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Ligand Effects on Membrane Lipids Associated with Sodium, Potassium-Activated Adenosine Triphosphatase: Comparative Spin Probe Studies with Rat Brain and Heart Enzyme Preparations

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SUMMARY

Physical properties of membrane lipids associated with rat or dog brain and heart Na^+,K^+-ATPase preparations were compared using an electron spin resonance probe, 5-doxyl stearate. The degree of acyl chain order of the membrane lipids was greater for brain enzyme than for heart enzyme preparations; membrane lipids in the rat heart enzyme preparations were the most disordered. In the absence of added ligands, membrane lipids did not appear to undergo a detectable temperature-dependent rearrangement or structural transition. An apparent transition was observed in the simultaneous presence of Na^+, Mg^{2+}, and ATP. These ligands increased lipid order at temperatures above the structural transition, but not below it. In the presence of the above ligands, K^+ caused a marked decrease in the transition temperature in the rat brain enzyme preparations, but only a modest decrease in rat heart enzyme preparations. Arrhenius plots of rat brain and heart Na^+,K^+-ATPase activity revealed a break point roughly corresponding to respective membrane lipid transition temperatures observed in the presence of Na^+, K^+, Mg^{2+}, and ATP. A low concentration of ouabain (1 μM) failed to affect either the lipid transition temperature estimated by the spin probe or the value of lipid order of the rat brain enzyme preparations observed in the presence of Na^+, Mg^{2+}, and ATP, but markedly reduced the effect of K^+ to lower the transition temperature observed in the presence of the above ligands. A high concentration (100 μM) of ouabain which was needed to completely inhibit rat heart enzyme eliminated the thermally induced structural rearrangement observed in the presence of Na^+, Mg^{2+}, and ATP, apparently through a nonspecific lipid perturbation. These results indicate that differences in the physical properties of the membrane lipids per se are unlikely to account for the low affinity of rat heart Na^+,K^+-ATPase for ouabain and also suggest that the use of high concentrations of ouabain required to completely inhibit Na^+,K^+-ATPase activity may cause nonspecific changes in addition to inhibition of Na^+,K^+-ATPase or the sodium pump.

It is widely recognized that the physical state of membrane lipids may influence the activity of many membrane-bound enzymes (1). In particular, the physical structure of the membrane lipids (2) and their ordering (3) are reported to be an important determinant of Na^+,K^+-ATPase activity (2). Membrane delipidation results in a reversible inactivation of the enzyme, associated with a slowing of the conformational change in phosphoenzyme from the ADP-sensitive to a K^+-sensitive form (4). Although the presence of either phosphatidylserine or phosphatidylinositol has been implicated by many as being necessary for maximal enzyme activity, neither an enzymatic conversion of all phosphatidylserine into phosphatidylethanolamine nor the complete hydrolysis of phosphatidylinositol has been shown to affect Na^+,K^+-ATPase activity (5). These results are consistent with earlier observations that Arrhenius plots of enzyme activity, observed in the presence of Na^+, K^+, Mg^{2+}, and ATP, have a break point at about 20°, presumably corresponding to a structural transition in the bulk membrane lipids (see Ref. 6). These results suggest that the physical state of the membrane lipids, rather than the presence of a specific lipid, influences the activity of Na^+,K^+-ATPase.

Sensitivity of Na^+,K^+-ATPase to the cardiac glycosides, such as ouabain, seems to be influenced by membrane lipids associated with the enzyme. An exposure of dog brain or dog heart enzyme preparations to high concentrations of deoxycholate or glycerol decreases affinity of the enzyme for ouabain (7). Differences in the protein structure have been suggested as the primary cause of the species-dependent variation in the glyco-

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ABBREVIATION: 5DS, 5-doxyl stearate.

side sensitivity (8, 9); however, membrane lipids have also been suggested to play a role in regulating accessibility of the glycoside-binding sites and thereby influencing affinity of Na^+,K^+-ATPase for the glycosides or aglycones (10–13).

