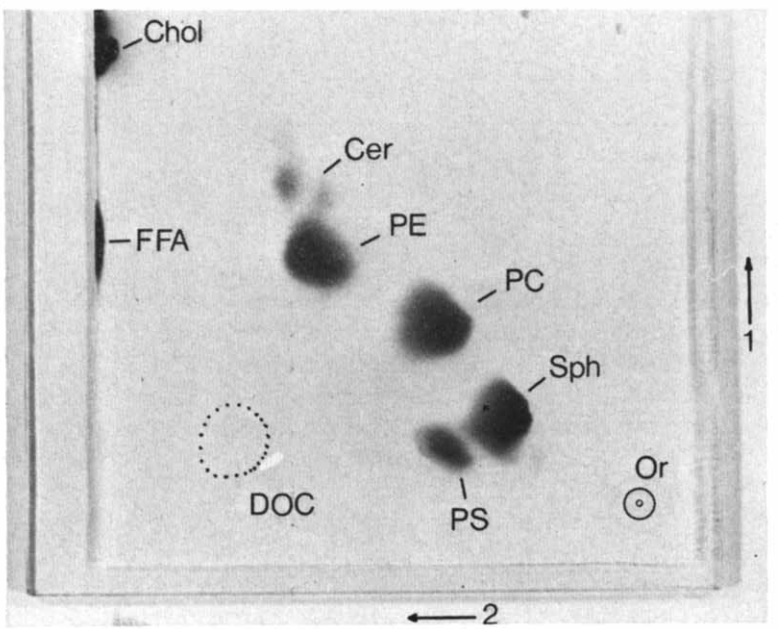

Fig. 2. Two-dimensional chromatography of microsomal lipid extract. 20% of the microsome extract containing approximately 1 μmole phospholipid was applied at the spot labelled Or. The plate was then run in both directions. Solvent 1 was chloroform–methanol–7 M NH_4OH (230:90:1515, v/v/v). Solvent 2 was chloroform–methanol–water–acetic acid–acetone (5:1:0.5:1:2, by vol.). The spots were then sprayed with H_2SO_4 and charred. The area labelled DOC (= deoxycholate) was its position in the chromatogram of the soluble enzyme lipid extract. Abbreviations: Chol, cholesterol; FFA, free fatty acids; Cer, cerebrosides; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; Sph, sphingomyelin. Phosphatidylinositol is probably a minor spot not completely resolved from the main sphingomyelin spot.

(upper left). The minor spots above phosphatidylethanolamine are probably cerebrosides.

Two-dimensional chromatography of the delipidized enzyme extracts showed a number of new minor spots, presumably due to breakdown products of the phospholipids and one large spot due to deoxycholate (marked in Fig. 2). The relative amounts of phosphatidylethanolamine, phosphatidylcholine, sphingomyelin and phosphatidylserine, however, remained the same as far as could be determined by visual inspection, and no selective removal of any one component was apparent.

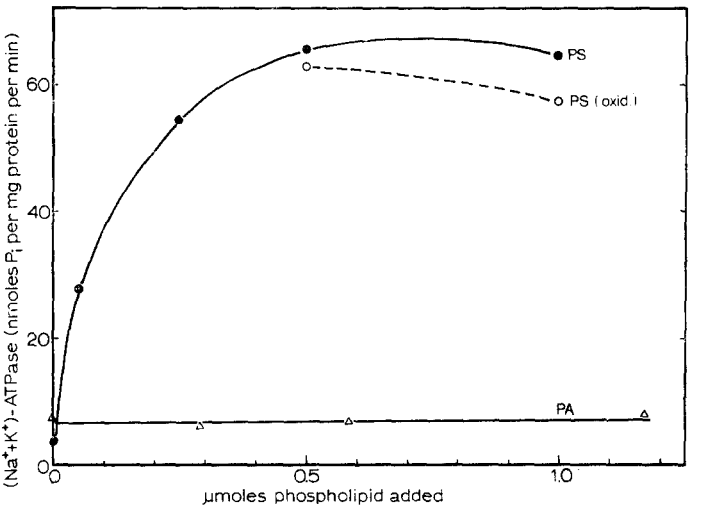

Fig. 3. Effect of varying phospholipid concentration on activation of $(Na^+ + K^+)$-ATPase. Conditions as described in Table I. 0.58 mg of protein was present in 1.5 ml reaction mixture. PS, phosphatidylserine; PA, phosphatidic acid.

