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Difference in phospholipid dependence between two isozymes of brain ($Na^+ + K^+$)-ATPase

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The effect of phospholipase C on two isozymes ($\alpha(+)$ and α forms) of rat brain ($Na^+ + K^+$)-ATPase and the temperature-dependence of their activities were investigated. Phospholipase C from *Clostridium welchii* inhibited the activities of the enzymes treated with and without pyrithiamin or *N*-ethylmaleimide, a preferential inhibitor of the $\alpha(+)$ form, but the extent of the inhibition was higher in the control enzyme than in the treated enzymes. The treatment of the ($Na^+ + K^+$)-ATPase with phospholipase C altered a ratio between high- and low-affinity components for ouabain inhibition. It also caused the similar change in a ratio between the $\alpha(+)$ and α forms of Na^+-stimulated phosphorylation from [γ-^{32}P]ATP. These findings indicate that the $\alpha(+)$ form of rat brain ($Na^+ + K^+$)-ATPase is more sensitive to phospholipase C than the α form. Analysis of Arrhenius plots of the activities of the control and pyrithiamin-treated enzymes showed that there was a difference between the two enzymes in a break point. We suggest that two isozymes of rat brain ($Na^+ + K^+$)-ATPase differ in the interaction with phospholipids or in the lipid-environment.

Introduction

It has been reported that there are two different molecular forms of the ($Na^+ + K^+$)-ATPase (EC 3.6.1.3) in the brain [1–7]. On the basis of the different apparent molecular weights of the catalytic subunits by SDS-polyacrylamide gel electrophoresis, they are called $\alpha(+)$ and α forms, corresponding to the larger and smaller molecular weights, respectively [1]. Both forms are also present in fat cells where the $\alpha(+)$ form is selectively stimulated by insulin [8–10]. They have different affinities for cardiac glycosides [1,11,12], and can be distinguished by their sensitivities to several biochemical probes, such as proteinases and *N*-ethylmaleimide [1], and by the antigen determinants [5]. We have recently found a new probe to distinguish two molecular forms of the ($Na^+ + K^+$)-ATPase: pyrithiamin, an antimetabolite of thiamin, inactivates preferentially the $\alpha(+)$ form [13–15]. On the other hand, it is accepted that phospholipids are important in the function of the ($Na^+ + K^+$)-ATPase: they are involved in the catalytic activity or in the conformational change of the enzyme [16,17]. The objective of this study was to examine whether two isozymes of brain ($Na^+ + K^+$)-ATPase differed in the interaction with phospholipids. We demonstrate here that the two isozymes have different sensitivity to phospholipase C treatment and that there is a difference in a break point of Arrhenius plots between the untreated and pyrithiamin-treated ($Na^+ + K^+$)-ATPase.

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Materials and Methods

Enzyme preparation and enzyme assay. ($Na^+ + K^+$)-ATPase was partially purified from rat cerebral cortex by the method of Jørgensen [18] with a modification [13], and the activity was measured as previously described [13,14]. The specific activity of the enzyme used here was 6.75–9.35 μmol P_i/mg protein per min. Protein was determined by the method of Lowry et al. [19] using bovine serum albumin as standard.

Treatment with pyrithiamin and N-ethylmaleimide. The enzyme suspension (50 μg/ml) was incubated with 1 mM pyrithiamin or 1 mM *N*-ethylmaleimide freshly dissolved in 20 mM imidazole buffer (pH 7.25) [14,15]. The resulting sediment was resuspended in 0.16 M sucrose/0.5 mM EDTA/10 mM imidazole buffer (pH 7.25). These treatments decreased the ($Na^+ + K^+$)-ATPase activity by 75–85% and SDS-polyacrylamide gel electrophoresis showed that the residual activity reflected mainly the activity of α form as previously described [14,15].

Treatment with phospholipase C and sphingomyelinase. Phospholipase C treatment was carried out for 30 min at 37°C in a medium consisting of the enzyme (0.5 mg/ml), phospholipase C at the specified concentrations, 20 mM imidazole buffer (pH 7.25) and 1 mM $CaCl_2$. In another experiment, the enzyme suspension (0.5 mg/ml) was treated with sphingomyelinase (0.5 U/ml) in the same way as the phospholipase C treatment except that 1 mM $MgCl_2$ was used instead of 1 mM $CaCl_2$. After treatment, 2 mM EGTA (for phospholipase C treatment) or EDTA (for sphingomyelinase treatment) was added to the mixture. The mixture was centrifuged at 279 000 × *g* for 20 min in a Beckman TL-100 ultracentrifuge. The resulting sediment was resuspended in 0.16 M sucrose/0.5 mM EDTA/10 mM imidazole buffer (pH 7.25) for the enzyme activity and phospholipid determinations.

