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Vanadate-sensitive phosphatidate phosphohydrolase activity in a purified rabbit kidney Na,K-ATPase preparation

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Reconstitution of purified rabbit kidney Na,K-ATPase in phosphatidylcholine/phosphatidic acid liposomes resulted in the absence of ATP in a time-, temperature- and protein-dependent formation of inorganic phosphate. This formation of inorganic phosphate could be attributed to a phosphatidate phosphohydrolase activity present in the Na,K-ATPase preparation. A close interaction of the enzyme with the substrate phosphatidic acid was important, since no or little P_i production was observed under any of the following conditions: without reconstitution, after reconstitution in the absence of phosphatidic acid, with low concentrations of detergent or at low lipid/protein ratios. The hydrolysis of phosphatidic acid was not influenced by the Na,K-ATPase inhibitor ouabain but was completely inhibited by the P-type ATPase inhibitor vanadate. Besides P_i diacylglycerol was also formed, confirming that a phosphatidate hydrolase activity was involved. Since the phosphatidate phosphohydrolase activity was rather heat- and *N*-ethylmaleimide-insensitive, we conclude that the phosphatidic acid hydrolysis was not due to Na,K-ATPase itself but to a membrane-bound phosphatidate phosphohydrolase, present as an impurity in the purified rabbit kidney Na,K-ATPase preparations.

Introduction

The sodium pump enzyme Na,K-ATPase is an integral membrane protein complex consisting of a catalytic α-subunit (114 kDa) and a non-catalytic β-subunit (35 kDa) [1] present in a 1:1 ratio. The natural environment of the protein complex is a phospholipid bilayer, the presence of which is essential for the functioning of the enzyme [2]. There has been a long-lasting dispute whether there is any phospholipid headgroup specificity for the function of Na,K-ATPase [2,3].

One possible approach to solve this controversy is by reconstitution of purified Na,K-ATPase with defined phospholipids and to measure partial and complete reactions of this enzyme. We therefore purified Na,K-ATPase from rabbit kidney outer medulla according to Jørgensen [4]. In this method a membrane preparation containing high enzyme activity is extracted with a low sodium dodecyl sulphate concentration, whereupon the residual membranes are purified by sucrose gradient centrifugation. Although this preparation has been used in very many studies, up to now all enzymatic activity measured in this preparation, with exception of a minor ouabain-insensitive Mg-ATPase activity, has been attributed to Na,K-ATPase.

In experiments with purified Na,K-ATPase, reconstituted in proteoliposomes with different composition, we observed that upon incubation of phosphatidic acid containing proteoliposomes inorganic phosphate was formed despite the absence of added ATP. In the absence of phosphatidic acid no inorganic phosphate was formed. The latter process was temperature dependent and was inhibited by vanadate.

In this paper we present evidence that the P_i formation is due to a phosphatidate phosphohydrolase activity, present in the enzyme preparation, which is distinct from Na,K-ATPase itself. This finding has consequences both for studies on the lipid dependency of Na,K-ATPase and on phosphatidate metabolism.

Materials and Methods

Enzyme preparation

Na,K-ATPase was purified from rabbit kidney outer medulla according to Jørgensen [4]. The SDS treated microsomal fraction (300 ml, 423 mg protein) was layered on a discontinuous sucrose gradient of 15% (150

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Abbreviation: P_i, inorganic phosphate.

ml), 20% (250 ml), 25% (150 ml), 30% (500 ml) and 50% (300 ml). After centrifugation overnight in a zonal rotor at 40000 × g the enzyme fractions with a density above 1.07 g/l were collected. After dilution (two times) with buffer and centrifugation at 100000 × g for 1 h a pellet was collected. The preparations were stored at −20°C in 0.25 M sucrose, 50 mM imidazole-acetate (pH 7.0). Protein was determined by the method of Lowry et al. [5] using bovine serum albumin as standard.

