Biochimica et Biophysica Acta, 508 (1978) 464—477

BBA 77995

ROLE OF NEGATIVELY CHARGED PHOSPHOLIPIDS IN HIGHLY PURIFIED ($Na^+ + K^+$)-ATPase FROM RABBIT KIDNEY OUTER MEDULLA

STUDIES ON ($Na^+ + K^+$)-ACTIVATED ATPase, XXXIX

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(Received September 23rd, 1977)

Summary

1. The requirement for specific polar head groups of phospholipids for activity of purified ($Na^+ + K^+$)ATPase from rabbit kidney outer medulla has been investigated.

2. Comparison of content and composition of phospholipids in microsomes and the purified enzyme indicates that purification leads to an increase in the phospholipid/protein ratio and in phosphatidylserine content.

3. The purified preparation contains 267 molecules phospholipid per molecule ($Na^+ + K^+$)-ATPase, viz. 95 phosphatidylcholine, 74 phosphatidylethanolamine, 48 sphingomyelin, 35 phosphatidylserine and 15 phosphatidylinositol.

4. Complete conversion of phosphatidylserine into phosphatidylethanolamine by the enzyme phosphatidylserine decarboxylase has no effect on the ($Na^+ + K^+$)-ATPase activity of the purified preparation.

5. Complete hydrolysis of phosphatidylinositol by a phospholipase C from *Staphylococcus aureus*, which is specific for this phospholipid, has no effect on the ($Na^+ + K^+$)-ATPase activity.

6. Hydrolysis of 95% of the phosphatidylcholine and 60—70% of the sphingomyelin and phosphatidylethanolamine by another phospholipase C (*Clostridium welchii*) lowers the ($Na^+ + K^+$)-ATPas activity by about 20%.

7. Combination of the phospholipid-converting enzymes has the same effect as can be calculated from the effects of the enzymes separately. Only complete conversion of both phosphatidylserine and phosphatidylinositol results in a loss of 44% of the ($Na^+ + K^+$)-ATPase activity and 36% of the potassium 4-nitrophenylphosphatase activity.

8. These experiments indicate that there is no absolute requirement for one of the polar head groups, although in the absence of negative charges the activity is lower than in their presence.

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid.

Introduction

The enzyme ($Na^+ + K^+$)-ATPase is involved in the active transport of Na^+ and K^+ across plasma membranes [1—4]. During the last few years the enzyme has been purified from a number of specialized tissues (see ref. 4). Even the purest active preparations contain phospholipids, but little attention has been devoted to the importance of these compounds for the enzyme activity in the purified preparations. However, from studies with crude preparations there is abundant evidence that the phospholipids are important for the enzyme activity. This is shown by the fact that removal of phospholipids by treatment with detergents, phospholipases and organic solvents causes partial or complete loss of enzyme activity (for references see ref. 5). Moreover, the physical state of the membrane lipids also has influence on the enzyme activity, as shown by discontinuities in the Arrhenius plots of the enzyme [6—11] and by studies with fluorescence labeling and electron spin labeling techniques [12—15].

In order to investigate the polar head group specificity of the phospholipid requirement, most authors have first delipidated the enzyme by one of the above-mentioned agents and then tried to reactivate it by addition of various phospholipids. Generally, these studies show that acidic phospholipids such as phosphatidylserine, phosphatidylinositol, phosphatidylglycerol and phosphatidic acid are better able to restore the enzyme activity than neutral phospholipids [7,10,16—22]. Restoration with phosphatidylcholine after detergent extraction was recently shown to be only possible when in addition a negatively charged compound, e.g. dicetylphosphate, was present [22].

From this type of studies the conclusion has been drawn that phosphatidylserine [17,20] or negatively charged phospholipids in general [7,10,23] are essential for the ($Na^+ + K^+$)-ATPase activity. Phosphatidylserine is the only negatively charged phospholipid present in all mammalian plasma membranes, which may suggest that in situ this phospholipid is in close contact with the enzyme protein [20]. A weakness of the approach of general delipidation followed by specific reconstitution is that the enzyme molecule may be altered in the process. It could be that the specificity for negatively charged phospholipids has only to do with the reconstitution process and that these phospholipids do not play a role in the functioning of the enzyme [5,21,24].