Therefore, possible differences in properties of membrane lipids associated with Na^+,K^+-ATPase preparations obtained from rat brain and rat heart were examined using an ESR probe, 5DS. Na^+,K^+-ATPase obtained from rat brain, dog brain, and dog heart has a high affinity for the glycoside, whereas that obtained from the rat heart has a low affinity (12, 14, 15). The results of the electron spin resonance probe studies were compared with those of the Na^+,K^+-ATPase activity assay.

Materials and Methods

Crude Na^+,K^+-ATPase preparations were obtained from the brain and heart of male Sprague-Dawley rats weighing 250–300 g as described previously for brain enzyme (16). Rats were decapitated, and brains and hearts were rapidly removed. Twelve brains or 24 hearts were pooled and homogenized. Preparations were obtained with deoxycholate and NaI treatment of "microsomal" fractions. The same procedure was used for both tissues, so that the observed differences in physical properties are due to differences in the composition or concentrations of the membrane lipids and not influenced by the purification procedures. Brain Na^+,K^+-ATPase has a specific activity of 51.8 ± 3.5 μmol of P_i/mg of protein/10 min (mean ± SE of six preparations) at 37°. Mg^{2+}-ATPase activity, assayed in the presence of 5 mM $MgCl_2$, 5 mM Tris-ATP, 50 mM Tris-HCl buffer (pH 7.5) and 0.1 mM ouabain, accounted for 6.4 ± 0.4% of the total ATPase activity, assayed in the presence of 100 mM NaCl, 15 mM KCl, 5 mM $MgCl_2$, 5 mM Tris-ATP, and 50 mM Tris-HCl buffer (pH 7.5). Na^+,K^+-ATPase activity is the difference between total and Mg^{2+}-ATPase activity. The specific Na^+,K^+-ATPase activity of three rat heart enzyme preparations used in the spin probe study was in the range of 2.12–3.67 μmol/mg of protein/10 min at 37°, with Mg^{2+}-ATPase activity accounting for 39–44% of the total ATPase activity. Na^+,K^+-ATPase preparations from dog brain or dog heart were obtained using the same procedure as above from tissues freshly excised from pentobarbital-anesthetized animals.

The fatty acid spin probe, 5DS, was dissolved in absolute ethanol to prepare a 30 mM stock solution. An aliquot was transferred to a test tube and dried under a stream of air at room temperature. Subsequently, a 0.2-ml aliquot of Tris-HCl buffer solution (pH 7.5) containing appropriate ligands was added and mixed vigorously using a Vortex mixer to resuspend 5DS attached to the test tube walls. The enzyme preparation was then added and mixed by gentle swirling.

The final protein concentration was 2 and 5 mg/ml for brain and heart enzyme preparations, respectively. The concentration of 5DS was adjusted to produce a final probe to protein weight ratio of less than 0.005. The mixture was preincubated at 37° for 60 min in the sample chamber of an ESR spectrometer to ensure equilibration of 5DS in membrane lipids. The loss of Na^+,K^+-ATPase activity during this incubation was 5–10%. Subsequently, the samples were cooled at a rate of 0.5°/min until the temperature reached 10°. ESR spectra were obtained after a 15-min equilibration at 10°. The sample was then warmed at a rate of 0.5°/min, and spectra were recorded at approximately 5° intervals. Experiments were repeated at least three times using three different enzyme preparations. All ESR data reported in this paper comprise the pooled results from these experiments. Regression lines are fitted to the pooled data such as those shown in Figs. 2–4.

A Varian Century Line ESR spectrometer (model E-112), equipped with a variable temperature controller, was used for ESR recordings. The temperature was monitored continuously using an externally calibrated thermistor probe. The lipid order is estimated from the order parameter of 5DS (17). Temperature dependency of the order parameter was analyzed using a computer curve-fitting program (18). Briefly, the transition points, which can be represented by an intersection of two regression lines, were determined for each set of data. A fourth degree spline was used to simplify the transition search process, and the final solution was obtained using a more rigorous interactive least squares procedure. Statistical significance of the difference in slopes of the regression lines above and below each transition point was estimated using the F test for parallelism. Confidence limits were set at $\alpha = 0.05$. When the differences in slopes of two regression lines were not statistically significant, a single line was fit by the least squares procedure. The same computer program was used to analyze the Arrhenius plots of the enzyme activity.