Preparation of liposomes

Liposomes were prepared by the reversed phase evaporation method as described by Szoka and Papahadjopoulos [6]. Mixtures of cholesterol, phosphatidylcholine and phosphatidic acid in chloroform were evaporated under a stream of nitrogen. After repeated washing with diethyl ether, a 1:1 mixture of diethyl ether and vesicle medium (1 mM EDTA, 50 mM imidazole-acetate (pH 7.0)) was added and the solution thoroughly mixed on a vortex mixer, while the diethyl ether was slowly evaporated in a stream of nitrogen. The liposomes then formed were sonicated for 30 min at 4°C in a Branson sonicator bath at maximal output. Generally the liposomes contained 40 mg phosphatidylcholine, 10 mg phosphatidic acid and 2 mg cholesterol per ml.

Reconstitution

Proteoliposomes were prepared as described by Van der Hijden et al. [7] with some slight modifications. Na,K-ATPase preparations (4 mg/ml) in 1 mM EDTA, 50 mM imidazole-acetate (pH 7.0) were partially solubilized by incubation with 1 or 2% cholate for 1 min at room temperature. A 9-fold volume of the liposomes was added, thoroughly mixed, frozen in liquid nitrogen and subsequently thawed at room temperature. The freeze and thaw procedure was repeated twice. The vesicle suspension was sonicated for 6 min at 0°C. In our experiments the cholate was not removed with the purpose to keep the vesicles permeable.

Phosphatidate phosphohydrolase activity

This activity was determined as the release of P_i from phosphatidic acid. To 50 μl proteoliposomes, containing 12.5–25 μg protein, 2.5 mg phosphatidylcholine/phosphatidic acid (4:1) and 0.1 mg cholesterol, 50 μl vesicle medium (1 mM EDTA in 50 mM imidazole-acetate (pH 7.0)) was added and this suspension was incubated for 60 min at 37°C. The hydrolysis was stopped by adding 2 ml chloroform. After thoroughly mixing 0.9 ml 3 M NaCl was added and the mixing was repeated. Blanks were prepared by adding the chloroform at time zero. The organic phase and the water phase were separated by centrifugation for 20 min at 1500 × g. The amount of inorganic phosphate in the upper phase was determined as the malachite green phosphomolybdate complex [8]. To 0.2 ml sample 1.0 ml 0.35% (w/v) ammonium heptamolybdate in 0.63 M H_2SO_4 was added and mixed. After 10 min at room temperature 0.2 ml 0.035% (w/v) malachite green in 0.35% (w/v) polyvinyl alcohol was added and mixed again. The absorbance at 610 nm was determined after 30 min at room temperature. Standards (0–20 nmol P_i) were also included.

Diacylglycerol detection

The organic phase (obtained during the phosphatidate phosphohydrolase test, see previous section) was evaporated in a stream of nitrogen dissolved in 50 μl dichloromethane and 20 μl was applied to thin-layer plates (type HPTLC silica gel 60). The chromatogram was developed with hexane/diethyl ether/formic acid in a ratio of 50:150:4 (v/v). The lipids were localized by staining with iodine vapour.

ATP phosphorylation

Proteoliposomes (4–10 μg protein in 20 μl) were preincubated for 5 min at 22°C with 20 μl 1% (w/v) cholate and 10 mM $MgCl_2$ in vesicle medium, in order to make all ATP-sites accessible for phosphorylation. The phosphorylation capacity of Na,K-ATPase was determined by adding 40 μl phosphorylation medium (200 μM [γ-^{32}P]ATP, specific activity 0.05 Ci/mmol (Radiochemical Centre Amersham, Amersham, UK), 50 mM imidazole-acetate (pH 7.0) and 200 mM NaCl. The high nucleotide concentration was required to overcome phosphorylation by P_i as released from phosphatidic acid. After 5 s the reaction was stopped by adding 5 ml 5% (w/v) trichloroacetic acid in 0.1 M phosphoric acid and filtered over a Schleicher & Schüll (Dassel, Germany) filter (type AE95, 1.2 μm). After washing four times with 5 ml trichloroacetic acid, the filters were analyzed for their ^{32}P-protein content. Blanks were prepared by denaturing the enzyme before the incubation with the stopping solution [9].