In an earlier study we have, therefore, introduced a gentler technique for investigating the phosphatidylserine requirement of ($Na^+ + K^+$)-ATPase [5]. We have treated an enzyme preparation from cattle brain with the enzyme phosphatidylserine decarboxylase from *Escherichia coli* in order to convert phosphatidylserine into phosphatidylethanolamine without changing the other phospholipids present [5]. We were able to convert all phosphatidylserine without loss of ($Na^+ + K^+$)-ATPase activity, which would seem to disprove the above hypothesis. However, because of the relatively low purity of the cattle brain preparation and the limited sensitivity of the lipid determination, we could not exclude that in the residual preparation some nine molecules phosphatidylserine per molecule of ($Na^+ + K^+$)-ATPase were left. Moreover in this preparation another negatively charged phospholipid, phosphatidylinositol, was still present in normal amounts.

Therefore, the investigation has been extended in two ways. A highly

purified ($Na^+ + K^+$)-ATPase preparation from rabbit kidney outer medulla has been used. In addition to phosphatidylserine decarboxylase two phospholipases C, one specific for phosphatidylinositol and the other for neutral phospholipids, have been applied. The results of these experiments permit more definitive conclusions about the phospholipid requirements of ($Na^+ + K^+$)-ATPase.

Materials and Methods

($Na^+ + K^+$)-ATPase preparation. ($Na^+ + K^+$)-ATPase (EC 3.6.1.3) is purified from rabbit kidney outer medulla according to Jørgensen [25]. The microsomes, obtained after dissection, homogenisation and differential centrifugation, have a ($Na^+ + K^+$)-ATPase activity (determined after 30 min preincubation at 20°C with 0.6 mg/ml deoxycholate) of 200—250 μmol ATP · h^{-1} · mg^{-1} protein. The final purified preparation is obtained after sodium dodecyl sulphate extraction of the microsomes, followed by isopycnic zonal centrifugation in a sucrose density gradient. In order to remove ATP present in the enzyme preparation [25] 10 ml of the ATPase incubation medium A (ref. 1, p. 261) without ATP is added to 2 ml of the enzyme preparation. After incubation at 37°C for 15 min the enzyme is sedimented by centrifugation for 10 min at 300 000 × g. In order to remove adhering ions and residual amounts of sodium dodecyl sulphate the enzyme is twice washed with imidazole (25 mM), pH 7.5. The enzyme is stored at —20°C in the latter buffer to whcih 0.25 mM sucrose is added. The final preparation has an activity of 1300 (S.E. 90; n = 15) μmol ATP · h^{-1} · mg^{-1} protein. It is free of ouabain-sensitive Mg^{2+}-ATPase activity and is 95% pure on a protein basis, as determined by sodium dodecyl sulphate gel electrophoresis.

Phospholipid converting enzymes. Phospholipase C, *Clostridium welchii*: A crude preparation, α toxin AD 1051 A, free of proteolytic activity, has been obtained as a generous gift from the Wellcome Research Laboratories, Beckenham, England. This preparation, which is a lyophilized powder, is stored at 4°C and is dissolved immediately before use.

Phosphatidylinositol specific phospholipase C, *Staphylococcus aureus*: This has been obtained as a generous gift from Dr. Martin Low, Department of Biochemistry, Birmingham, U.K. It is prepared from the culture supernatant of *S. aureus* by chromatography on Amberlite CG-50, as described by Low and Finean [26], followed by precipitation with saturated $(NH_4)_2SO_4$. This preparation which has a specific activity of 300 units/mg protein (units defined as in ref. 26), is centrifuged for 20 min at 10 000 × g at 4°C, is dissolved in 2 ml 20 mM imidazole · HCl (pH 7.0) and is dialysed against the imidazole buffer. The final preparation is stored at —20°C.

Phosphatidylserine decarboxylase: This has been partially purified from a cell-free extract of *E. coli* frozen cells (Strain B, Med Log Harvest, Miles Laboratories Inc. Elkhart, Ind., U.S.A.). The purification is carried out essentially as described by Dowham et al. [27] up to step 4. The eluate from the DEAE-cellulose column is used as the enzyme preparation. It has a specific activity of 260 units/mg (units defined as in ref. 27) and is stored at —20°C.

Treatment with phospholipid converting enzymes. Treatment with phospholipase C (*Cl. welchii*): The ($Na^+ + K^+$)-ATPase preparation is centrifuged for

30 min at 4°C at 321 000 × *g* and is taken up at a final concentration of 1.5 mg/ml in a medium prepared according to Stahl [28]. This medium contains 4 mM histidine (pH 7.4), 0.6 mM $CaCl_2$, 0.5 mM Na_2ATP, 0.4 mg/ml bovine serum albumin, 25% (v/v) dimethylsulfoxide and additionally 0.1 mM $ZnCl_2$. To 1 ml of this suspension 100 µl phospholipase C (20 mg/ml of the Stahl medium) or buffer alone is added and the mixture is incubated for 10 min at 30°C. Thereafter 3 ml 0.1 M EGTA/Tris (pH 7.4) is added and the mixture is centrifuged for 30 min at 4°C at 321 000 × *g*. The pellet is resuspended in double-distilled water and the centrifugation is repeated. Thereafter the pellet is taken up in 1 ml double-distilled water. In this suspension, after appropriate dilution, the $(Na^+ + K^+)$-ATPase and potassium 4-nitrophenylphosphatase activities are immediately determined. Protein analysis and lipid extraction, followed by lipid phosphorus determination and phospholipid analysis, are carried out as described under Analytical Methods.

