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LIPID DEPENDENCE OF ACTIVITY–TEMPERATURE RELATIONSHIP OF (Na^+, K^+)-ACTIVATED ATPase

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SUMMARY

The lipid moieties of (Na^+, K^+)-dependent ATPases of bovine cerebral cortex and bullfrog kidney were partly replaced with extraneous lipids, and temperature effects on their activities were investigated. The effects were found to depend on the lipid moiety, and to be constant with a given lipid regardless of the enzyme source.

INTRODUCTION

In our previous studies[1,2] it has been demonstrated that the presence of a variety of phospholipids and alkyl phosphates was an absolute requirement for the activity of (Na^+, K^+)-dependent ATPase (EC 3.6.1.3) which had been prepared from membrane components of the cerebral cortex by partial removal of the lipid moiety through deoxycholate treatment. Alkyl phosphate showed the highest activation capacity at 25 °C when its single carbon chain contained ten carbon atoms.

In addition to their molecular structures, however, the physical properties of both phospholipids and alkyl phosphates may possibly be of importance for activation of the membrane enzymes, since the interaction between these amphiphilic compounds and other molecules are partly determined by physical properties and states of the former. If their physical properties played a role in the activation, then the activating effects of lipids would be expected to vary with the temperature, because the physical state is a function of the temperature. The temperature effects on the physical properties differ from lipid to lipid. The activity–temperature curve, therefore, is also expected to change according to the used lipid.

The curve may also vary according to the source of the enzyme, inasmuch as the enzyme protein itself may affect the activity–temperature relationship. The degree of the activity control by the lipid may be reflected in this variation; *i.e.* if the activity control by the lipid were very strong, the activity–temperature curves of the enzymes from different sources would vary little.

The present work is an attempt to investigate these points, using two different (Na^+, K^+)-ATPase preparations of two sources and three different types of lipids.

METHODS

The method of preparation of the deoxycholate-treated (Na^+, K^+)-ATPase was the same as reported elsewhere[3,4]. The preparation of (Na^+, K^+)-ATPase

was inactive without the added phospholipids or the related compounds. The NaI-treated ATPase was prepared according to Nakao *et al.*[5]. The enzyme sources were a membrane fraction (heavy microsomal fraction) of bovine cerebral cortex and bullfrog (*Rana catesbeiana*) kidney. Didecyl and didodecyl phosphates were synthesized by the method of Brown *et al.*[6]. A commercial preparation of crude animal phospholipids was "animal lecithin" of Nutritional Biochemicals Corporation; the preparation contained lecithin (27.2%), phosphatidylethanolamine (21.9%), phosphatidylserine (17.5%), lysophospholipids (6.0%), unidentified phospholipids (12.1%) in addition to neutral lipids and cholesterol. The figures in parentheses are the percentage of phosphorus contained in the specified fractions of the preparation of crude animal phospholipids. Lipids were separated by two-dimensional thin-layer chromatography, using Silica gel-H and the solvent system of Rouser *et al.*[7]. Lipids of NaI-treated ATPase were extracted with a chloroform–methanol mixture (2:1, v/v)[3]. After methylation, the fatty acyl moieties of lipids were analyzed by gas–liquid chromatography, using diethylene glycol succinate columns, Perkin–Elmer gas chromatogram, Model 881. The determination of (Na^+, K^+)-ATPase activity was the same as described previously[3].

RESULTS AND DISCUSSION

(Na^+, K^+)-ATPase of rat tissues shows a large decline of the activity with temperature decrease[8–10]. The activity below 10 °C was considerably lower than that of the ATPase of frog brain, which animal is more active at low temperatures than warm-blooded animals[11]. This was the case also with (Na^+, K^+)-ATPases prepared from bovine brain and from bullfrog kidney, which animal had been kept at 24 °C for more than 3 months. The NaI-treated (Na^+, K^+)-ATPase of bullfrog kidney was still active at low temperatures (Fig. 1a, open circles), whereas the enzyme of bovine brain showed little activity at temperatures below 10 °C (Fig. 1a, closed circles). The discontinuities in Arrhenius plots were found at approximately 10 and 17 °C with frog kidney (Na^+, K^+)-ATPase and bovine brain enzyme, respectively (Fig. 1b).