Determination of phospholipids. Phospholipids of the enzyme preaprations were isolated by extraction with chloroform/methanol (1 : 2, v/v) in the presence of 0.05% butylated hydroxytoluene, and separated by two-dimensional thin-layer chromatography on silica-gel by the method of Esko and Raetz [20]. The spots were scraped off the sheet and the phosphorus content was assayed by the method of Bartlett [21]. Total phospholipid was determined as described previously [22].

Phosphorylated intermediate. The enzyme was phosphorylated with [γ-^{32}P]ATP and subjected to SDS-polyacrylamide gel electrophoresis as previously described [13,14]. The gel was stained, destained, and dried on filter paper. The two bands, corresponding to the α(+) and α, were cut off and their radioactivity was counted using a liquid scintillation spectrometer.

Arrhenius plot analysis. The log specific activity of the enzyme was plotted against the reciprocal of the absolute temperature of the incubation medium and the T_d value was calculated as previously reported [23].

Materials. Vanadate-free ATP, pyrithiamin, ouabain, phospholipase C (Type XII, from *Clostridium welchii*) and sphingomyelinase (from *Staphylococcus aureus*) were obtained from Sigma Chemical Co. Silica-gel aluminium sheet was purchased from E. Merk AG. [γ-^{32}P]ATP was from Amersham. All other chemicals used here were of the highest purity commercially available. The disodium salt of ATP was converted to the Tris salt by passage over an AG50W-X8 cation exchanger resin.

Results

The ($Na^+ + K^+$)-ATPase preparations pretreated with and without pyrithiamin or *N*-ethylmaleimide were incubated with phospholipase C from *Clostridium welchii*. These enzyme preparations showed a similar sensitivity to phospholipid hydrolysis by phospholipase C (Fig. 1), while the inhibition of the enzyme activity by phospholipase C was greater in the control enzyme than in the pyrithiamin- and *N*-ethylmaleimide-treated enzymes (Fig. 2). In all of the enzymes, the effects of phospholipase C on the enzyme activity and the phospholipid content were dose-dependent and biphasic. The effect of phospholipase C on each phospholipid in rat brain ($Na^+ + K^+$)-ATPase preparation was examined to study which phospholipid species might be related to the decrease in the activity by phospholipase C (Fig. 3). Phosphatidylcholine was readily hydrolyzed by phospholipase C at lower concentrations, and sphingo-

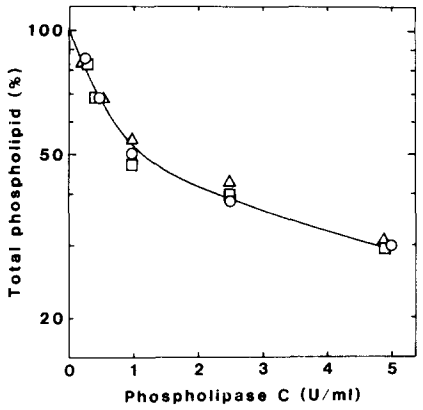

Fig. 1. Hydrolysis of phospholipid in the enzyme preparations by phospholipase C. Rat brain ($Na^+ + K^+$)-ATPase was pretreated with pyrithiamin (△) or *N*-ethylmaleimide (□). The control (○) and treated enzymes were then incubated with phospholipase C at the indicated concentrations for 30 min at 37°C and centrifuged. Total phospholipid in the enzyme preparations was determined as described under Materials and Methods. Total phospholipid content (μmol P_i/mg protein) of these enzyme preparations were 3.00 ± 0.11 (control), 2.67 ± 0.17 (pyrithiamin-treated) and 3.20 ± 0.12 (*N*-ethylmaleimide-treated) ($n = 4$–8). Each point is the mean of four experiments.