Treatment with phosphatidylinositol-specific phospholipase C: The $(Na^+ + K^+)$-ATPase is pelleted as described above and the pellet is taken up in 20 mM imidazole · HCl buffer, pH 7.0 (2 mg/ml). To 800 µl of this suspension 200 µl of the phospholipase suspension or imidazole buffer alone is added and the mixture is incubated for 30 min at 37°C. The reaction mixture is diluted and the $(Na^+ + K^+)$-ATPase and the potassium 4-nitrophenylphosphatase activities are determined immediately. Samples are taken for protein analysis and lipid extraction, followed by lipid phosphorus determination and phospholipid analysis is carried out as described under Analytical Methods.

When the treatment with the phosphatidylinositol-specific phospholipase C is followed by treatment with phospholipase C of *Cl. welchii*, 500 µl of the above reaction mixture is added to 500 µl of a double concentrated Stahl buffer. The further procedure is carried out as described for phospholipase C (*Cl. welchii*) alone.

Treatment with phosphatidylserine decarboxylase: The $(Na^+ + K^+)$-ATPase preparation, which is or is not pretreated with one of the phospholipases, is sedimented by centrifugation for 30 min at 4°C at 321 000 × *g*. The pellet is washed once and is taken up in 0.1 M Tris · HCl (pH 7.0), 5 mM ATP, 5 mM EDTA, 0.1% Triton X-100. To 100 µl of this suspension 200 µl phosphatidylserine decarboxylase or as a control 200 µl heat-inactivated preparation (5 min at 100°C) is added. After 3 h incubation at 30°C samples are taken for the determination of $(Na^+ + K^+)$-ATPase and potassium 4-nitrophenylphosphatase activities and for lipid extraction, followed by phospholipid analysis.

The phospholipid contents of the treated preparations are expressed as percent of those of the untreated preparation. For this purpose the values for the individual phospholipids (measured as percent of the total amount of phospholipids in the same preparation) are multiplied with the ratio of the lipid phosphorus content of the treated $(Na^+ + K^+)$-ATPase preparation to that of the untreated preparation.

Analytical methods. $(Na^+ + K^+)$-ATPase and potassium-stimulated 4-nitrophenylphosphatase activities are determined as previously described [29].

The lipid phosphorus content and the phospholipid pattern of a preparation are determined after extraction of total lipids with chloroform/methanol (2 : 1, v/v) and washing with 0.1 M KCl [30]. The phosphorus content of the lipid

extract is determined by a modified Fiske-Subbarow method after H_2SO_4/$HClO_4$ digestion [29]. Phospholipid analysis of the lipid extract is carried out by two-dimensional thin-layer chromatography on silica gel containing 4% alkaline magnesium silicate [31]. The spots are detected with iodine vapour, scraped off and their phosphorus content is determined in the same way as that of the total lipid extract.

Protein is determined according to Lowry et al. [32] after trichloroacetic acid precipitation as described by Jørgensen [25]. Bovine serum albumin is used as a standard.

Results

Lipid composition of the ($Na^+ + K^+$)-ATPase preparations

The lipid content and the phospholipid composition of both the "microsomal" and the highly purified ($Na^+ + K^+$)-ATPase preparations, prepared from rabbit kidney outer medulla, are listed in Table I. During purification the lipid/protein ratio increases by 80%, indicating that sodium dodecyl sulphate extracts more protein than phospholipids from the membranes or, less likely, that the membranes which contain the ($Na^+ + K^+$)-ATPase have a relatively higher phospholipid/protein ratio than the microsomal fraction. Expressed as percent of total phospholipids the purified preparation contains more phosphatidylserine and less phosphatidylcholine than the "microsomal" fraction. The percentage of the other three important phospholipids, sphingomyelin, phosphatidylinositol and phosphatidylethanolamine, is not significantly changed.

TABLE I

PHOSPHOLIPID CONTENT AND COMPOSITION OF MICROSOMES AND PURIFIED ($Na^+ + K^+$)-ATPase FROM RABBIT KIDNEY OUTER MEDULLA

The results are presented as averages with standard errors and in parentheses the number of preparations.