After membrane phospholipids had been partly removed by deoxycholate treatment and the ATPase protein had been recombined with extraneous lipids, the activity–temperature curves were entirely different from the initial one. In the presence of didecyl phosphate, bovine (Na^+, K^+)-ATPase was active even at as low a temperature as 10 °C and exhibited an activity peak between 25 and 30 °C (Fig. 2, open circles; compare with Fig. 1a). With didodecyl phosphate the ATPase activity was hardly measurable at 15 °C but it increased abruptly above 20 °C as the incubation temperature was enhanced. The activity curve was still increasing between 30 and 35 °C (Fig. 2, closed circles). In the presence of a commercial preparation of crude animal phospholipids, the activities were already noticeable at 10 °C and increased smoothly from 10 up to 35 °C without inflection point (Fig. 2, squares). Thus, the relation between bovine (Na^+, K^+)-ATPase activity and incubation temperature was shown to depend on the lipid which activated the enzyme.

Since the commercial preparation of animal phospholipids may have been prepared from mammalian tissues, one would wonder why (Na^+, K^+)-ATPase activated by the preparation did not show a similar pattern to that of bovine brain

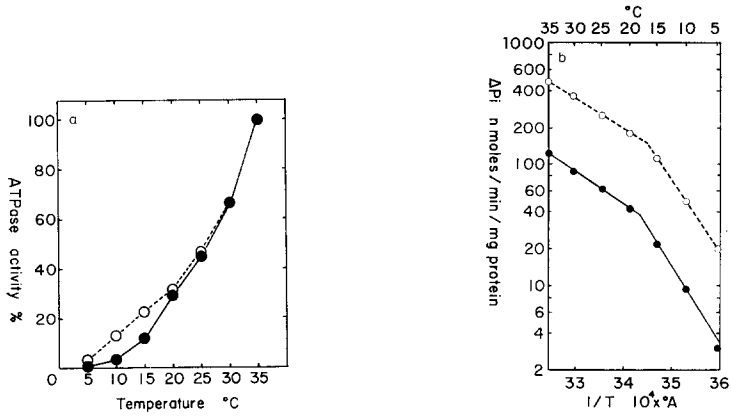

Fig. 1. Temperature effects on activities of NaI-treated (Na^+, K^+)-ATPases of bovine cerebral cortex and bull frog kidney. (a) The incubation medium contained 150 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, 10 mM cysteine, 50 mM Tris (pH 7.4), 2 mM ATP and 0.2 ml of the enzyme preparation in a final volume of 2 ml. For Mg^{2+}-dependent ATPase, NaCl, and KCl are omitted and 0.5 mM ouabain was added. The difference between the amounts of P_i liberated in those two incubation media was assumed to be due to (Na^+, K^+)-ATPase. Incubation time varied from 10–60 min with the temperature. The hydrolysis rate of ATP at each temperature was linear within the incubation period, and the amount of hydrolyzed ATP did not exceed 20% of the initial quantity. The values obtained at 35 °C were arbitrarily set as 100% for the purpose of comparison. Each point represents a mean of values obtained by 3 separate experiments; and the values agreed within 7%. ○– – –○, bullfrog kidney ATPase; ●—●, bovine brain enzyme. (b) Arrhenius plots for NaI-treated (Na^+, K^+)-ATPase of bovine cerebral cortex and bullfrog kidney. The values are taken from the experiment for Fig. 1a and are rearranged in order to show the discontinuity in the plots. ○– – –○, activity of the frog kidney enzyme; ●—●, bovine brain enzyme.