Na,K-ATPase and Mg-ATPase assay

To 0.4 ml medium, which contained either 110 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 0.1 mM EDTA and 30 mM imidazole-HCl, pH 7.4 (medium A) or 110 mM NaCl, 5 mM $MgCl_2$, 5 mM ATP, 0.1 mM ouabain and 30 mM imidazole-HCl, pH 7.4 (medium B), 0.5–3 μg Na,K-ATPase was added. After 30 min incubation at 37°C or at 0°C, 1.5 ml 8.6% (w/v) trichloroacetic acid and 1.5 ml ammonium heptamolybdate (1.15%, w/v) in 0.66 M sulphuric acid with 9.2% (w/v) $FeSO_4 \cdot 7H_2O$ was added. The blue phosphomolybdate complex was analyzed after 30 min at 700 nm and the Na,K-ATPase activity calculated as the ouabain sensitive ATPase activity (difference in medium A and B). The Mg-ATPase activity was calcu-

lated as the difference between the ATPase activity at 37°C and 0°C in medium B.

Heat inactivation

Na,K-ATPase preparations (5 mg/ml, in 50 mM imidazole-acetate, 1 mM EDTA (pH 7.0)) were incubated for the indicated time intervals at either 55°C or 100°C and cooled to 0°C.

N-Ethylmaleimide treatment

Na,K-ATPase (6.25 mg/ml) was incubated at 37°C with 0–10 mM *N*-ethylmalcimide in 50 mM imidazole-acetate, 1 mM EDTA (pH 7.0) [10]. After 30 min the treatment was stopped by adding dithioerythritol (final concentration 30 mM).

Materials

Both phospholipids (phosphatidylcholine 840051 and phosphatidic acid 840101) used in this study were from Avanti Polar Lipids, Birmingham AL, USA. Phosphatidylcholine was purified from eggs and was used for preparation of phosphatidic acid by means of a phospholipase D. Both phospholipids had thus the same fatty acid composition. Cholesterol was purchased from Sigma, St. Louis, MO, USA and cholate, from Matheson Coleman & Bell, Norwood, OH, USA. The latter compound was recrystallised in 50% ethanol, following destaining with charcoal and dissolved as a Tris salt (pH 7.0). *N*-Ethylmaleimide and the HPTLC silica gel 60 plates were obtained from Merck, Darmstadt, Germany. Propranolol and sphingosine were from Sigma, St Louis, MO, USA. All other chemicals were of analytical grade.

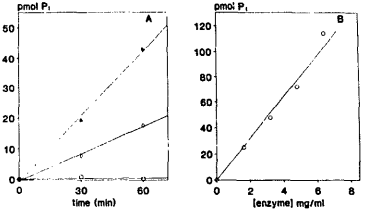

Fig. 1. Effect of the incubation time, temperature (A) and the protein concentration (B) on the amount of P_i formed from phosphatidic acid and phosphatidylcholine Na,K-ATPase proteoliposomes. (A) Proteoliposomes (22 μg Na,K-ATPase and 2.22 mg phosphatidylcholine/phosphatidic acid; ratio 4:1) were incubated in 0.1 ml of 1 mM EDTA and 50 mM imidazole-acetate, pH 7.0 at 0°C (○——○), 22°C (◇——◇) or 37°C (•——•) for the indicated times. The amount of P_i formed is plotted vs. time. (B) Na,K-ATPase, in the indicated concentrations, was reconstituted in phosphatidylcholine/phosphatidic acid liposomes (50 mg/ml) containing 1 mM EDTA and 50 mM imidazole-acetate (pH 7.0). The 21-times diluted samples were incubated for 60 min at 37°C and the P_i formation in 0.1 ml was determined.