	Microsomes (μg/mg protein)	Purified ($Na^+ + K^+$)-ATPase	
		μg/mg protein	mol phospholipid per mol ($Na^+ + K^+$)-ATPase
	Phospholipid content		
	18.5 ± 1.7 (7)	33.1 ± 2.4 (15)	267 ± 19 (15)
Phospholipid	Phospholipid composition		
	Percent of total phospholipids	Percent of total phospholipids	mol/mol ($Na^+ + K^+$)-ATPase *
Sphingomyelin	15.9 ± 1.1 (7)	17.9 ± 0.6 (15)	48 ± 4 (15)
Phosphatidylcholine	43.6 ± 2.3 (7)	35.6 ± 0.7 (15)	95 ± 7 (15)
Phosphatidylserine	7.9 ± 0.6 (7)	13.1 ± 0.9 (15)	35 ± 4 (15)
Phosphatidylinositol	6.6 ± 0.4 (7)	5.5 ± 0.3 (15)	15 ± 2 (15)
Phosphatidylethanolamine	25.8 ± 2.6 (7)	27.9 ± 1.0 (15)	74 ± 6 (15)

* Calculated from phospholipid compostion and lipid/phosphorus content, assuming a molecular weight of 250 000 for ($Na^+ + K^+$)-ATPase.

Assuming a molecular weight of 250 000 for $(Na^+ + K^+)$-ATPase [33], the total number of phospholipid molecules per molecule $(Na^+ + K^+)$-ATPase is calculated to be 267 (S.E. 19; $n = 15$). The same calculation can be made for each of the individual phospholipids (Table I). This table shows that there are about 50 acidic phospholipid molecules per molecule of $(Na^+ + K^+)$-ATPase.

Phosphatidylserine conversion

When the purified $(Na^+ + K^+)$-ATPase preparation is treated with phosphatidylserine decarboxylase, there is a complete conversion of phosphatidylserine into phosphatidylethanolamine (Table II). The increase in the content of the latter phospholipid (9.8 ± 2.2%) is equal within the experimental error to the original phosphatidylserine content. This treatment does not significantly lower the $(Na^+ + K^+)$-ATPase activity.

Phosphatidylinositol conversion

Treatment of the $(Na^+ + K^+)$-ATPase preparation with a phosphatidylinositol specific phospholipase C leads to complete hydrolysis of phosphatidylinositol (Table III, column 3). The conversion of the latter phospholipid completely accounts for the decrease in lipid phosphorus content of the preparation. The contents of the other phospholipids are not changed. This treatment does not change the activities of $(Na^+ + K^+)$-ATPase or potassium 4-nitrophenylphosphatase.

Conversion of neutral phospholipids

Treatment of the $(Na^+ + K^+)$-ATPase preparation with phospholipase C (*Cl. welchii*) leads to a very marked breakdown of phospholipids (Table III, column 4). Only 5% of phosphatidylcholine is left, whereas 30—40% of sphingomyelin and phosphatidylethanolamine remains unconverted. Phosphatidylserine and phosphatidylinositol are not converted by this phospholipase. Incubation for longer time periods or with more phospholipase C does not result in further phospholipid breakdown. This treatment, which reduces

TABLE II

EFFECTS OF TREATMENT OF PURIFIED $(Na^+ + K^+)$-ATPase WITH PHOSPHATIDYLSERINE DECARBOXYLASE ON PHOSPHOLIPID COMPOSITION AND $(Na^+ + K^+)$-ATPase ACTIVITY

The results are expressed as averages with standard errors and in parentheses the number of experiments.

Phospholipid	Control	Treated with phosphatidylserine decarboxylase
	Phospholipid content (in percent of total phospholipids)	
Sphingomyelin	17.9 ± 0.6 (15)	18.0 ± 1.6 (6)
Phosphatidylcholine	35.6 ± 0.7 (15)	39.0 ± 0.8 (6)
Phosphatidylserine	13.1 ± 0.9 (15)	0.0 ± 0.0 (6)
Phosphatidylinositol	5.5 ± 0.3 (15)	5.1 ± 0.3 (6)
Phosphatidylethanolamine	27.9 ± 1.0 (15)	37.7 ± 2.0 (6)
	Percent of control activity	
$(Na^+ + K^+)$-ATPase activity	≡100	87 ± 6.8 (7)

TABLE III

EFFECTS OF TREATMENT OF $(Na^+ + K^+)$-ATPase WITH PHOSPHOLIPASE C FROM TWO DIFFERENT SOURCES, SEPARATELY AND COMBINED, ON PHOSPHOLIPID COMPOSITION, $(Na^+ + K^+)$-ATPase ACTIVITY AND POTASSIUM 4-NITROPHENYLPHOSPHATASE ACTIVITY

The results are presented as averages with standard errors and in parentheses the number of experiments.

