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THE EFFECT OF PHOSPHOLIPIDS ON THE APPARENT ACTIVATION ENERGY OF (Na^+–K^+)-ATPase

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SUMMARY

The Arrhenius plots of ATPase activities by (Na^+–K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) preparation treated with phospholipase A (phosphatide acylhydrolase, EC 3.1.1.4) gave a single straight line and the apparent activation energy was approximately 30 kcal/mole, while the plots of the control preparation gave two straight lines intersecting at about 20 °C with apparent activation energies of approximately 15 and 30 kcal/mole above and below 20 °C, respectively. Both of the Arrhenius plots of ATPase activities in the presence of both phosphatidylserine and phosphatidylinositol by phospholipase A-treated ATPase and control ATPase gave two straight lines intersecting at about 20 °C. Those apparent activation energies were 13–14 and 31 kcal/mole above and below 20 °C, respectively. The experiments suggested that the phospholipid molecules were prerequisite for the abrupt change in the activation energy at about 20 °C.

INTRODUCTION

When an (Na^+–K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) preparation from ox brain microsome was treated with phospholipase A (phosphatide acylhydrolase, EC 3.1.1.4), its ATPase activity was reduced to 20–25 % of the control value; ATPase activity was reactivated to nearly the control value by additions of both phosphatidylinositol and phosphatidylserine[1]. On the basis of both studies of phospholipid dependency on the partial reaction of (Na^+–K^+)-ATPase[1] and on the binding of ouabain to the enzyme[2], we have proposed that the effect of phospholipids on (Na^+–K^+)-ATPase is to create a negative charge and hydrophobicity in the vicinity of the active site of ATPase rather than to act as components of the active site itself[1,2].

The effect of temperature on (Na^+–K^+)-ATPase activity has been studied by many workers[3–8]. Post *et al.*[3] reported that the apparent activation energy value compared favourably with the values for Na^+ efflux and K^+ influx in human erythrocytes (at relatively high temperatures). Gruener *et al.*[4] and Kanazawa *et al.*[6] reported that the Arrhenius plots of (Na^+–K^+)-ATPase activity consisted of three and two intersecting straight lines. Quite recently Charnock *et al.*[9–11] made a precise study on the temperature dependency of (Na^+–K^+)-ATPase activity and reported that the point of inflection or the transition temperature was about 20 °C. Takenaka[12] previ-

ously observed that the temperature coefficient of Na^+ transport across frog skin changed at about 14 °C. But the reason for those transitions has not been clarified as yet.

In this report, on the basis of the measurements of the temperature dependency of the (Na^+–K^+)-ATPase activities in a phospholipase A-treated preparation, the authors have suggested that phospholipid molecules are prerequisite for the transition.

METHODS

The method of partial purification of (Na^+–K^+)-ATPase from ox brain microsome was reported previously[1]. The ATPase preparation was treated with phospholipase A from *Naja naja* venom according to the methods of Imai and Sato[13] in an incubation mixture containing 1 mg/ml ATPase protein, 0.1 mg/ml phospholipase A, 14.7 mg/ml bovine serum albumin, 1.1 M sucrose, 29.5 mM Tris, 67.5 mM 2-mercaptoethanol, 0.07 mM EDTA and 11.2 mM $CaCl_2$ (pH 7.4) at 37 °C for 12 min. The treatment was terminated by addition of glycoletherdiaminetetraacetic acid–Tris–HCl buffer (pH 7.4) at a final concentration of 28.6 mM glycoletherdiaminetetraacetic acid and 190 mM Tris. The control was treated without $CaCl_2$. Both samples were diluted with an 8-fold volume of cold distilled water and centrifuged at 77 477 × *g* for 3 h at 1 °C. The precipitates were suspended in 0.75 M sucrose and 0.5 mM EDTA–Tris (pH 7.4) to give 2 mg/ml of protein and were stored at −20 °C in small aliquots. (Na^+–K^+)-ATPase activities were approximately 13 and 60 μmoles ADP per mg protein per h under the conditions described below for the phospholipase A-treated and control preparations, respectively (37 °C). Other details, except for the measurement of ATPase activity, were similar to our previous report[1].