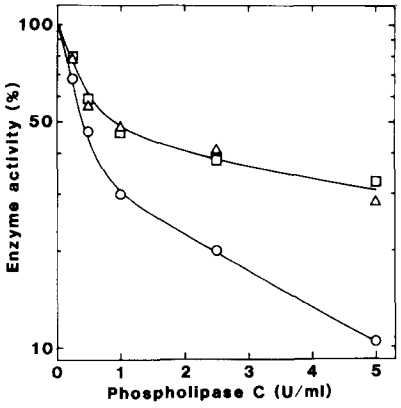

Fig. 2. Effect of phospholipase C on the activities of ($Na^+ + K^+$)-ATPase pretreated with pyrithiamin or *N*-ethylmaleimide. Rat brain ($Na^+ + K^+$)-ATPase was pretreated with pyrithiamin (△) or *N*-ethylmaleimide (□) for 15 min at 37°C and washed as described under Materials and Methods. The pretreatment resulted in the decrease in the enzyme activity by 75–85%. The control (○) and treated enzyme preparations were then treated with phospholipase C at the indicated concentrations for 30 min, and the enzyme activities were determined. Each point represents the mean of three to six experiments with different enzyme preparations. The standard error was within 10% of the mean.

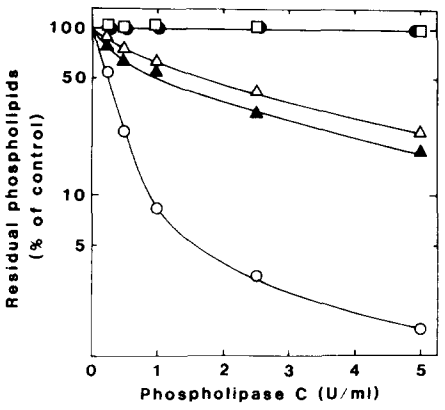

Fig. 3. Effect of phospholipase C on each phospholipid content in rat brain ($Na^+ + K^+$)-ATPase preparation. The enzyme was treated with phospholipase C at the indicated concentrations for 30 min and centrifuged. Each phospholipid was separated and the content was determined as described under Materials and Methods. Phospholipid contents (μmol/mg protein) of the control enzyme were 0.140 ± 0.010 (sphingomyelin, ▲). 1.270 ± 0.040 (phosphatidylcholine, ○), 0.075 ± 0.002 (phosphatidyl inositol, ●), 0.421 ± 0.019 (phosphatidylserine, □) and 1.135 ± 0.052 (phosphatidylethanolamine, △) (mean ± S.E. of 14 determinations). Each point represents the mean of three or four experiments. The standard error was within 10% of the mean.

myelin and phosphatidylethanolamine were hydrolyzed to a lesser extent. In contrast, phosphatidylinositol and phosphatidylserine were resistant to hydrolysis by phospholipase C even at 5 U/ml.

The effect of phospholipase C on the isozymes in the brain was further examined by analysis of their sensitivities to ouabain inhibition and by resolving the two forms by SDS-polyacrylamide gel electrophoresis. The effects of the treatment with phospholipase C and sphingomyelinase on sensitivity of rat brain ($Na^+ + K^+$)-ATPase to ouabain inhibition are illustrated in Fig. 4. Two components of ouabain inhibition which reflected the presence of two molecular forms [1,14,15] were observed in the control and treated enzymes. Phospholipase C treatment significantly changed a ratio between the high- (inhibition by $1 \cdot 10^{-8}$–$5 \cdot 10^{-6}$ M ouabain) and low- (inhibition by $5 \cdot 10^{-6}$–$1 \cdot 10^{-3}$ M ouabain) affinity components of the inhibition: the high-affinity component of the inhibition was about 80% of the total in the control, while it was about 67% in the phospholipase

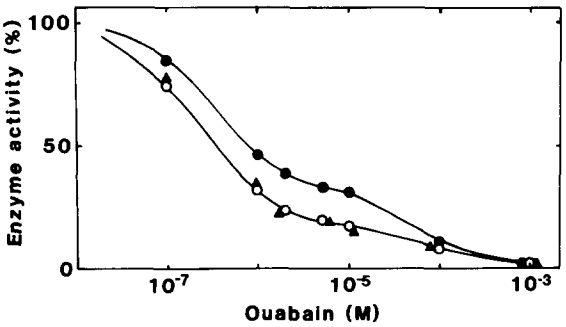

Fig. 4. Effect of phospholipase C and sphingomyelinase on the dose-response curve of brain $(Na^+ + K^+)$-ATPase versus ouabain concentrations. The enzyme suspension (0.5 mg/ml) was treated with 2.5 U/ml phospholipase C (●) or 0.5 U/ml sphingomyelinase (▲) for 30 min and centrifuged as described under Materials and Methods. The ATPase reaction of the control (○) and treated enzymes was carried out for 30 min in a final volume of 0.1 ml in which protein concentration was adjusted to 2 μg/tube by addition of bovine serum albumin as previously reported [13,14]. Points are values from a representative experiment which has been repeated three times.