Metal ion concentrations in the incubation mixture derived from Na^+,K^+-ATPase preparations were estimated from the metal concentrations in enzyme preparations assayed by plasma emission spectroscopy using a Jarrell-Ash model 955 Atomcomp spectrometer. Rat brain or heart enzyme preparations (2 or 5 mg of protein/ml, respectively) were wet-ashed by mixing with equal volumes of concentrated nitric acid and incubating at 70–75° for 24 hr. The mixture was then diluted such that the final concentration of the acid was less than 15% (v/v). The wavelength of the N+1 channel of the spectrometer was set at 766.5 nm for the determination of potassium concentrations.

The fatty acid spin probe, 5DS, was purchased from Syva Corp., Palo Alto, CA. Ouabain octahydrate and Tris-ATP were obtained from Sigma Chemical Co., St. Louis, MO. Other chemicals were of reagent grade, except for the nitric acid (Ultra grade, Atomergic Chemetals Corp., Plainview, NY), which was used in the wet-ashing.

Results

ESR spectra in the absence of ligands. Rat brain and rat heart Na^+,K^+-ATPase preparations were labeled with the fatty acid spin probe, 5DS. This probe has been shown to be useful for measuring membrane lipid order, quantified as the order parameter (17). A typical ESR spectrum obtained with a rat brain enzyme preparation suspended in a 50 mM Tris-HCl buffer (pH 7.5) at 37° is shown in Fig. 1. The absence of sharp spectral lines which are characteristic of the free probe indicate that virtually all of the spin label was membrane bound under these conditions. The absence of an excessive broadening of the low field peak suggests that the spin probe is in a relatively homogeneous environment (19). Similar results were obtained with rat heart enzyme preparations (data not shown), indicating that the conditions of the present study are adequate to assess changes in the ordering of the lipids using calculated

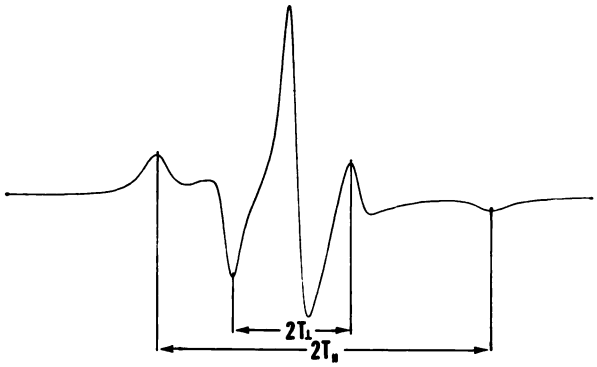

Fig. 1. Typical ESR spectrum of rat brain Na^+,K^+-ATPase preparations. The enzyme preparation was suspended in 50 mM Tris-HCl buffer solution (pH 7.5) yielding a final protein concentration of 2 mg/ml. The ESR spectrum was recorded at 37.4° with the microwave frequency of 9.014409 GHz and microwave power 11 mW. Spectral range = 100 G. In this and in all following ESR experiments, the probe (5DS) to protein weight ration was 0.005.

order parameters. Analysis of both brain and heart enzyme preparations indicated that concentrations of Na^+, K^+, and Mg^{2+} (Table 1) were substantially lower than those required for enzyme activation (20). Therefore, the spectrum shown in Fig. 1, and those obtained in the following studies, may be regarded as those of ligand-free enzyme preparations, unless these ions were added to the incubation medium.

ESR spectra taken at 15-min intervals during a 60-min preliminary incubation at 37° indicated no appreciable time-dependent change in the order parameter, either with brain or heart enzyme preparations (data not shown). Furthermore, comparison of the mid-field peak height at 37° at the beginning and at the end of a temperature scan indicated that the loss of the spin probe signal was less than 5% during the entire experimental period.