Results

P_i formation in phosphatidylcholine / phosphatidic acid proteoliposomes

Incubation of proteoliposomes containing Na,K-ATPase and the phospholipids phosphatidylcholine and phosphatidic acid (ratio of 4:1) in a 100-fold excess of phospholipids on weight basis resulted in a time and temperature dependent formation of P_i (Fig. 1A). The

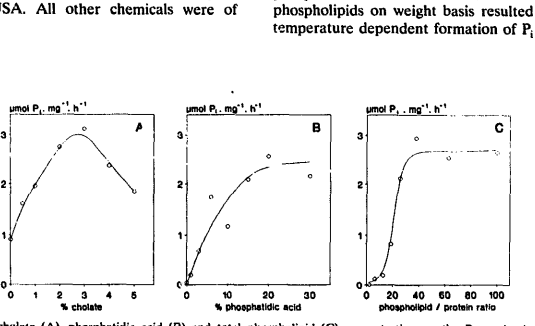

Fig. 2. The effect of cholate (A), phosphatidic acid (B) and total phospholipid (C) concentration on the P_i production in phosphatidylcholine/phosphatidic acid Na,K-ATPase proteoliposomes. (A) Proteoliposomes as described in Materials and Methods were prepared using cholate concentrations as indicated. Subsequently they were incubated in 1 mM EDTA and 50 mM imidazole-acetate (pH 7.0). (B) Proteoliposomes as described in Materials and Methods, but using variable amounts phosphatidic acid (indicated as percentage of total phospholipids), were incubated in 1 mM EDTA and 50 mM imidazole-acetate (pH 7.0), for 60 min at 37°C. (C) Proteoliposomes as described in Materials and Methods, but with variable phospholipid/protein ratios, were incubated in 5 mM $MgCl_2$, 1 mM EDTA and 50 mM imidazole-acetate (pH 7.0) for 60 min at 37°C. In all experiments the amount of P_i formed by the proteoliposomes during 60 min incubation at 37°C was determined.

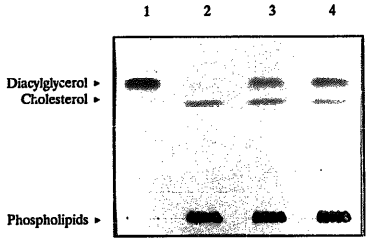

Fig. 3. Thin-layer chromatogram of the lipid extract. Phosphatidylcholine/phosphatidic acid proteoliposomes (containing Na,K-ATPase 0.24 mg/ml, phosphatidylcholine 40 mg/ml, phosphatidic acid 10 mg/ml and cholesterol 2 mg/ml) in 50 mM imidazole acetate, 5 mM $MgCl_2$ and 1 mM EDTA (pH 7.0) were incubated for 0 (lane 2), 1 (lane 3) and 2 h (lane 4) at 37°C. After extraction, part of the organic fraction (see Materials and Methods) was chromatographed on silica gel plates with hexane/diethyl ether/formic acid (50:150:4, by vol.) and stained with iodine vapour. Lane 1 contained only diacylglycerol ($R_f = 0.55$). Cholesterol and the phospholipids originated from the proteoliposomes.

rate of P_i formation was linear with the Na,K-ATPase concentration used (Fig. 1B). Heat-inactivation (5 min at 100°C) of the Na,K-ATPase preparation before reconstitution precluded P_i formation. When phosphatidylcholine/phosphatidic acid liposomes were sonicated and added to a membrane suspension of Na,K-ATPase or when sucrose (100 mM) was present during the reconstitution procedure no P_i formation occurred indicating that a close interaction of the enzyme preparation with phosphatidic acid is important.

In the reconstitution procedure Na,K-ATPase is generally treated with 1% cholate, whereupon a freeze-thaw sonication step is applied [7]. Fig. 2A shows the effect of the cholate concentration during the reconstitution step. Maximal P_i formation was found with 2–3% cholate. Upon varying the phosphatidic acid concentration in the proteoliposomes, keeping the lipid/protein ratio at 100:1, a dose dependent P_i formation was observed, the maximal activity being reached between 15 and 30% phosphatidic acid (Fig. 2B). When during reconstitution, at a constant phosphatidylcholine/phosphatidic acid ratio of 4:1, the lipid/protein ratio was decreased from 100:1 to 25:1 the rate of P_i formation did not decrease (Fig. 2C). At lower lipid/protein ratios the activity decreased resulting in a negligible activity below a ratio of 10:1. In proteoliposomes containing only phosphatidylcholine no P_i or any water soluble organic phosphate compound was formed. This indicates that no phospholipase activity was involved in P_i formation.