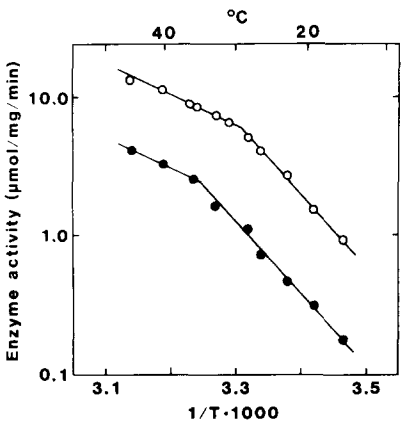

Fig. 5. Arrhenius plots of brain $(Na^+ + K^+)$-ATPase activity. Rat brain $(Na^+ + K^+)$-ATPase was pretreated with (●) and without (○) 0.4 mM pyrithiamin for 15 min at 37°C and the activity was determined. This treatment inhibited the enzyme activity by 70–75% and SDS-polyacrylamide gel electrophoresis showed that the predominant form of the treated enzyme was α, in agreement with the previous result [14]. The log specific activity of each enzyme was plotted against the reciprocal of the absolute temperature of incubation. Each point represents the mean of three to five experiments.

C-treated enzyme. Sphingomyelinase treatment, which decreased sphingomyelin in the enzyme preparation by 84% (mean of four determinations), affected neither the enzyme activity nor the dose-response curve for ouabain inhibition. This finding suggests that sphingomyelin does not contribute to the different sensitivity of the isozymes to phospholipase C. The control and treated (2.5 U/ml phospholipase C for 30 min at 37°C) enzymes were phosphorylated by [γ-^{32}P]ATP in the presence of Mg^{2+} and Na^+ [14,15] and then the α(+) and α forms were distinguished by SDS-polyacrylamide gel electrophoresis. The distribution ratios of the radioactivity incorporated into the α(+) were 86.1 ± 1.3 (the control enzyme) and 74.9 ± 3.3 (phospholipase C-treated ezyme) % of the total (mean ± S.E. of three determinations, $P < 0.05$).

The temperature-dependence of membrane-bound activity has often been studied because it reflects changes in lipid-protein or lipid-lipid interaction [24–27]. Fig. 5 shows Arrhenius plots of the activities of brain $(Na^+ + K^+)$-ATPase pretreated with and without pyrithiamin. A discontinuity in the plots was observed in both preparations. T_d values in the control and the treated enzymes were 29.1 and 34.2°C, respectively. The activation energies above and below T_d in the treated enzyme were calculated from the slopes of the line to be 27.5 and 94.1 kJ/mol, respectively, in the two preparations.

Discussion

It is estimated that a ratio of the α(+) and α forms in rat brain $(Na^+ + K^+)$-ATPase is about 4:'1 and that more than 80% of the total activity in the enzyme treated with pyrithiamin or *N*-ethylmaleimide is due to the α form, on the basis of the results on sensitivity to the ouabain inhibition and the phosphorylated intermediates of the enzyme. Then, it may be roughly considered that the control and pyrithiamine-treated enzymes are the α(+) and α forms, respectively. In this study, we first examined the effect of phospholipase C on the $(Na^+ + K^+)$-ATPase treated with and without pyrithiamin or *N*-ethylmaleimide to clarify sensitivity of the two isozymes to phospholipase C. Phospholipase C inhibited the activities of these enzymes, but the extent of the inhibition was higher in the control enzyme than in pyrithiamin- and *N*-ethylmaleimide-treated enzymes (Fig. 2). It

is unlikely that the difference may be due to that between the control and treated enzymes in hydrolysis of phospholipids by phospholipase C, since the treatment with pyrithiamin or *N*-ethylmaleimide did not affect the hydrolysis of phospholipids in the enzyme preparation (Fig. 1). Then, the finding suggests that there is a difference betwene the two isozymes in sensitivity to inhibition by phospholipase C. This point was subsequently examined by analysis of sensitivity of the enzyme to ouabain inhibition. Brain $(Na^+ + K^+)$-ATPase shows two components of ouabain inhibition which reflect the presence of two moelcular forms [1,14]. Phospholipase C treatment caused a change in a ratio between high- and low-affinity components for ouabain inhibition (Fig. 4), indicating that the treatment inhibited the activity of the $\alpha(+)$ form more than that of the α. The similar conclusion was obtained by the experiment with SDS-polyacrylamide gel electrophoresis, which could distinguish the two forms. Phospholipase C increased the proportion of the phosphorylation of the α form, and, conversely, decreased that of the phosphorylation of the $\alpha(+)$. It is thus concluded that two isozymes of rat brain $(Na^+ + K^+)$-ATPase have different sensitivity to inhibition by phospholipase C.