Effect of varying phospholipid concentration on activation of $(Na^+ + K^+)$*-ATPase*

In order to establish the correct concentration range for the previous experiments, the effects of different phospholipid concentrations on $(Na^+ + K^+)$-ATPase activity were studied. As can be seen from Fig. 3, maximal activation is obtained at 0.50 μmole phosphatidylserine corresponding to approximately 1 μmole phosphatidylserine per mg protein. The results for different concentrations of phosphatidic acid are also plotted and, as can be seen, it was ineffective throughout this concentration range. Also shown in Fig. 3 is the slightly lowered activation by phosphatidylserine when it was sonicated for 5 min in the presence of air. This procedure caused a 50 % decrease in the K^+/Na^+ permeability ratios[17], as well as higher permeability rates, presumably due to oxidation of the polyunsaturated fatty acid chains[26].

Effects of temperature on the activation of $(Na^+ + K^+)$*-ATPase*

In view of the recent demonstration of the effect of membrane lipid fluidity on several transport properties of living cells[27] it was of interest to study the effects of phospholipid phase transitions on the activation of the $(Na^+ + K^+)$-ATPase. Dipalmitoyl phosphatidylcholine has been shown to undergo a phase transition[28] at approx. 42–45 °C, corresponding to the melting of the fatty acid chains. Dipalmitoyl

TABLE III

EFFECT OF TEMPERATURE ON THE ACTIVATION OF $(Na^+ + K^+)$-ATPase BY DIPALMITOYL PHOSPHATIDYLGLYCEROL AND EGG PHOSPHATIDYLGLYCEROL

Assay procedures and the addition of phospholipid was as described in Methods and Materials and in Table I. Preincubation with phospholipid for 15 min at the designated temperatures.

Phospholipid added	*ATPase activity (nmoles P_i per mg protein per min)*		
	24 °C	*29 °C*	*36 °C*
	$(Na^+ + K^+)$-ATPase		
—	1.6	2.2	3.5
Dipalmitoyl phosphatidylglycerol	2.9	10.1	28.9
Egg phosphatidylglycerol	29.5	35.6	51.1
	Mg^{2+}-ATPase		
—	5.4	9.0	17.4
Dipalmitoyl phosphatidylglycerol	9.6	15.4	37.9
Egg phosphatidylglycerol	32.1	47.3	54.4

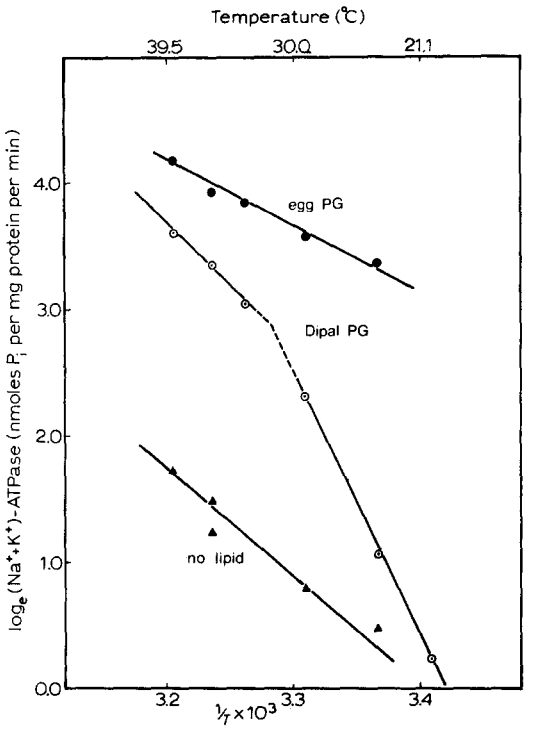

Fig. 4. Arrhenius plot of dipalmitoyl and egg phosphatidylglycerol-activated $(Na^+ + K^+)$-ATPase. $(Na^+ + K^+)$-ATPase activity measured as described in Methods and Table I. PG = phosphatidylglycerol.

phosphatidylglycerol has not been studied as yet, but it can be anticipated that it undergoes the same transition at somewhat lower temperatures, due to the charge–charge repulsion. Permeability studies with dipalmitoyl phosphatidylglycerol vesicles show a 250-fold increase in Na^+-efflux rate between 27.6 and 36.0 °C (midpoint 32 °C) indicating a phase transition within this temperature range (unpublished observations).