Phosphatidate phosphohydrolase activity

To confirm the existence of a phosphatidate phosphohydrolase activity we also measured the formation of diacylglycerol. Fig. 3 shows a iodine stained thin-layer chromatogram of the lipids present in the medium before and after incubating the phosphatidylcholine/phosphatidic acid proteoliposomes at 37°C. This experiment clearly shows that after 1 and 2 h (lane 3 and 4) diacylglycerol was formed. There was no indication for a further hydrolysis of diacylglycerol to monoacylglycerol and free fatty acids.

Comparison of Na,K-ATPase and phosphatidate phosphohydrolase

The above mentioned experiments indicate that there is a phosphatidate phosphohydrolase activity present in the Na,K-ATPase preparation. To test whether this phosphohydrolase activity was either due to Na,K-ATPase itself or a separate enzyme present we treated the Na,K-ATPase preparation by various physical and chemical means in order to inactivate one of either activities and to keep the other enzyme activity intact. We measured the steady-state phosphorylation level in the presence of Na^+, Mg^{2+} and ATP for 5 s, as parameter for the activity of Na,K-ATPase. Since Na,K-ATPase can also be phosphorylated by P_i, which process inhibits and excludes ATP from phosphorylation [9], high Na^+ (100 mM) and ATP (100 μM) concentrations were used in order to suppress P_i phosphorylation. Ouabain, the specific inhibitor of Na,K-

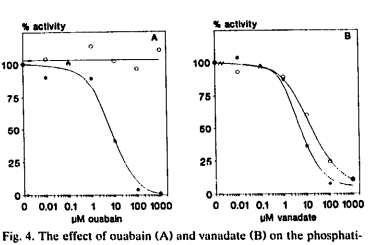

Fig. 4. The effect of ouabain (A) and vanadate (B) on the phosphatidate phosphohydrolase activity and the ATP phosphorylation level of phosphatidylcholine/phosphatidic acid Na,K-ATPase proteoliposomes. Proteoliposomes as described in Materials and Methods were incubated in 1 mM EDTA, 5 mM $MgCl_2$ and 50 mM imidazole-acetate (pH 7.0) for 60 min at 37°C (phosphatidate phosphohydrolase activity, ○——○) or preincubated for 5 min at 22°C in the presence of 0.5% cholate (ATP phosphorylation, •——•) with the indicated ouabain (A) or vanadate (B) concentrations. During the ATP phosphorylation assay (5 s) the concentrations of ouabain or vanadate were kept constant. The 100% value for phosphatidate phosphohydrolase activity corresponds to 3.6 μmol P_i per mg per h and for the ATP phosphorylation capacity to 1.0 nmol P per mg protein.

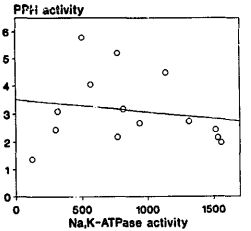

Fig. 5. Correlation between Na,K-ATPase activity and phosphatidate phosphohydrolase activity in various enzyme preparations. The Na,K-ATPase activity (in μmol ATP hydrolysed per mg protein per h) was determined in the original Na,K-ATPase preparations, whereas the phosphatidate phosphohydrolase (PPH) activity (in μmol P_i formed per mg protein per h) was determined after reconstitution in phosphatidylcholine/phosphatidic acid liposomes, see Materials and Methods. The values of 14 different Na,K-ATPase preparations are plotted and from these data the correlation ($r = -0.17$, approximate 95% confidence interval, calculated according to Ref. 12: −0.64, 0.40) was calculated by linear regression analysis.