Phospholipase C from *C. welchii* affected each phospholipid species in the $(Na^+ + K^+)$-ATPase preparation in a different way, in agreement with the previous reports [28–30]. Phosphatidylcholine was highly sensitive to the treatment, phosphatidylethanolamine and sphingomyelin were moderately sensitive, and phosphatidylinositol and phosphatidylserine were insensitive. The activities of the control and pyrithiamin- or *N*-ethylmaleimide-treated enzymes were inhibited by the hydrolysis of phospholipids, though their inhibitions differed in an extent as described above. In contrast to phospholipase C, sphingomyelinase treatment did not affect the activities of these enzymes. These findings suggest that most of the activity seems to depend on phosphatidylcholine or phosphatidylethanolamine in the two isozymes, though phosphatidylinositol or phosphatidylserine may be involved in the activity remaining after phospholipase C treatment, as reported previously in the kidney enzyme [31]. The finding observed here is in conflict with the previous suggestion [16,17,28] that phosphatidylserine or more generally acidic phospholipids are essential for the activity. But, De Pont et al. [30,32] provided evidence against the essentiality of the phospholipids using phosphatidylserine decarboxylase and phosphatidylinositol-specific phospholipase C. Furthermore, Hilden and Hokin [33] demonstrated that coupled transport of Na^+ and K^+ could be found in phospholipid vesicles containing purified $(Na^+ + K^+)$-ATPase with phosphatidylcholine as the only phospholipid. They also suggested that the specificity for phosphatidylserine was not for $(Na^+ + K^+)$-ATPase activity but for some other process such as combination of delipidated protein and phospholipid in aqueous solution.

The present study demonstrates that the $\alpha(+)$ form is sensitive to phospholipase C more than the α. The difference suggests that the two forms have different regulations by phospholipids. It is alternatively considered that there is a difference between the two forms in the phospholipid-environment. For example, phosphatidylcholine and phosphatidylserine, which are required for the activity and easily hydrolyzed by phospholipase C, may be localized more around the $\alpha(+)$ than around the α. Though the exact mechanism is not yet known, the finding suggests that the two isozymes differ in the phospholipid-dependence or in the interaction with phospholipids. It is known that the discontinuity in Arrhenius plot of the enzyme activity is attributed to the interaction of the enzyme protein with a tightly associated lipid annulus [24–27]. The analysis of the temperature-dependence of the enzyme activity then provides information on the lipid environment of the enzyme [23]. We have demonstrated here that the treatment of the $(Na^+ + K^+)$-ATPase with pyrithiamin, an inhibitor of the $\alpha(+)$ form [14], altered T_d value without a change in the activation energies above and below T_d. The finding may support the idea that two forms of rat brain $(Na^+ + K^+)$-ATPase differ in their interactions with phospholipids.

The isozymes of $(Na^+ + K^+)$-ATPase are thought to serve different functions in the regulation of ion transport. It has been reported that the $\alpha(+)$ form is selectively regulated by insulin in fat cells [8–10]. A similar difference in the regulation of the isozymes could be considered in

the brain: they might have different sensitivity to regulation by hormone or neurotransmitter. However, there is no evidence for this idea, and the physiological function of each form in the brain is not clarified so far. The study on the difference between the two isozymes will obviously contribute to clarification of their functions. The most striking difference between the isozymes reported so far is sensitivity to inhibition by ouabain [1,6,11,12,14]. We demonstrate here that there is a difference between the $\alpha(+)$ and α forms in the interaction with phospholipids. It is accepted that the reaction of the ATPase occurs accompanying conformational changes which may be condensed into four states as follows [17,34–37].

$$E_1 \rightleftharpoons E_1P \rightleftharpoons E_2P \rightleftharpoons E_2 \rightleftharpoons E_1$$

Since phospholipids seem to be important in the conversion of E_1P to E_2P [38–40], the present finding suggests that the two isozymes differ in the transition of the two phosphorylated states. In accord with this suggestion, we have found a difference between the two isozymes in sensitiivty of their phosphorylated intermediates to ADP under certain conditions (unpublished data). A similar suggestion has been recently provided by Sweadner [6]. She has examined the basic kinetic properties of the two $(Na^+ + K^+)$-ATPases purified from rat kidney and brainstem axolemma, and proposed from the difference in kinetic cooperativity that the isozymes may differ in their conformational transitions during enzyme turnover.

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