Consequently, we have investigated the activation of $(Na^+ + K^+)$-ATPase at different temperatures by dipalmitoyl phosphatidylglycerol, synthesized as described in Methods. The results are shown in Table III. There is a sharp temperature dependence corresponding to a 10-fold difference in activation of $(Na^+ + K^+)$-ATPase activity between 24 and 36 °C. Egg phosphatidylglycerol is derived from egg phosphatidylcholine (see Methods) which contains both unsaturated and saturated fatty acids, and exhibits a phase transition at temperatures[28] below 0 °C. As expected, egg phosphatidylglycerol shows a much smaller temperature dependence (2-fold difference between 24 and 36 °C). The effects are seen more clearly in the Arrhenius plot of Fig. 4 where the logarithm of $(Na^+ + K^+)$-ATPase activity is plotted against $1/T$. A discontinuity at approximately 32 °C is seen for dipalmitoyl phosphatidylglycerol, with a decrease in activation energy from 42 kcal/mole below this temperature to 20 kcal/mole above. For egg phosphatidylglycerol a linear curve is obtained, with an activation energy of 10 kcal/mole. In the absence of added lipid, the activation energy is approx. 17 kcal/mole. The activation energy for the Mg^{2+}-ATPase shows no abrupt change corresponding to a phase transition in the presence of either egg ($E_a = 8$ kcal/mole) or dipalmitoyl ($E_a = 22$ kcal/mole) phosphatidylglycerol. In terms of the relative levels of activity, it can be seen from Table III and also Fig. 4 that at temperatures below the transition point, the difference between the $(Na^+ + K^+)$-ATPase activity in the presence of dipalmitoyl phosphatidylglycerol and in the presence of egg phosphatidylglycerol is much greater (10-fold) than at 36 °C (1.8-fold).

The question also arises as to whether the failure of dipalmitoyl phosphatidylglycerol to activate at 24 °C is due only to the relative immobility of the fatty acid chains, or to a failure of the protein to bind and penetrate the phospholipid membranes at this temperature. Table IV shows the effect of preincubating the dipalmitoyl phosphatidylglycerol–enzyme mixture at 36 °C for 30 min, which would allow the

TABLE IV

EFFECT OF PREINCUBATION AT 36 °C AND TRANSFER TO 24 °C ON THE ACTIVATION OF $(Na^+ + K^+)$-ATPase ACTIVITY BY DIPALMITOYL PHOSPHATIDYLGLYCEROL

Average of two experiments. 0.5 μmole phospholipid was added. For 24 → 24 °C the mixture was kept at 24 °C for 40 min and the reaction was then run at 24 °C for 20 min. Similarly for 36 → 36 °C the mixture was kept at 36 °C for 40 min and then run at 36 °C. For 36 → 24 °C the mixture was kept at 36 °C for 30 min, equilibrated at 24 °C for 10 min, and then, the reaction was run at 24 °C for 20 min as usual.

	ATPase activity (nmoles P_i per mg protein per min)		
	24 → 24 °C	*36 → 24 °C*	*36 → 36 °C*
$(Na^+ + K^+)$-ATPase	2	1	23
Mg^{2+}-ATPase	21	31	36

protein to interact with the lipid above its liquid–crystalline transition point. The mixture was then re-equilibrated at 24 °C and the reaction then run at this temperature. However, as shown in Table IV, pre-equilibration at high temperature did not enhance the ability of dipalmitoyl phosphatidylglycerol to activate the enzyme at 24 °C.

Effect of cholesterol on activation of $(Na^+ + K^+)$-ATPase

It has been reported that cholesterol can activate delipidized $(Na^+ + K^+)$-ATPase[16] when recombined at −75 °C in cholesterol–methanol but is ineffective when recombined at 35 °C in aqueous solution. Therefore, it was of interest to study the