ATPase, did not inhibit the phosphatidate phosphohydrolase activity (Fig. 4A), but inhibited the ATP phosphorylation of these proteoliposomes with an I_{50} value of 7 μM. The absence of an effect of ouabain suggests that the phosphatidate phosphohydrolase activity is not due to Na,K-ATPase itself. Vanadate, an inhibitor of P-type ATPases, probably because of its phosphate like configuration [11], decreased the ATP phosphorylation level with an I_{50} of 7 μM (Fig. 4B). The phosphatidate phosphohydrolase activity was also inhibited by vanadate with a similar I_{50} value of 15 μM. These apparently contradictory results led us to further investigations. If the phosphatidate phosphohydrolase activity were an inherent property of the Na,K-ATPase one would expect a positive correlation between both activities in enzyme preparations of different purity. Both the Na,K-ATPase activity and the phosphatidate phosphohydrolase activity were therefore measured in a large series ($n = 14$) of Na,K-ATPase preparations. Fig. 5 shows that there is no significant correlation ($r = -0.17$).

We also traced the phosphatidate phosphohydrolase activity during the Na,K-ATPase purification procedure. In the microsomal fraction the phosphatidate phosphohydrolase activity reached about the same level (2–3 μmol P_i per mg protein per h at 37°C) as in our purified Na,K-ATPase preparations. After separation of the SDS-treated microsomes on a sucrose gradient the fractions with densities from 1.07 up to 1.18 g/ml were analyzed for their specific Na,K-ATPase, Mg-ATPase and phosphatidate phosphohydrolase activities. Fig. 6B shows that the phosphatidate phosphohydrolase activity, in contrast to Na,K-ATPase and Mg-ATPase activity, is present throughout the gradient. The highest specific phosphatidate phosphohydrolase activity (13.5 μmol P_i per mg protein per h) was observed in the membrane fraction with a density of 1.09 g/ml (fraction 6), whereas the highest Na,K-ATPase activity (773 μmol P_i per mg protein per h) was found at a density of 1.16 g/ml (fraction 21). The Mg-ATPase, also maximal at $d = 1.09$ g/ml, decreased nearly to zero at $d = 1.16$ g/ml, whereas the specific phosphatidate phosphohydrolase activity at this density was still 40% of its maximal activity. Analysis of these fractions by means of SDS-PAGE did not show any increase or decrease of a specific protein other than the two subunits of Na,K-ATPase (data not shown).

A number of putative inhibitors of phosphatidate phosphohydrolase activity were also tested (Table I). No effects of propranolol (1 mM), and sphingosine-

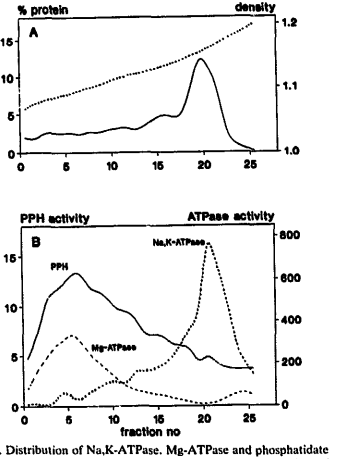

Fig. 6. Distribution of Na,K-ATPase, Mg-ATPase and phosphatidate phosphohydrolase activities after zonal gradient centrifugation of SDS treated rabbit kidney microsomes. After centrifugation 40-ml fractions of density above 1.07 g/l were diluted two times and the 100000 × g pellet was collected. (A) Percentage distribution of the collected protein (———) and the density (g/ml) profile (·····). (B) Distribution of the specific phosphatidate phosphohydrolase (PPH) (———), Mg-ATPase (— — —) and Na,K-ATPase (·····) activities. The phosphatidate phosphohydrolase activity was determined after reconstitution, whereas the Na,K-ATPase and Mg-ATPase activities were determined on the original fractions. The specific activities are given in μmol P_i per mg protein per h.

TABLE I

Effect of various substances on the phosphatidate phosphohydrolase activity

Na,K-ATPase proteoliposomes prepared in 50 mM imidazole-acetate (pH 7.0) as described in Materials and Methods were incubated at 37°C for 1 h in the presence of the substances as indicated. The phosphatidate phosphohydrolase activity (mean values ± S.D.) of four different Na,K-ATPase preparations is given in μmol P_i per mg protein per h.