A gradual warming from 10 to 47° decreased the order parameter of 5DS-labeled brain or heart enzyme preparations suspended in a 50 mM Tris-HCl buffer solution in the absence of added Na^+, K^+, Mg^{2+}, and ATP (Fig. 2). A linear inverse relationship was observed between the temperature and the order parameter. Either brain or heart enzyme preparation exposed to temperatures above 47° and then cooled to 37° gave values of the order parameter which were significantly greater than the corresponding value observed before heating (data not shown). If the temperature did not exceed 47°, changes in the order parameter were reversible. A brief exposure of rat brain or heart enzyme preparations to 45° had negligible effects on Na^+,K^+-ATPase activity, whereas an exposure to 50° markedly reduced the enzyme activity (data not shown). The order parameters for rat heart enzyme preparations observed in the absence of added ligands were lower than those for the rat brain enzyme preparations (Fig. 2, Table 2).

TABLE 1

Ion concentrations in Na^+,K^+-ATPase preparations

Element	Rat brain enzyme	Rat heart enzyme
	μM[a]	μM[a]
Na	934	1205
K	8	85
Mg	16	58
Ca	24	105
Al	<1	<2.5

[a] Protein concentrations of rat brain and rat heart enzyme preparations were adjusted to 2 mg/ml and 5 mg/ml, respectively. Therefore, the above values represent metal ion concentrations in the incubation mixture derived from enzyme preparations.

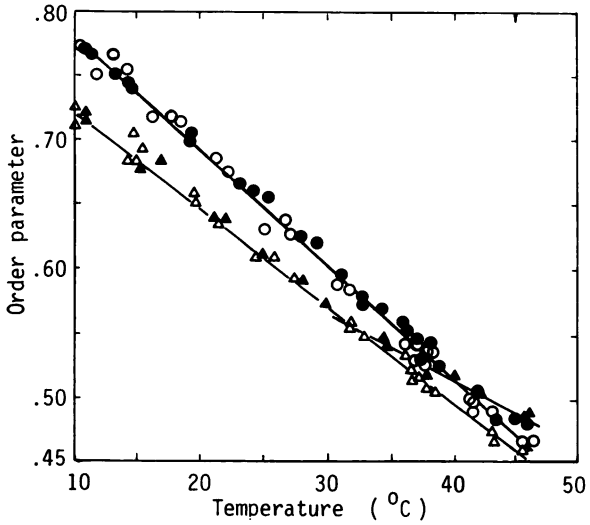

Fig. 2. Temperature dependence of 5DS order parameter in rat brain and rat heart Na^+,K^+-ATPase preparations. Rat brain (○, ●) or rat heart (△, ▲) enzyme preparations were suspended in 50 mM Tris-HCl buffer solution (pH 7.5) in the absence (○, △) or presence (●, ▲) of 15 mM KCl. In this and following studies, order parameters were calculated as described by Schreier *et al.* (17), and regression lines were fitted by the least squares method.

ESR spectra in the presence of ligands. The addition of K^+ significantly increased the membrane order parameter of rat heart enzyme preparations at high temperatures in a medium containing 50 mM Tris-HCl buffer without added Mg^{2+}, Na^+, or ATP (Fig. 2). This effect of K^+ was not observed in the rat brain enzyme preparations. In rat brain enzyme preparations, no indication of an abrupt change in the temperature dependence of the membrane order parameter was observed in the presence of K^+ in the temperature range of 10–47°, whereas an apparent structural transition was detected at 31.8° in the heart enzyme preparations.

In the presence of Na^+, Mg^{2+}, and ATP, but not K^+, nearly 100% of the enzyme molecules exist in the phosphorylated form (21). The order parameter of the membrane lipids associated with the rat brain or rat heart enzyme preparations observed under these conditions at temperatures below 30° was not significantly different from that observed at the corresponding temperature in the absence of added ligands (Table 2). In the presence of the above ligands, however, an abrupt change in the temperature dependence of the order parameter occurred at approximately 30° and 35° for the brain and heart enzyme preparations, respectively (Fig. 3A, Table 2). Therefore, above these temperatures, the order parameter was higher in the presence of the added ligands compared to corresponding values observed in their absence.