(Na^+, K^+)-ATPase. This may be ascribable to the difference in nature between the commercial crude phospholipids and the lipid composites of NaI-treated (Na^+, K^+)-ATPases. Since phosphatidylserine is known to be most effective in activation of (Na^+, K^+)-ATPase[1,12–14], it is likely that the phospholipid, among other lipids contained in the commercial preparation, plays a main role in activating the enzyme. Phosphatidylserine isolated from the commercial preparation, therefore, was analyzed with regard to its fatty acid composition by gas–liquid chromatography, and the analysis was compared with those of the lipid composites of NaI-treated (Na^+, K^+)-ATPases of both bovine brain and frog kidney. The whole lipids contained in NaI-treated ATPases were analyzed, because it is not clear yet what type of phospholipid plays a main role in maintaining the (Na^+, K^+)-ATPase activity in either the intact membrane or NaI-treated preparation[16]. The data are shown in Table I.

The ratio of unsaturated fatty acids to saturated ones of major fatty acid components (over 5%) in phosphatidylserine of the crude phospholipid preparation was 1.3:1. The ratio was 1.6:1 with the lipids contained in frog kidney (Na^+, K^+)-ATPase, whereas it was 0.7:1 with the bovine brain enzyme. In regard to fatty acid composition, the crude phospholipid preparation resembled the lipids of frog ATPase rather than those of the bovine enzyme. It is obvious from the previous[2]

and present work that not only lipid type but the nature of acyl chains of lipid are decisive factors in (Na^+, K^+)-ATPase activation. In addition, there are a few reports that the fluidity (or the degree of unsaturation) of membrane lipids is intimately correlated to the enzyme activity of mitochondrial membrane, sarcoplasmic reticulum and bacterial membrane[15,17–19]. The crude phospholipids and the frog ATPase lipids are likely to maintain more fluidity at low temperatures than the bovine

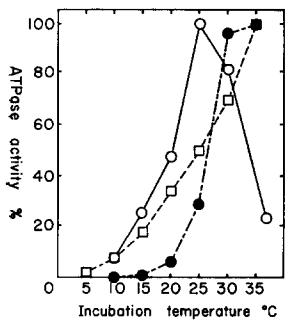

Fig. 2. Temperature effects on activity of deoxycholate-treated (Na^+, K^+)-ATPase of bovine cerebral cortex. The incubation conditions were as described for Fig. 1, except that the medium contained either 0.3 mM didecyl phosphate (○—○), or 0.6 mM didodecyl phosphate (●– – –●), or 250 μg crude phospholipids per ml (□– – –□). Both NaCl and KCl were omitted and 0.5 mM ouabain was added for Mg^{2+}-dependent ATPase. The difference between the amount of P_i liberated in those two incubation media were assumed to be due to the (Na^+, K^+)-ATPase activity. The highest values obtained in the used temperature range were set arbitrarily as 100%. At 25 °C the actual activities in the presence of didecyl phosphate and didodecyl phosphate were 94 and 59%, respectively, of that in the presence of crude phospholipids. Each point represents a mean of values obtained by 3 experiments agreeing within 10%.

TABLE I

FATTY ACID COMPOSITION OF LIPIDS OF NaI-TREATED (Na^+, K^+)-ATPases AND PHOSPHATIDYLSERINE OF CRUDE ANIMAL PHOSPHOLIPIDS

Fatty acids are identified by total number of carbon atoms and double bonds. The results are expressed in weight percentage. Since fatty acids that have longer chains and more double bonds than 22:1 are difficult to identify with certainty by simple gas–liquid chromatography only[7] and their amounts were detected to be small, the fatty acids of 14:0 to 20:4 only are shown in the table.