	Phosphatidate phosphohydrolase activity	
	μmol P_i per mg protein per h	(%)
Control	2.11 ± 0.22	100
+ 1 mM EDTA	2.33 ± 0.29	111
+ 1 mM propranolol	2.33 ± 0.27	111
+ 1 mM sphingosine	2.30 ± 0.30	109
+ 10 mM $MgCl_2$	2.40 ± 0.42	113
+ 10 mM $CaCl_2$	1.20 ± 0.17	57
+ 100 mM NaCl	2.76 ± 0.27	131
+ 100 mM sucrose	1.83 ± 0.19	87

(1 mM) on the activity was found, despite the fact that these compounds inhibit the membrane bound phosphatidate phosphohydrolase activity from rat liver [13]. Ca^{2+} decreased the phosphatidate phosphohydrolase activity while Mg^{2+} had no effect. The latter finding explains why the phosphatidate phosphohydrolase activity in Fig. 2 (no Mg^{2+}) and Fig. 4 (Mg^{2+} present)

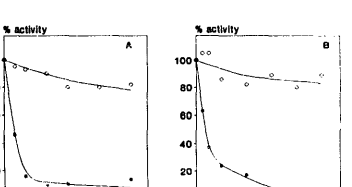

Fig. 7. The effect of heat-inactivation (A) or *N*-ethylmaleimide treatment (B) on the phosphatidate phosphohydrolase activity and the ATP phosphorylation capacity of phosphatidylcholine/phosphatidic acid Na,K-ATPase proteoliposomes. (A) Na,K-ATPase preparations (5 mg/ml, in 50 mM imidazole-acetate, 1 mM EDTA, (pH 7.0)) were incubated at 55°C for the indicated time intervals and reconstituted in phosphatidylcholine/phosphatidic acid liposomes as described in Materials and Methods. (B) Na,K-ATPase (6.25 mg/ml) was incubated at 37°C with 0–10 mM *N*-ethylmaleimide (NEM) in 50 mM imidazole acetate, 1 mM EDTA (pH 7.0). After 30 min the treatment was stopped by adding dithioerythritol (final concentration 30 mM). The enzyme was reconstituted in phosphatidylcholine/phosphatidic acid liposomes as described in Materials and Methods. The 100% value for phosphatidate phosphohydrolase activity (○——○) corresponds to 4.0 (A) and 3.9 (B) μmol P_i per mg per h and for the ATP phosphorylation capacity (•——•) to 1.3 (A) and 1.8 (B) nmol P per mg protein.

were about equal. There was a small increase in the phosphatidate phosphohydrolase activity in the presence of NaCl, whereas EDTA and sucrose, unless used during reconstitution (see above), had no effect.

In lung and liver a membrane bound phosphatidate phosphohydrolase activity has been described, which is relatively heat-stable and insensitive towards *N*-ethylmaleimide [13,14]. We, therefore, preincubated the Na,K-ATPase preparation for various times at 55°C (Fig. 7A) or pretreated the enzyme with varying concentrations of *N*-ethylmaleimide for 30 min at 37°C (Fig. 7B). Subsequently the enzyme was reconstituted in phosphatidylcholine/phosphatidic acid liposomes and both the P_i formation from phosphatidic acid and the steady-state ATP phosphorylation level (E-P) were measured. The E-P level was decreased under each condition, whereas the phosphatidate phosphohydrolase activity was only slightly inhibited. These experiments give the final proof that the two activities originate from distinct enzymes.

Discussion

The findings reported in this paper indicate that a phosphatidate phosphohydrolase activity is present in a purified Na,K-ATPase preparation from rabbit kidney outer medulla and that the activity has to be attributed to an enzyme which is distinct from Na,K-ATPase. The theoretical possibility that phosphatidate is an ATP analogue and so a substrate for Na,K-ATPase has been excluded, despite the fact that both enzyme activities are inhibited by vanadate. The lack of effect of ouabain on the phosphatidate phosphohydrolase activity and the different sensitivities of the two activities towards heat and *N*-ethylmaleimide are the main objections against this possibility.