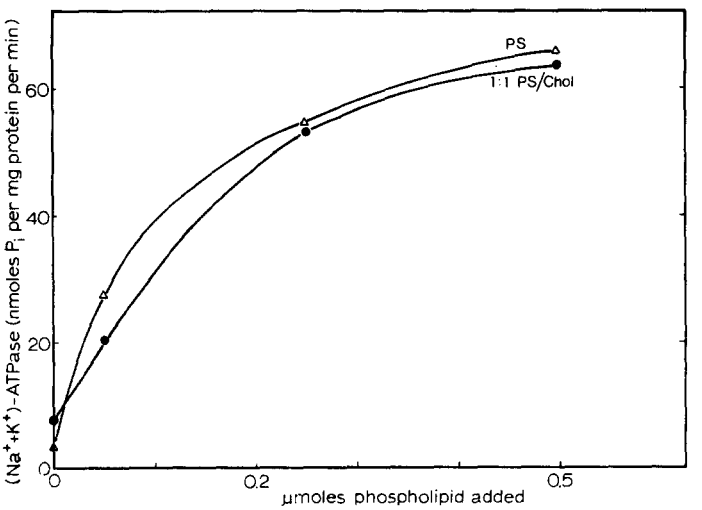

Fig. 5. Effect of cholesterol (Chol) on activation of $(Na^+ + K^+)$-ATPase by phosphatidylserine (PS). Conditions as described in the text and Table I.

TABLE V

EFFECT OF CHOLESTEROL ON THE ACTIVATION OF $(Na^+ + K^+)$-ATPase BY DIPALMITOYL PHOSPHATIDYLGLYCEROL

Conditions were the same as for Tables I and III. The cholesterol and phospholipid were mixed as chloroform solutions at a 1:1 molar ratio (0.6 μmole of each). These mixtures were then evaporated and sonicated as described in text.

Phospholipid added	*ATPase activity (nmoles per mg protein per min)*			
	25 °C	*29 °C*	*33 °C*	*36 °C*
	$(Na^+ + K^+)$-ATPase			
—	0	1.0	4.1	5.8
Dipalmitoyl phosphatidylglycerol–cholesterol (1:1)	1.3	2.3	0.6	3.5
	Mg^{2+}-ATPase			
—	15.0	18.9	23.0	28.3
Dipalmitoyl phosphatidylglycerol–cholesterol (1:1)	17.0	22.4	26.0	33.6

effect of cholesterol under the present conditions. We also wanted to see whether cholesterol altered the activation by phosphatidylserine. As shown in Fig. 5, cholesterol at a 1:1 molar ratio to phosphatidylserine had only a small inhibitory effect when compared to phosphatidylserine alone on the basis of the same amount of phosphatidylserine. However, cholesterol had a significant effect on the activation of the enzyme by dipalmitoyl phosphatidylglycerol. As can be seen from a comparison of Tables 3 and 5, the presence of cholesterol completely inhibits the stimulation of $(Na^+ + K^+)$-ATPase activity by dipalmitoyl phosphatidylglycerol, in contrast to minimal inhibition with phosphatidylserine. This effect is especially marked at higher temperatures.

DISCUSSION

Delipidation and reactivation

As mentioned in the introduction, a variety of different results for phospholipid activation of $(Na^+ + K^+)$-ATPase in different delipidized preparations have been obtained by various workers. Some of these discrepancies are undoubtedly due to the heterogeneity of the phospholipids used[1,12]. Some discrepancies, however, are also probably due to differences in the degree of phospholipid extraction. Our data indicate that preparations with considerable residual $(Na^+ + K^+)$-ATPase activity show less specific activation by phospholipids. As shown in Table II, the detergent treatment used here reduced the total phospholipid content to 7 % of that in the original microsomes. Previous workers employing this extraction procedure have not documented the degree of phospholipid extraction. The amount that we found to be extracted is somewhat greater than the total decrease obtained with venom or phospholipase C (ref. 11). Taniguchi and Tonomura[11] found a general decrease in all phospholipids after phospholipase C treatment and a specific requirement for phosphatidylserine and phosphatidylinositol for full activation after venom treatment. In our study, phosphatidylinositol did show one third the activation given by phosphatidylserine and phosphatidylglycerol in Prepn 2. It is noteworthy also that phosphatidylinositol does show a slight preference for K^+ over Na^+ in terms of permeability. The results obtained after treatment with phospholipases[11] may be unreliable due to the presence of high levels of degradation products, such as lysophospholipids and free fatty acids, and also because of the considerable residual $(Na^+ + K^+)$-ATPase activity present.

The specific activity of our $(Na^+ + K^+)$-ATPase preparations (8 μmoles P_i per mg protein per h + phosphatidylserine) agrees with the preparations of several workers using deoxycholate treatment[1-3,50], but is up to 10-fold lower than others[11]. The deoxycholate concentrations used may be somewhat inhibitory so that a compromise has to be obtained between inhibition and solubilization. Jorgensen and Skou[29] have shown that treatment of rabbit kidney outer medulla with low concentrations of deoxycholate (0.6 mg/ml or 0.06 %, w/v) result in preparations showing very high specific activities (270 μmoles P_i per mg protein per h). Uesugi *et al.*[30] have also reported high specific activity with a Lubrol-solubilized preparation which could be 25–50 % pure in terms of the protein component. The protein to phospholipid ratio in this preparation was still quite high (2:1), and the amount of bound Lubrol was approximately 17 % of total dry weight.