Fatty acids	*Crude animal phospholipids* (%)	*ATPase of bovine brain* (%)	*ATPase of frog kidney* (%)
14:0	3.3	1.1	4.1
16:0	14.5	23.2	19.1
16:1	1.6	4.7	2.0
18:0	24.7	25.2	13.7
18:1	10.4	22.8	13.5
18:2	30.6	1.3	26.0
20:4	8.5	12.9	14.5
Unidentified	6.4	8.8	7.1

ATPase lipids. Consequently, the higher contents of unsaturated fatty acids in both the crude phospholipids and the frog ATPase lipids may explain, at least in part, the fact that the phospholipids activated the enzyme at low temperature in a fashion that is similar to the activity–temperature relation of frog ATPase rather than bovine ATPase.

The activity–temperature curve was not only dependent on the type of lipid, but it was constant regardless of the source of (Na^+, K^+)-ATPase. NaI-treated (Na^+, K^+)-ATPases prepared from two different tissues of different species of animals differed markedly in temperature sensitivity (Fig. 1a). The reconstituted (Na^+, K^+)-ATPases from these tissues, however, displayed very similar patterns in all three cases of activation by didecyl phosphate, didodecyl phosphate, and crude phospholipids (Figs 2 and 3a). Thus, the activity–temperature curve of (Na^+, K^+)-ATPase seemed to be determined not by the enzyme source but by the nature of the lipid activating the enzyme. These findings suggest (a) that the control of $(Na,^+ K^+)$-ATPase activity by lipids is sufficiently effective to cover the influence of enzyme protein, or alternatively (b) that the potential activity–temperature relations of (Na^+, K^+)-ATPase proteins are the same regardless of the enzyme source and (c) that the physical state of lipid in aqueous solutions is a decisive factor in the activation of (Na^+, K^+)-ATPase.

The discontinuity in Arrhenius plots is considered to be explicable by a conformational difference of the enzyme protein on the both sides of the break[10–15]. Since phospholipid activation of (Na^+, K^+)-ATPase could be attributed, at least in part, to conformational change[20], the physical state of activating lipid may wel

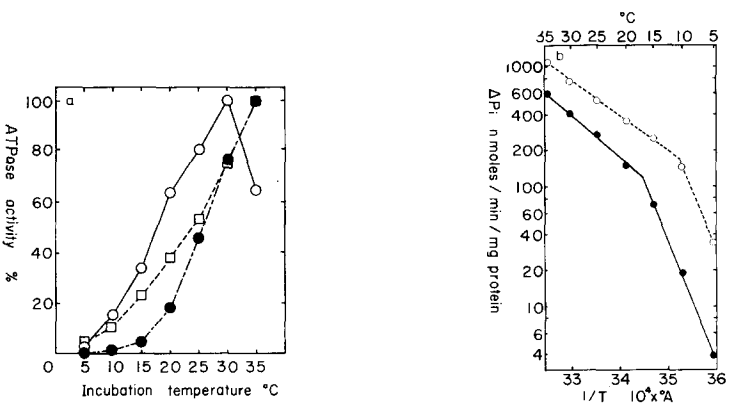

Fig. 3. Temperature effects on activity of deoxycholate-treated (Na^+, K^+)-ATPase of bullfrog kidney. (a) The incubation conditions were as given for Fig. 2. ○—○, apparant activity in the presence of 0.3 mM didecyl phosphate; ●– – –●, apparent activity in the presence of 0.6 mM didodecyl phosphate; □– – –□, apparent activity in the presence of 250 μg crude brain phospholipids per ml. The highest values obtained in the used temperature range were set arbitrarily as 100%. At 25 °C the actual activities in the presence of didecyl phosphate and didodecyl phosphate were 64 and 58%, respectively, of that in the presence of crude phospholipids. The points on the curves represent means of values from 3 separate experiments. Under the same conditions, the values agreed within 9%. (b) Arrhenius plots for bovine cerebral cortex and bullfrog kidney (Na^+, K^+)-ATPase reconstituted with crude phospholipids. The values are taken from the results for Figs 2 and 3a. ○---○, frog kidney enzyme; ●—●, bovine cerebral enzyme.