	Control	Treated with phosphatidylinositol-specific phospholipase C	Treated with phospholipase C (*Cl. Welchii*)	Combined treatment
Lipid phosphorus	≡100	93.0 ± 0.9 (6)	37.3 ± 1.8 (7)	33.6 ± 1.4 (3)
Phospholipid	Phospholipid composition (in percent of total phospholipids in untreated preparation)			
Sphingomyelin	17.9 ± 0.6 (15)	17.2 ± 0.9 (6)	6.5 ± 0.6 (7)	9.0 ± 1.7 (3)
Phosphatidylcholine	35.6 ± 0.7 (15)	37.3 ± 1.4 (6)	1.7 ± 0.2 (7)	2.8 ± 1.2 (3)
Phosphatidylserine	13.1 ± 0.9 (15)	10.8 ± 0.7 (6)	12.7 ± 1.0 (7)	15.7 ± 1.2 (3)
Phosphatidylinositol	5.5 ± 0.3 (15)	0.0 ± 0.5 (6)	6.6 ± 0.4 (7)	0.2 ± 0.4 (3)
Phosphatidylethanolamine	27.9 ± 1.0 (15)	27.8 ± 1.4 (6)	9.6 ± 0.8 (7)	5.9 ± 1.0 (3)
	Percent of total activity			
$(Na^+ + K^+)$-ATPase activity	≡100	101 ± 2.8 (11)	80 ± 5.6 (7)	82 ± 6.1 (3)
Potassium 4-nitrophenyl phosphatase activity	≡100	99 ± 3.5 (11)	90 ± 8.4 (12)	85 ± 6.2 (3)

the total number of phospholipid molecules from 267 to 100 per molecule of $(Na^+ + K^+)$-ATPase, leads to a 20% loss of $(Na^+ + K^+)$-ATPase activity, whereas the potassium 4-nitrophenylphosphatase activity is not significantly diminished.

Conversion of phosphatidylinositol and neutral phospholipids

When the $(Na^+ + K^+)$-ATPase activity is treated successively with the phosphatidylinositol-specific phospholipase C and the phospholipase C from *Cl. welchii*, the changes in phospholipid composition and enzyme activities are the sum of the changes resulting from treatments with the two phospholipases separately (Table III, column 5). The $(Na^+ + K^+)$-ATPase activity is still 82% of the control, and the potassium 4-nitrophenylphosphatase activity 85%. The control in this case has been carried through both treatments but with phospholipase C (*Cl. welchii*) omitted in the second step.

Conversion of phosphatidylserine and neutral phospholipids

In a further experiment the $(Na^+ + K^+)$-ATPase preparation is first treated with phospholipase C, followed by treatment with phosphatidylserine decarboxylase. In this experiment the total number of phospholipid molecules is reduced to about 100 per molecule of $(Na^+ + K^+)$-ATPase, as is also the case after treatment with phospholipase C alone. Phosphatidylserine is completely converted to phosphatidylethanolamine. Table IV shows that the decrease in phosphatidylserine content is equal within the experimental error to the increase in phosphatidylethanolamine content. The second step of this treatment does not lead to a significant reduction in either $(Na^+ + K^+)$-ATPase or potassium 4-nitrophenylphosphatase activity as compared to a control prepara-

TABLE IV

EFFECT OF TREATMENT OF $(Na^+ + K^+)$-ATPase WITH PHOSPHATIDYLSERINE DECARBOXYLASE AFTER PREVIOUS TREATMENT WITH PHOSPHOLIPASE C (*Cl. Welchii*) ON PHOSPHOLIPID COMPOSITION AND $(Na^+ + K^+)$-ATPase AND POTASSIUM 4-NITROPHENYLPHOSPHATASE ACTIVITIES

The results are presented as averages with standard errors and in parentheses the number of experiments.