ATPase activity was measured by the method of difference spectrum and oxidation of NADH in a Hitachi 124 double-beam spectrophotometer equipped with a constant temperature cell holder, at a wavelength of 340 nm, employing a linked enzyme system. The spectrophotometer used had a stability of 0.002 absorbance/h. The advantages of this assay procedure are that ATP hydrolysis can be monitored continuously and an inhibitory buildup of ADP is prevented. Both sample and reference cells contained 5 mM $MgCl_2$, 140 mM NaCl, 14 mM KCl, 20 mM Tris–HCl, 150 mM sucrose, 0.1 mM EDTA, 3 mM ATP, 1.1 mM phosphoenolpyruvate, 0.3 mM NADH, 0.05 mg (7.5 I.U.)/ml pyruvate kinase (EC 2.7.1.40), 0.0025 mg (9 I.U.)/ml lactic dehydrogenase (EC 1.1.1.27) and ATPase protein and phospholipids as indicated in the figures and in the absence and presence of 0.17 mM ouabain in a final volume of 1.55 ml (pH 7.4), respectively. Care was taken to ensure that adequate substrate (MgATP) was provided during the assay so that the observed rate of ADP liberation was effectively the maximum velocity of the reaction. Pyruvate kinase and lactic dehydrogenase activities were always maintained in at least 1000-fold excess over ATPase activity. The ATPase reaction was started by addition of ATP at 0 °C. The reference cell containing ouabain was incubated at 37 °C for 2 min to enable ouabain to bind to the ATPase. After both cells were set in a cell holder, approximately 10 min was required for equilibration to the indicated temperatures. The temperatures were controlled to ± 0.1 °C with a Coolnics circulator.

Pyruvate kinase from rabbit muscle and lactic dehydrogenase from pig muscle were obtained from Boehringer Mannheim, Tokyo, Japan. Phosphatidylserine from

ox brain and phosphatidylinositol from plant were obtained from Applied Science Laboratories, Pa., U.S.A. The other reagents were of reagents grade.

RESULTS

NADH was oxidized linearly with time until the concentration of NADH in the reaction mixture became approximately 0.02 mM and NADH oxidations were directly proportional to the amount of ATPase added in the range of 1–50 μg ATPase protein per ml at various temperatures (8.4–40 °C). All experiments described below were performed with those ranges of protein concentrations and temperatures. A typical experiment was shown in Fig. 1. NADH oxidations were measured in the presence of 5.65 and 34.5 μg ATPase protein per ml at 8.4 °C. NADH oxidations were linear with time for over 1 h (data not cited here) and were directly proportional to the amount of ATPase added. From these slopes (Na^+–K^+)-dependent ouabain-sensitive ATPase activities were calculated to be approximately 1.26 μmoles ADP liberated per mg protein per h at 8.4 °C.

(Na^+–K^+)-dependent, ouabain-sensitive ATPase activities were measured at various temperatures as described above and Arrhenius plots were constructed from the experiments. The Arrhenius plots of ATPase activities by phospholipase A-treated ATPase preparation gave a single straight line for all temperatures examined as shown

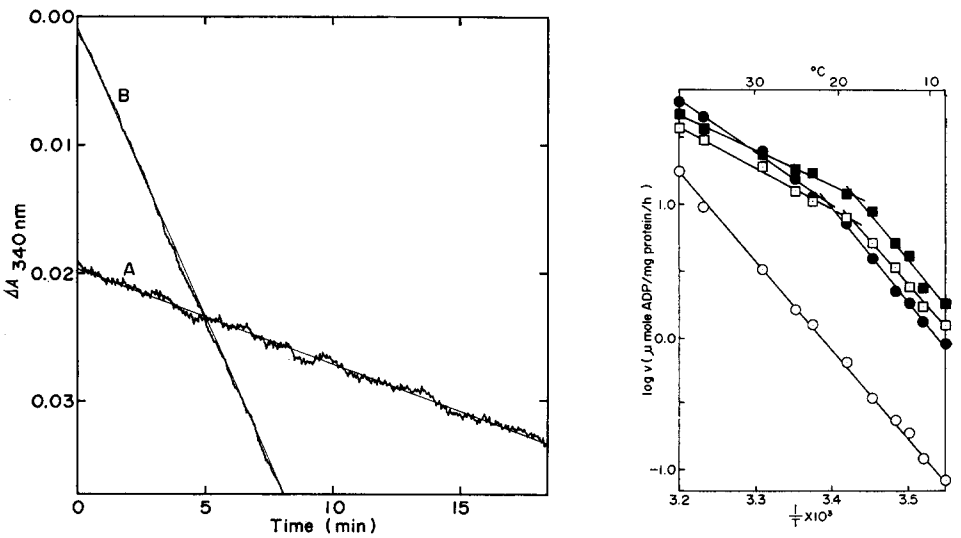

Fig. 1. Measurement of (Na^+–K^+)-ATPase activity at 8.4 °C by a linked enzyme system. ATPase activities of the control enzyme preparation were measured in 5.65 and 34.5 μg protein per ml. Other details are described in the text. A, 5.65 μg protein per ml; B, 34.5 μg protein per ml.