affect the manner in which the lipid induces the conformational change of the enzyme protein. If this were the case, the reconstituted (Na^+, K^+)-ATPases with the same lipid would exhibit the discontinuity in Arrhenius plots at the same temperature, regardless of the enzyme sources. Indeed, the break in Arrhenius plots for frog kidney (Na^+, K^+)-ATPase shifted from approximately 10 to 17 °C when crude phospholipids were used to reconstitute the enzyme (Fig. 3b). The break in the plots of bovine brain (Na^+, K^+)-ATPase was about 18 °C when reconstituted with crude phospholipids (Fig. 3b). In spite of the different enzyme sources, the breaks were almost at an identical temperature. It is just as if the result indicated that the conformational change of (Na^+, K^+)-ATPase protein was dictated by activating lipids.

When reconstituted with alkyl phosphates, the temperature–activity curves were not as simple as those of (Na^+, K^+)-ATPase activated by crude phospholipids, probably because three different types of the alkyl phosphate effects on (Na^+, K^+)-ATPase appeared in various proportions. These effect types are activation[2,3], protection against thermal inactivation[3,4], and inhibition[1,2]. Since the mechanisms of these effects are not well known and the proportion of the effects at a given temperature is also unknown, Arrhenius plots of these curves seem not to be helpful to interpret the results.

There are two possible manners in which the physical state of lipids affect the degree of (Na^+, K^+)-ATPase activation by lipids. (a) The degree of lipid binding to the enzyme protein depends on the physical state of lipids (therefore, on the temperature at which the binding takes place). (b) Although the degree of lipid binding is independent of the temperature, the activity of reconstituted (Na^+, K^+)-ATPase is determined by the physical state of lipids.

In order to distinguish between these two possibilities, the following experiments were carried out. Two sets of solubilized bovine brain (Na^+, K^+)-ATPase were preincubated for 3 min at 0 and 35 °C, respectively, in the presence of crude phospholipids. A previous experiment demonstrated that the (Na^+, K^+)-ATPase activation by lipids occurred within 30 s after the addition of lipids at 0 °C (Tanaka, R., unpublished data). (Na^+, K^+)-ATPase activity of each set was determined at 5, 15, 25 and 35 °C after a 1-min period of temperature equilibrium immediately following preincubation. The determination was started by adding ATP to the enzyme solution. If the degree of lipid binding was dependent on the temperature, then there would be a difference in the observed ATPase activity between the preparation which has been preincubated at 0 °C and the one which has been preincubated at 35 °C. Two separate experiments were carried out at each preincubation temperature and the results are shown in Fig. 4 (therefore, there were four values at each incubation temperature; two, preincubated at 0 °C and two, at 35 °C). At each incubation temperature, the activities of the enzyme preparations preincubated at 0 and 35 °C agreed reasonably well. Consequently, the possibility that lipid binding to (Na^+, K^+)-ATPase protein depends on the temperature may be ruled out in the tested temperature range.

The second experiment was centrifugation of deoxycholate-treated (Na^+, K^+)-ATPase on a sucrose density gradient at 0 and 20 °C. The sedimentation patterns were determined in the presence of added palmitoyl lysolecithin, which was a good activator and had been well studied with regard to its activation properties[2,21].

If the temperature affected the protein–lipid binding, then the sedimentation patterns of the bound phospholipid would be different at two centrifugation temperatures. As shown in Fig. 5, profiles of lipid phosphorus (broken lines) when centrifuged alone were unchanged at centrifugation temperatures of 0 and 20 °C. The proteins of (Na^+, K^+)-ATPase preparation (solid lines) were sedimented slightly faster at 20 °C than they were at 0 °C. This difference is presumably due to the fact that the proteins of (Na^+, K^+)-ATPase preparation, which are hydrophobic membrane proteins, tend to aggregate each other to form larger particles during a 6-h period of centrifugation and the aggregation rate is faster at higher temperatures. When