The phospholipids found in our microsome preparations are in general agreement with the more quantitative work of Morgan *et al.*[31] on whole rabbit kidney. From the data shown in Table II it can be calculated that the protein to phospholipid ratio in the original microsomes is approx. 2.4:1. However, this ratio is increased to 30:1 following treatment with deoxycholate. The remaining lipids in the delipidized preparation were qualitatively similar to the lipids of the original microsomes.

Functional roles for phospholipids

Our present report supports previous work on the subject that, despite some variability, phosphatidylserine is a specific requirement for the restoration of $(Na^+ + K^+)$-ATPase activity to preparations inhibited by the removal of phospholipids[3,11–13]. In addition, we report the new finding that phosphatidylglycerol is equally as effective as phosphatidylserine. The fact that these two phospholipids are most effective correlates with recently reported work that these are the only two phospholipids which show a significant discrimination for K^+ over Na^+ permeability of up to 10-fold (see ref. 17 and Fig. 2 of this paper). This suggests that phosphatidylserine (or phosphatidylglycerol in membranes lacking phosphatidylserine such as *Escherichia coli*) may actually function as a cation-specific site of the $(Na^+ + K^+)$-ATPase (perhaps a K^+ site). Conversely, and even more speculatively, sulfatide which shows preference for Na^+ in terms of permeability, could then function as the Na^+ site. It is of interest to note that a specific requirement for phosphatidylserine has also been demonstrated for basal and glucagon-stimulated adenyl cyclase activity[32], and phosphatidylglycerol appears to be required for bacterial phosphotransferase activity[33].

An objection to such a specific role for the phospholipids is the large amount required, *e.g.* 1 μmole phosphatidylserine per mg protein (see Fig. 3). Preliminary binding studies also indicate that the activated $(Na^+ + K^+)$-ATPase can be centrifuged down with almost all the added phosphatidylserine, so that approximately 10 % of the added protein is bound to phosphatidylserine, or 30 μg ATPase protein per μmole phosphatidylserine. Similar results have been reported recently[11]. However, an estimation of the molar ratio of phosphatidylserine per ATPase from the saturation point in Fig. 3 (assuming a $3 \cdot 10^5$ molecular weight and 5 % purity) gives a figure of 3000. In view of the assumptions involved, this figure corresponds surprisingly well with the estimated number of phosphatidylserine molecules per vesicle[39], and indicates that maximal activation is obtained when each ATPase molecule interacts with one phosphatidylserine vesicle.

The view that phospholipids can provide an ion-specific site or even perhaps function as the cation-specific "carrier" or "pore" is in contrast to a non-specific function such as providing the correct milieu for the enzyme. This latter role has been suggested recently[11]. The same study presented evidence[11] that both the formation and breakdown of the phosphorylated enzyme intermediate is inhibited when phospholipid has been removed, although there was some evidence that the degradation products were inhibitory. Goldman and Albers[34], have also suggested that the formation of this complex specifically requires phosphatidylserine.

All the above observations, namely the specificity of the head group requirement, the necessity for fluid fatty acid chains, and the excess of phospholipid required, can be reconciled in the viewpoint that the phospholipid fatty acid chains provide

the appropriate (low dielectric) environment, and the head groups provide a specific "functional" requirement such as cation binding or transport. The fluidity of the chains would presumably increase the degree of hydrophobic association between the phospholipid and the protein and allow freedom for conformational and topographical changes.

Effects of cholesterol

Since cholesterol is an almost ubiquitous component of plasma membranes which are generally considered to be the site of the $(Na^+ + K^+)$-ATPase, the effect of cholesterol on the phospholipid activation is of considerable interest. The effect of cholesterol on phospholipid monolayers[35] and vesicles[36–39] is rather complex but in summary it appears to induce an "intermediate fluid" state[28]. This means that in bilayers below their transition temperature the presence of cholesterol causes them to be more fluid. At temperatures above their transition point, when they are in a liquid crystalline state, the presence of cholesterol makes them less fluid. This can explain the results of table V, where the effect of dipalmitoyl phosphatidylglycerol is completely inhibited at a 1:1 molar ratio of cholesterol. It would appear, however, that the "intermediate" fluid state that would be expected to occur with 1:1 phosphatidylserine/cholesterol (Fig. 5) has a much smaller effect on the ability of phosphatidylserine to activate. A variety of soluble and membrane proteins[22,40–44] have been shown to penetrate fluid phospholipid monolayers and bilayers forming hydrophobic associations and leading to large increases in the permeability of these membranes[43]. Current, preliminary work in this laboratory indicates that such penetration and subsequent permeability changes are also inhibited by the presence of cholesterol.