	Treated with phospholipase C (*Cl. Welchii*) alone	Treated with phospholipase C (*Cl. Welchii*) followed by phosphatidylserine decarboxylase
Phospholipid	Phospholipid composition (in percent of total phospholipids in untreated preparation)	
Sphingomyelin	6.5 ± 0.6 (7)	6.6 ± 0.7 (4)
Phosphatidylcholine	1.7 ± 0.2 (7)	3.1 ± 0.9 (4)
Phosphatidylserine	12.7 ± 1.0 (7)	0.2 ± 0.2 (4)
Phosphatidylinositol	6.6 ± 0.4 (7)	6.9 ± 0.7 (4)
Phosphatidylethanolamine	9.6 ± 0.8 (7)	20.1 ± 2.0 (4)
	Percent of activity in phospholipase C-treated preparation	
$(Na^+ + K^+)$-ATPase activity	≡100	90 ± 14.5 (7)
Potassium 4-nitrophenylphosphatase activity	≡100	75 ± 16.1 (7)

TABLE V

EFFECT OF TREATMENT OF $(Na^+ + K^+)$-ATPase WITH PHOSPHATIDYLSERINE DECARBOXYLASE AFTER PREVIOUS TREATMENT WITH A PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C ON PHOSPHOLIPID COMPOSITION, AND $(Na^+ + K^+)$-ATPase AND POTASSIUM 4-NITROPHENYLPHOSPHATASE ACTIVITIES

The results are presented as averages with standard errors and in parentheses the number of experiments.

	Treated with phosphatidylinositol-specific phospholipase C alone	Treated with phosphatidylinositol-specific phospholipase C followed by phosphatidylserine decarboxylase
Phospholipid	Phospholipid composition in percent of total phospholipids in untreated preparation	
Sphingomyelin	17.2 ± 0.9 (6)	18.9 ± 0.9 (4)
Phosphatidylcholine	37.3 ± 1.4 (6)	39.0 ± 1.5 (4)
Phosphatidylserine	10.8 ± 0.7 (6)	0.6 ± 0.5 (4)
Phosphatidylinositol	0.0 ± 0.5 (6)	0.2 ± 0.3 (4)
Phosphatidylethanolamine	27.8 ± 1.4 (6)	36.3 ± 1.2 (4)
	Percent of activity in phospholipase C-treated preparation	
$(Na^+ + K^+)$-ATPase activity	≡100	56 ± 2.4 (4)
Potassium 4-nitrophenylphosphatase activity	≡100	64 ± 7.9 (4)

tion, where a preheated phosphatidylserine decarboxylase preparation has been used in the second step.

Conversion of both phosphatidylserine and phosphatidylinositol

Finally, the $(Na^+ + K^+)$-ATPase preparation is first treated with the phosphatidylinositol-specific phospholipase, followed by treatment with phosphatidylserine decarboxylase. Table V shows that this combined treatment leads to virtually complete hydrolysis of phosphatidylinositol and also virtually complete conversion of phosphatidylserine to phosphatidylethanolamine. The resulting preparation thus contains essentially only neutral phospholipids. The second step of the treatment leads to significant losses of $(Na^+ + K^+)$-ATPase activity (44%) and of potassium 4-nitrophenylphosphatase activity (36%) as compared to a control preparation, where a preheated phosphatidylserine decarboxylase preparation has been used in the second step.

Discussion

Phospholipid composition of the enzyme preparation

$(Na^+ + K^+)$-ATPase has been purified in recent years from a variety of tissues by means of different procedures (see ref. 4). With one exception [34], there is general agreement on the presence of two protein subunits in the preparations. However, the phospholipids, which are always present in these preparations, have been investigated much less extensively.

Published values for the lipid phosphorus content (expressed in μg lipid phosphorus/mg protein, using an average molecular weight of 750 for phospholipids) vary considerably: 18 from the enzyme preparation from bovine brain [35], 24 from rabbit kidney outer medulla [25], 31 from pig kidney outer medulla [23], 209 from electric organ of *Electrophorus electricus* [36] and 389 from rectal gland of *Squalus acanthius* [36]. Our value for the rabbit kidney outer medulla preparation (33 μg lipid phosphorus/mg protein) is slightly higher than that given by Jørgensen [25] for the same preparation and is approximately equal to the value reported by Wheeler et al. [21] for the corresponding preparation from pig kidney.

The phospholipid composition of highly purified $(Na^+ + K^+)$-ATPase preparations has been reported in a few cases [19,21,34,37], but without statistical data. There is qualitative agreement between the results of these studies, except that no phosphatidylinositol was found in a beef brain preparation [19]. In addition, Kawai et al. [37] find in a pig brain preparation lysophosphatidylethanolamine, and Wheeler et al. [21] find in a preparation from pig kidney outer medulla the lysoproducts of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine. Quantitatively the differences between the various analyses are rather large. The most striking difference is that our preparation from rabbit kidney outer medulla contains more sphingomyelin and phosphatidylinositol than the other preparations. Since both sphingomyelin and $(Na^+ + K^+)$-ATPase are plasma membrane components, the presence of sphingomyelin is to be expected.