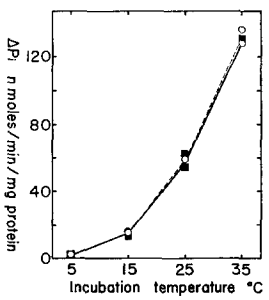

Fig. 4. Effects of preincubation temperature on (Na^+, K^+)-ATPase activity. Bovine brain (Na^+, K^+)-ATPase was preincubated in the presence of crude phospholipids at either 0 °C (■) or 35 °C (○) for 3 min. The enzyme activity was determined at 5, 15, 25, and 35 °C. Two separate sets of experiments were carried out; and therefore, there are four values at each incubation temperature, although the overlapping of the values does not allow to show all four.

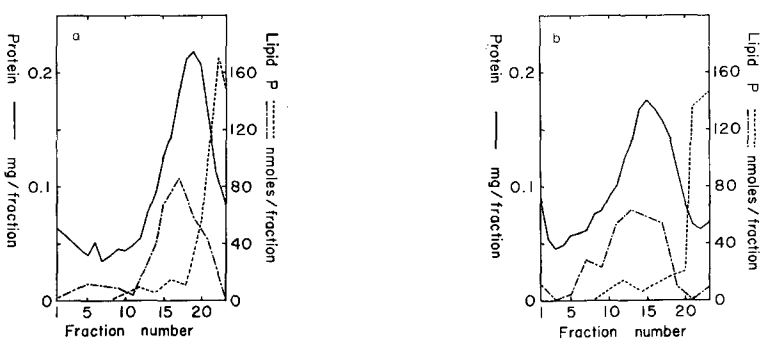

Fig. 5. Gradient centrifugation profile of (Na^+, K^+)-ATPase and lysolecithin. The deoxycholate-treated preparations of bovine brain (Na^+, K^+)-ATPase and palmitoyl lysolecithin were centrifuged on a sucrose gradient at 200000 × *g* for 6 h using rotor SW 40Ti (Spinco Division, Beckman Instrument Co.). The gradient is continuous from 0 to 8% and the solution contained 5 mM Tris buffer, pH 7.4. After the centrifugation 0.5-ml fractions were collected starting from the bottom of the centrifuge tubes. Protein (—) was determined by measuring absorbance at 280 nm using bovine serum albumin as a standard. The ATPase preparation was mixed with lysolecithin at 0 °C 10 min prior to the start of the centrifugation and was layered on the top of the gradient. Lipid phosphorus (– – – and · –· in the absence and presence of the ATPase protein, respectively) was determined by a minor modification of the method of Youngberg and Youngberg[22] after phospholipid had been extracted with a chloroform–methanol (2:1 v/v) mixture and digested using $HClO_4$. The centrifugation was carried out at 0 °C (a) and 20 °C (b). The values are averages of two separate experiments.

centrifuged with the ATPase preparation, the peak of lipid phosphorus (interrupted lines) shifted toward the bottom of the centrifuge tube, and was located slightly left of the protein peak. The peak of the lipid phosphorus at 20 °C was shifted more to the left as compared to the lipid peak at 0 °C, together with protein peak. Therefore, the relationship between the protein profile and the lipid profile remained to be unchanged by temperature shift. That (Na^+, K^+)-ATPase activity was found under the lipid peak was consistent with the results obtained using gel filtration column chromatography[21]. These results indicate that the degree of binding between (Na^+, K^+)-ATPase protein and the phospholipid was not affected by the temperature up to, at least, 20 °C. The contention is also substantiated by our previous experiment with gel filtration chromatography[21], showing that the amount of palmitoyl lysolecithin which was bound to deoxycholate-treated bovine brain (Na^+, K^+)-ATPase at 0 °C roughly equaled the amount of the lipid required for optimum activationat 25 °C.

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