When the Na^+,K^+-ATPase preparations were incubated in the presence of 15 mM KCl in addition to 5 mM $MgCl_2$, 100 mM NaCl, and 5 mM Tris-ATP, the temperature dependence of the order parameter of either brain or heart enzyme preparation did not follow a simple linear function (Fig. 3B). An apparent transition temperature observed with brain enzyme preparations was lower than that obtained with heart enzyme preparations (Table 2).

ESR spectra in the presence of ouabain. Several investigators (22, 23) have proposed that cardiac glycosides, such as ouabain, alter certain properties of membrane lipids associated with Na^+,K^+-ATPase by combining with the enzyme and indirectly affecting a large number of lipid molecules. Therefore, the effect of ouabain on the order parameter was studied in an attempt to determine whether ouabain induces a change in the physical state of the associated lipids. The addition of 1 μM ouabain (final concentration) to the incubation medium containing Na^+, Mg^{2+}, and ATP failed to cause appreciable changes in the order parameter of rat brain enzyme preparations (Table 2). A slight increase in the transition temperature for the brain enzyme lipids was noted; however, it was not possible to determine if ouabain significantly affected the transition temperature as confidence limits could not be assigned to the position of the transition. The addition of 15 mM KCl (final concentration) to the above incubation mixture did not affect the order parameter significantly (Table 2). In the absence of ouabain, the transition temperature of the brain enzyme preparation observed in an incubation mixture containing Mg^{2+}, Na^+, and

TABLE 2

Order parameters and their transition temperatures

Concentrations of ligands were: $MgCl_2$, 5 mM; NaCl, 100 mM; Tris-ATP, 5 mM; KCl, 15 mM. Tris-HCl buffer (pH 7.5, 50 mM) was present in all incubation mixtures.

Enzyme preparations	Ligands	Order parameters		Transition temperature
		at 20°	at 40°	
				°C
Rat brain	None	0.693	0.514	None
	K^+	0.693	0.514	None
	Mg^{2+}, Na^+, ATP	0.707	0.580	30.4
	Mg^{2+}, Na^+, ATP, K^+	0.700	0.580	20.0
	Mg^{2+}, Na^+, ATP, 1 μM ouabain	0.700	0.574	33.2
	Mg^{2+}, Na^+, ATP, K^+, 1 μM ouabain	0.708	0.577	30.9
Rat heart	None	0.650	0.500	None
	K^+	0.650	0.519	31.8
	Mg^{2+}, Na^+, ATP	0.663	0.542	34.5
	Mg^{2+}, Na^+, ATP, K^+	0.657	0.536	31.6
	Mg^{2+}, Na^+, ATP, 100 μM ouabain	0.665	0.529	None
	Mg^{2+}, Na^+, ATP, K^+, 100 μM ouabain	0.666	0.540	None
Dog brain	Mg^{2+}, Na^+, ATP	0.724	0.584	26.4
	Mg^{2+}, Na^+, ATP, 100 μM ouabain	0.730	0.579	None
Dog heart	Mg^{2+}, Na^+, ATP	0.679	0.564	28.4
	Mg^{2+}, Na^+, ATP, 100 μM ouabain	0.694	0.563	None