The Na,K-ATPase preparation used in the present study is purified according to Jørgensen [4]. This preparation has a maximal Na,K-ATPase activity around 2000 μmol per mg per h, but this very high activity is only seldom reached. Since the preparations used in this study have generally a somewhat lower activity, although they appear to be pure on basis of SDS gel electrophoresis, one could argue that the presence of phosphatidate phosphohydrolase activity is due to the use of these preparations. We find it very likely that the phosphatidate phosphohydrolase activity would also be present in the most active preparations of Na,K-ATPase, prepared according to this method, since (i) there is no positive correlation between the Na,K-ATPase and phosphatidate phosphohydrolase activities over a wide range of Na,K-ATPase activities (Fig. 5) and (ii) the pattern of the activity over the sucrose gradient (Fig. 6) does not give any indication for the possibility to remove phosphatidate phosphohydrolase activity completely from Na,K-ATPase activity.

The enzyme phosphatidate phosphohydrolase plays a key role in lipid metabolism. It is involved in the synthesis of triacylglycerols, phosphatidylcholine and phosphatidylethanolamine [15]. The enzyme has been reported to be present both in the cytosol and in the endoplasmic reticulum. In addition a phosphatidate phosphohydrolase activity has recently been reported to be present in rat liver plasma membranes [13]. The latter enzyme, which might play a role in signal transduction processes, is in contrast to the enzyme from the cytosol and the endoplasmic reticulum relatively stable to heat, is insensitive towards *N*-ethylmaleimide and not stimulated by Mg^{2+}. These properties were also found in the kidney enzyme described in the present study. The kidney enzyme, however, was in contrast to the liver counterpart not inhibited by either sphingosine or propranolol. Whether this is due to species or tissue differences or to different enzyme assay conditions (much higher phosphatidate concentration in our study) is not yet known.

The phosphatidate phosphohydrolase activity from rabbit kidney is apparently not very sensitive towards SDS used in the purification procedure of Na,K-ATPase to extract loosely bound proteins. The activity is neither very sensitive towards cholate: it is even activated and is optimally active at 2–3% cholate. This is in line with the properties of the rat liver plasma membrane enzyme which is stimulated by 0.5% Triton X-100 and still active at a 5% concentration of this detergent [14].

Vanadate has been described as an inhibitor of a number of enzymes involved in phosphorylation and dephosphorylation reactions such as Na,K-ATPase [11], alkaline phosphatase [16], acid phosphatase [17], phosphofructokinase [18], adenylate kinase [19] and phosphotyrosyl-protein phosphatase [20]. In the present study we show that it also inhibits the phosphatidate phosphohydrolase activity at about the same concentration. The inhibitory effect of vanadate on P-type ATPases has been attributed to structural similarities between vanadate and phosphate, explaining that it prevents phosphorylation of these enzymes [11]. This suggests that vanadate also interacts with the site where the phosphatidate phosphohydrolase interacts with the phosphate residue of phosphatidic acid.

The presence of phosphatidate phosphohydrolase activity in purified Na,K-ATPase from rabbit kidney has no consequences for most studies with the latter preparation. Phospholipid analysis of this Na,K-ATPase preparation has not shown any detectable amount of phosphatidic acid [2]. It is even likely that any phosphatidic acid formed in kidney membranes is hydrolysed by the phosphatidate phosphohydrolase activity. Only in studies with reconstituted enzyme using phosphatidic acid the phosphohydrolase activity might have an effect. One has either to avoid this phospholipid in reconstitution studies with Na,K-ATPase or to take into account that during incubation P_i may become formed. Since P_i has effects on the properties of Na,K-ATPase (phosphorylation, ouabain binding) knowledge of this phenomenon is relevant.

Vanadate is often used to inhibit P-type ATPase in either permeabilized cells or membrane preparations. The fact that this compound inhibits phosphatidate phosphohydrolase activity might have an effect on the phospholipid composition of the preparation during such experiments and could theoretically lead to an accumulation of phosphatidic acid. Whether this is indeed the case has to be at least taken into mind in carrying out experiments with this inhibitor.

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