Fluidity requirement for reactivation, and fluidity in biological membranes

The work of Steim and his associates[45,46] has indicated that a high proportion of the phospholipids in some natural membranes are in a fluid state. Even more pertinent are the findings of Fox[47,48] that the incorporation of induced transferase enzymes into bacterial membranes requires the concomitant presence of unsaturated, fluid, fatty acids. Thus the fluidity of phospholipids appears to be essential for the functioning of some membrane-bound enzymes, and as we have shown here for the solubilized $(Na^+ + K^+)$-ATPase. The discontinuities seen in the Arrhenius plot in Fig. 4 are reminiscent of those reported by Wilson *et al.*[27] and Wilson and Fox[47] for the temperature dependence of glycoside transport in *E. coli* mutants grown on different fatty acid supplements. The temperatures at which these discontinuities or "kinks" occurred, correspond approximately to the temperatures of the phase transitions of the respective fatty acids. Another point of similarity is that the activation energy below the "kink" is greater than that above. Similar results have also been reported by Esfahani *et al.*[49], for proline transport and succinic dehydrogenase activity in unsaturated fatty acid auxotrophs of *E. coli*.

It is of interest that Priestland and Whittam[59] also using a preparation treated with deoxycholate have found a "kink" in the Arrhenius plot for phosphatidylserine-activated $(Na^+ + K^+)$-ATPase at around 15 °C. The two activation energies found, 34 kcal/mole below the "kink" and 15 kcal/mole above, are similar to our results of 42 and 20 kcal/mole for dipalmitoyl phosphatidylglycerol which we have attributed to a gel to liquid–crystalline phase-change of the phospholipid. Priestland and

Whittam[50] also attribute their results with phosphatidylserine to temperature-dependent phase-changes, although the temperature for the phase transition in their preparations of phosphatidylserine was not stated. Discontinuities or "kinks" in Arrhenius plots have also been reported for ATPase membrane preparations where there is no requirement for added phospholipids. Gruener and Avi-Dor[51] found "kinks" at 20 and 6 °C for both the $(Na^+ + K^+)$- and Mg^{2+}-ATPase activity of rat brain microsomes. Smith[52] reported that the $(Na^+ + K^+)$-ATPase activity of preparations from goldfish intestinal mucosa showed "kinks" that reflected the previous acclimatization temperature of the fish. Thus fish which had been acclimatized at 8 °C showed a "kink" at 12 °C, whereas fish acclimatized at 30 °C showed a "kink" at 21 °C. It seems possible that this might reflect the different degrees of unsaturation of the fatty acid chains of the membrane phospholipids associated with the $(Na^+ + K^+)$-ATPase, which vary with the acclimatization temperature.

We are thus led to the picture of a fluid phospholipid membrane backbone which allows the interdigitation of different membrane proteins to varying degrees, as suggested in recent concepts of membrane structure[53]. An alternative complementary view of hydrophobic protein–phospholipid interactions is given by the "deformation" model of Kimelberg and Papahadjopoulos[43] and the bilayer "thinning" suggested by Haydon and colleagues[54]. In these models hydrophobic association between protein and phospholipid takes place at the lipid–water interface, accommodated by an increase in the area per phospholipid molecule and a consequent thinning of the lipid bilayer. The fluidity of the fatty acyl chains may also provide the required motional freedom, allowing enzymes within membranes to undergo conformational changes and movements associated with their activity. We would like to suggest the term "viscotropic" to describe the influence of membrane fluidity on enzyme activity. Finally, the head-groups of certain phospholipids, particularly acidic ones such as phosphatidylserine and phosphatidylglycerol, appear to provide specific functional sites in association with various enzymes. It would be anticipated that such phospholipids would be preferentially grouped in the vicinity of these membrane-bound enzymes, constituting special "domains" involved in transport functions[49].

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