Fig. 2. Arrhenius plots of log maximum velocity against 1/(absolute temperature). ATPase preparation (1 mg/ml) was preincubated with 0.375 M sucrose, 0.5 mM EDTA and 20 mM Tris–HCl (pH 7.4) only or in the presence of 0.75 mg/ml phosphatidylinositol or 2.75 mg/ml phosphatidylserine, or both, for 3 min at 37 °C. Aliquots of the incubation mixtures were withdrawn and ATPase activities were measured in 14.2 and 3.55 μg protein per ml at temperatures below and above 28 °C, respectively. Other details are described in the text. ○—○, phospholipase A-treated; ●—●, control; □—□, phospholipase A-treated + phosphatidylserine and phosphatidylinositol; ■—■, control + phosphatidylserine and phosphatidylinositol.

in Fig. 2, while the plots of the control preparation gave two straight lines intersecting at about 20 °C, as reported by several workers[4,6,10,11]. Both of the Arrhenius plots of ATPase activities in the presence of phospholipids by phospholipase A-treated ATPase and the control ATPase gave two straight lines intersecting at about 20 °C.

The apparent activation energies obtained from these plots were given in Table I. The apparent activation energy of the activity by the phospholipase A-treated enzyme was approximately 30 kcal/mole for all temperatures examined. But the activation energies of the activities in the presence of both phosphatidylinositol and phosphatidylserine were approximately 14 and 28 kcal/mole at temperatures over and under 20 °C, respectively, and similar values for the apparent activation energies were obtained from the ATPase activities in the presence of either phospholipid.

TABLE I

THE ACTIVATION ENERGIES OBTAINED FROM THE ARRHENIUS PLOTS

All values of E are mean kcal/mole ± S.E. obtained from five observations from two different enzyme preparations.

Sample	*Phospholipids added*		*Apparent activation energy E (kcal/mole ± S.E.)*	
	Phosphatidyl-inositol	*Phosphatidyl-serine*	*Above 20 °C*	*Below 20 °C*
Treated	—	—	29.5 ± 2.5	29.5 ± 2.5
	+	+	13.6 ± 2.0	28.4 ± 2.8
	—	+	13.8*	31.5*
	+	—	14.7*	29.4*
Control	—	—	14.9 ± 1.8	30.1 ± 3.0
	+	+	13.3 ± 1.7	30.6 ± 3.0

* Values are means of two observations from the same enzyme preparations.

The ATPase activities in the presence of either phospholipid were lower than those activities in the presence of both phospholipids (data not cited here), as reported previously[1]. The apparent activation energies of the control ATPase activity were approximately 15 and 30 kcal/mole, while those for control ATPase activity in the presence of both phospholipids were approximately 13 and 31 kcal/mole, at temperatures over and under 20 °C, respectively. The apparent activation energies of approximately 13 to 15 kcal/mole at higher temperatures compare favourably with values of 14 to 15 kcal/mole for Na^+ efflux and K^+ influx in human erythrocytes[3,14,15].

(Na^+–K^+)-ATPase activities at temperatures under 20 °C were reduced to approximately 9 % of the control by phospholipase A treatment and these activities were remarkably increased in the presence of phospholipids without any remarkable changes in activation energies as shown in Fig. 2 and Table I.

DISCUSSION

The phospholipase A treatment caused a marked increase in the apparent activation energy of (Na^+–K^+)-ATPase from 15 kcal/mole to 30 kcal/mole at temperatures over 20 °C, and the activation energy of the activity in the presence of

phospholipids from the phospholipase A-treated preparation was decreased from 30 kcal/mole to 14 kcal/mole, approximately the same level as that of the control. These facts indicate that phospholipids are prerequisites for the abrupt change in the activation energy at about 20 °C and, of course, for (Na^+–K^+)-ATPase activity, and possibly are also required for the Na^+ and K^+ transport across the cell membranes.

Approximately 70% of the phospholipids in the ATPase preparation were changed to lyso compounds by the phospholipase A treatment[1]. Chapman[16,17] described that the unsaturated hydrocarbon chain is usually found to be on the 2 position of the glycerol part of the phospholipids and that the unsaturation of the hydrocarbon chain appears to be related to the overall fluidity of the membrane, possibly controlling the diffusion processes into and out of the cell. Phospholipase A from snake venom specifically attacks the (C-2)-ester position of phospholipids[18,19]. Luzzati[20] reported that the phase transitions of phospholipids take place in the lipid–water systems at various physico-chemical conditions (concentration and temperature) and that those conditions may not be too different from those that prevail in the living cell. Wilson *et al.*[21] suggested that an *Escherichia coli* membrane may exist in two physical states, one above and the other below a transition temperature determined by the liquidity of the lipid components. Esfahani *et al.*[22] reported that membranes of *E. coli* are in a fluid state and in a more condensed state above and below transition temperature, respectively.

Our results may suggest that the abrupt change in the activation energy at 20 °C is a reflection of the phase transition of phospholipids which are essential for the (Na^+–K^+)-ATPase activity. The reason why Arrhenius plots of ATPase activity by phospholipase A-treated ATPase gave only a single straight line may be explained as follows. The phase transition does not occur on account of the lack of phospholipids whose glycerol parts contain hydrocarbon parts unsaturated in the 2-position within the temperature range examined. The phase transition of the phospholipid molecules of the enzyme may be related to some regulatory mechanism for active translocation of Na^+ and K^+ across the cell membrane.

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