Purification of $(Na^+ + K^+)$-ATPase from a microsomal fraction leads to an increase in the lipid phosphorus/protein ratio, which has likewise been observed

by Jørgensen [25] and Wheeler et al. [21]. We also find an increase in the phosphatidylserine content from 7.9 to 13.1%. Previous reports on a change in the phosphatidylserine content upon purification are contradictory [19,21,37], which may be due to differences in method and degree of purification, whereas the lack of statistical data in these reports may also contribute.

From the total lipid phosphorus content and the phospholipid composition the molecular ratio for each type of phospholipid can be calculated, assuming a value of 250 000 [33] for the molecular weight of the enzyme. In this way, our preparation is found to contain per molecule of $(Na^+ + K^+)$-ATPase 217 neutral phospholipid molecules (95 phosphatidylcholine, 74 phosphatidylethanolamine and 48 sphingomyelin) and 50 acidic phospholipid molcules (35 phosphatidylserine and 15 phosphatidylinositol).

Phosphatidylinositol requirement

We have studied the specific requirements of the enzyme for phospholipid headgroups by treating the preparation by three phospholipid-converting enzymes, singly and in combination, and by comparing the effects on the enzyme activity and the phospholipid composition. The phosphatidylinositol-specific phospholipase C is here to our knowledge applied for the first time to investigate the phosphatidylinositol requirement of an enzyme. This enzyme does seem not to work very well on liver plasma membranes [38], and gives incomplete breakdown of phosphatidylinositol in pig or sheep erythrocytes but not in ox erythrocytes [26]. However, it works surprisingly well on the purified $(Na^+ + K^+)$-ATPase preparation. All phosphatidylinositol is converted without loss of enzyme activity, indicating that phosphatidylinositol by itself is not required for enzyme activity.

Phosphatidylserine requirement

Phosphatidylserine decarboxylase has been applied by us [5] and other investigators [20,39] to study the requirements for phosphatidylserine. In applying the enzyme to the purified $(Na^+ + K^+)$-ATPase preparation, we have confirmed our earlier finding from a crude preparation from cattle brain that phosphatidylserine decarboxylase is able to convert all phosphatidylserine to phosphatidylethanolamine without significant loss of $(Na^+ + K^+)$-ATPase activity. In the previous study with the pure preparation we could not exclude the presence of up to nine molecules of residual phosphatidylserine per molecule of $(Na^+ + K^+)$-ATPase. Since the detection limit of each type of phospholipid is below 0.2% of total phospholipids, we can now state that the residual amount of phosphatidylserine (and also of phosphatidylinositol in the above-mentioned experiments) is less than 0.54 molecule per molecule of $(Na^+ + K^+)$-ATPase. This means that $(Na^+ + K^+)$-ATPase has no absolute requirement for either phosphatidylserine or phosphatidylinositol by itself.

Neutral phospholipid requirement

The specificity in the phospholipid breakdown pattern of the phospholipase from *Cl. welchii* is in agreement with that observed by previous investigators 28,40]. The enzyme hydrolyzes the neutral phospholipids, particulary phosphatidylcholine, without affecting the acidic phospholipids. In contrast to

earlier studies with impure preparations of $(Na^+ + K^+)$-ATPase [18,28,40] the treatment with this phospholipase has practically no effect on the $(Na^+ + K^+)$-ATPase activity in our purified preparation.

The effects of sequential treatment with phospholipase C (*Cl. welchii*) and phosphatidylinositol-specific phospholipase C are shown to be additive and the slight reduction in $(Na^+ + K^+)$-ATPase activity is completely due to the treatment with phospholipase C from *Cl. welchii*. This indicates that $(Na^+ + K^+)$-ATPase can function with a phospholipid/$(Na^+ + K^+)$-ATPase molar ratio down to about 90, in which phosphatidylinositol is absent and in which only five molecules of phosphatidylcholine are present.

The experiment in which $(Na^+ + K^+)$-ATPase is treated sequentially with phospholipase C and phosphatidylserine decarboxylase shows that the latter enzyme can also completely convert phosphatidylserine, when the major part of the neutral phospholipids has been hydrolysed. Since this combined treatment has no further effect on the $(Na^+ + K^+)$-ATPase activity, it can be concluded that the $(Na^+ + K^+)$-ATPase can function with down to 100 phospholipid molecules per molecule $(Na^+ + K^+)$-ATPase and the complete absence of phosphatidylserine.

Acidic phospholipid requirement

The most interesting experiment is the sequential treatment with phosphatidylinositol-specific phospholipase and phosphatidylserine decarboxylase. The residual preparation still contains some 250 molecules of phospholipids, but the acidic phospholipids are virtually absent. This treatment is the only one which has a marked effect on the $(Na^+ + K^+)$-ATPase activity, but the residual activity is still 56% of the control activity. This suggests that the enzyme can function in the absence of any acidic phospholipids, although the activity is significantly lower than in their presence.

The latter interpretation must be qualified in one respect. During purification the enzyme preparation is extracted with sodium dodecyl sulphate. The residual amount of this detergent in the preparation after zonal centrifugation, followed by sedimentation and washing, has been determined by Jørgensen [25] to be 9 ± 4 μg/mg protein, which is 7 mol/mol ATPase. In our case the preparation has been centrifuged five additional times after the treatments with phosphatidylinositol-specific phospholipase C and phosphatidylserine decarboxylase, and thus the residual sodium dodecyl sulphate content will be less than that determined by Jørgensen. Nevertheless, we cannot exclude that a few molecules of this detergent per enzyme molecule are still present in the preparation, and that in theory they could provide sufficient negative charge for enzyme activity.

Effect on nitrophenylphosphatase activity

The potassium-activated 4-nitrophenylphosphatase activity is an inherent property of the $(Na^+ + K^+)$-ATPase enzyme system, probably representing the dephosphorylation step of the enzyme. There are indications that this activity is less phospholipid dependent than the $(Na^+ + K^+)$-ATPase activity. The discontinuity observed in the Arrhenius plot for $(Na^+ + K^+)$-ATPase, which is attributed to changes in the fluidity of the phospholipids with temperature, is

absent in the phosphatase activity [41,42]. Moreover, delipidation with Lubrol has much less effect on the potassium 4-nitrophenylphosphatase activity than on the $(Na^+ + K^+)$-ATPase activity [43]. In our delipidation studies the potassium 4-nitrophenylphosphatase activity is generally changed to the same degree as the $(Na^+ + K^+)$-ATPase activity. This indicates that the polar head groups of the phospholipids are not concerned with the difference in lipid dependence between the potassium 4-nitrophenylphosphatase activity and the $(Na^+ + K^+)$-ATPase activity.

Effects of delipidation

Our finding that phosphatidylserine by itself is not essential for $(Na^+ + K^+)$-ATPase activity apparently contradicts the finding of several investigators that only this and other negatively charged phospholipids can reactivate $(Na^+ + K^+)$-ATPase after inactivation by delipidation with detergents or phospholipases [7,10,16—24]. However, these experiments only show a specificity for the reactivation process and do not prove that acidic phospholipids interact with the $(Na^+ + K^+)$-ATPase in the native membrane. As long as we do not exactly know what is happening to the enzyme protein during delipidation, the specificity for acidic phospholipids in the reactivation process cannot satisfactorily be explained.

Duck-Chong [44] has suggested that lipid depletion leads to formation of inactive protein aggregates and that the ionic binding by negatively charged dispersions of phosphatidylserine may aid subsequent hydrophobic bond formation between proteins and lipids. The finding that cholesterol is an effective reactivating agent after delipidation with organic solvents at low temperatures [45,46], can also be explained in terms of changes in protein conformation caused by delipidation [44]. Jensen and Ottolenghi [47] have recently found that upon delipidation with deoxycholate followed by gel filtration [48] the ADP binding capacity of the $(Na^+ + K^+)$-ATPase remains unchanged but the K^+ sensitivity of the binding has disappeared, which can be interpreted as an effect on protein conformation.

After this particular delipidation with deoxycholate activation can be obtained with phosphatidylcholine [47] and sodium transport takes place in vesicles prepared from $(Na^+ + K^+)$-ATPase in which the original phospholipids have been replaced by phosphatidylcholine [49] with the substitution method according to Warren et al. [50]. This supports our finding that there is no absolute requirement for negatively charged phospholipids for the functioning of $(Na^+ + K^+)$-ATPase.

Conclusions

From these experiments the following three conclusions can be drawn concerning the phospholipid requirements of the $(Na^+ + K^+)$-ATPase system. First, the enzyme does not need more than 90 phospholipid molecules per mol $(Na^+ + K^+)$-ATPase for proper functioning. Secondly, the enzyme is maximally active, when either phosphatidylserine or phosphatidylinositol is present. Thirdly, the enzyme can function without negatively charged phospholipids present, but at somewhat lower activity than in the presence of one or

both of the acidic phospholipids. A preliminary report of part of these experiments has appeared [51].

Acknowledgments

The authors would like to thank Dr. Thompson (Wellcome Research Laboratories) and Dr. Low (Department of Biochemistry, University of Birmingham) for their generous gifts of the phospholipases used in this study.

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