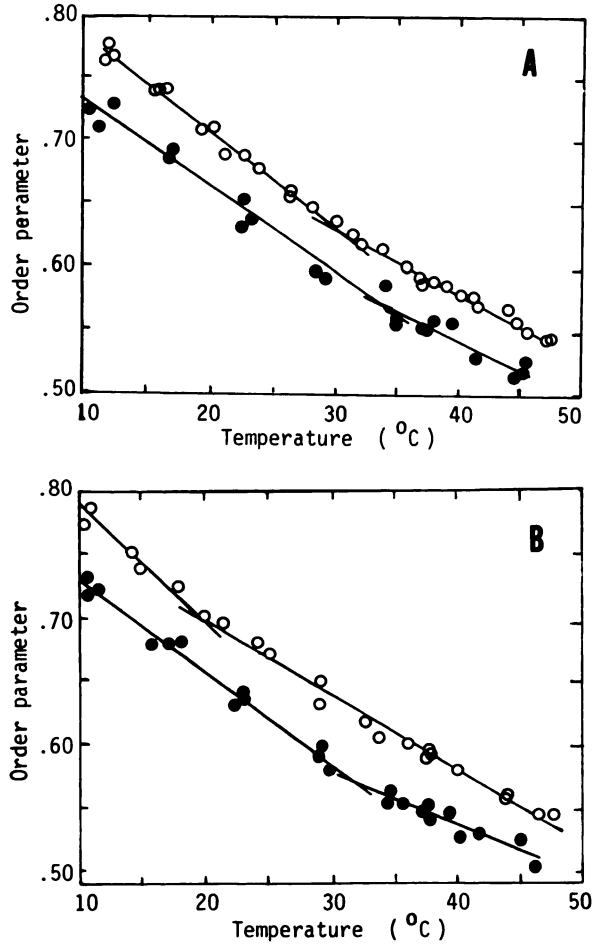

Fig. 3. Temperature dependence of 5DS order parameter in rat brain and rat heart Na^+,K^+-ATPase preparations in the presence of added ligands. Rat brain (○) or rat heart (●) enzyme preparations were suspended in a medium containing 5 mM $MgCl_2$, 100 mM NaCl, and 5 mM Tris-ATP (A) or 5 mM $MgCl_2$, 100 mM NaCl, 5 mM Tris-ATP, and 15 mM KCl (B).

ATP decreased by 10.4° with the addition of 15 mM KCl; however, in the presence of 1 μM ouabain, K^+ caused only a 2.3° decrease in the transition temperature.

Rat heart Na^+,K^+-ATPase has a substantially lower affinity for ouabain compared to the rat brain enzyme (12, 14, 15), necessitating the use of a high concentration of ouabain for complete enzyme inhibition. When 100 μM ouabain (final concentration) was added to the rat heart Na^+,K^+-ATPase preparations in the presence of 100 mM NaCl, 5 mM $MgCl_2$, and 5 mM Tris-ATP, the structural transition could no longer be detected, although the order parameters of probed lipids were not markedly affected (Table 2). The effects of 100 μM ouabain observed in a medium containing 15 mM KCl, in addition to Na^+, Mg^{2+}, and ATP, were virtually the same as those observed in a medium without K^+. These effects of ouabain addition, i.e., elimination of the apparent structural transition with little alteration in the lipid order parameter, were observed also with rat brain Na^+,K^+-ATPase preparations when the concentration of the glycoside was 100 μM (data not shown).

The lipid order parameters of dog brain and heart enzyme preparations were similar to those of rat brain and heart enzyme preparations, respectively (Table 2). The structural transition temperatures of dog brain and heart enzyme preparations observed in the presence of Mg^{2+}, Na^+, and ATP appear to be lower than the corresponding values for rat brain and heart enzyme preparations. The addition of 1 μM ouabain failed to cause marked changes in the order parameter or the lipid phase transition temperature with either dog brain or heart enzyme preparations (data not shown). A higher concentration (100 μM) of ouabain, however, eliminated the apparent structural transition with little alteration in the lipid order parameter in either dog brain or heart enzyme preparation (Table 2).

Na^+,K^+-ATPase activity. Spin probe studies yield information on bulk lipids contained in these relatively crude enzyme preparations. The present results indicate that spin probes are in a relatively homogeneous environment. It is possible, however, that those membrane lipids closely associated with Na^+,K^+-ATPase have different physical characteristics compared to the bulk lipids. Therefore, the effect of tem-

perature on membrane lipids closely associated with Na^+,K^+-ATPase was estimated from the transition temperature on Arrhenius plots of enzyme activity. Arrhenius plots of Na^+,K^+-ATPase activity observed with rat brain enzyme preparations showed a clear break point at 25.6° (Fig. 4A). The statistical probability that the observed values are represented by two intersecting lines is greater than 95%. The results obtained with rat heart enzyme preparations were less clear (Fig. 4B). The computer-assisted curve-fitting technique indicates that the probability of having a break point is 77% and the break point is approximately 32°.

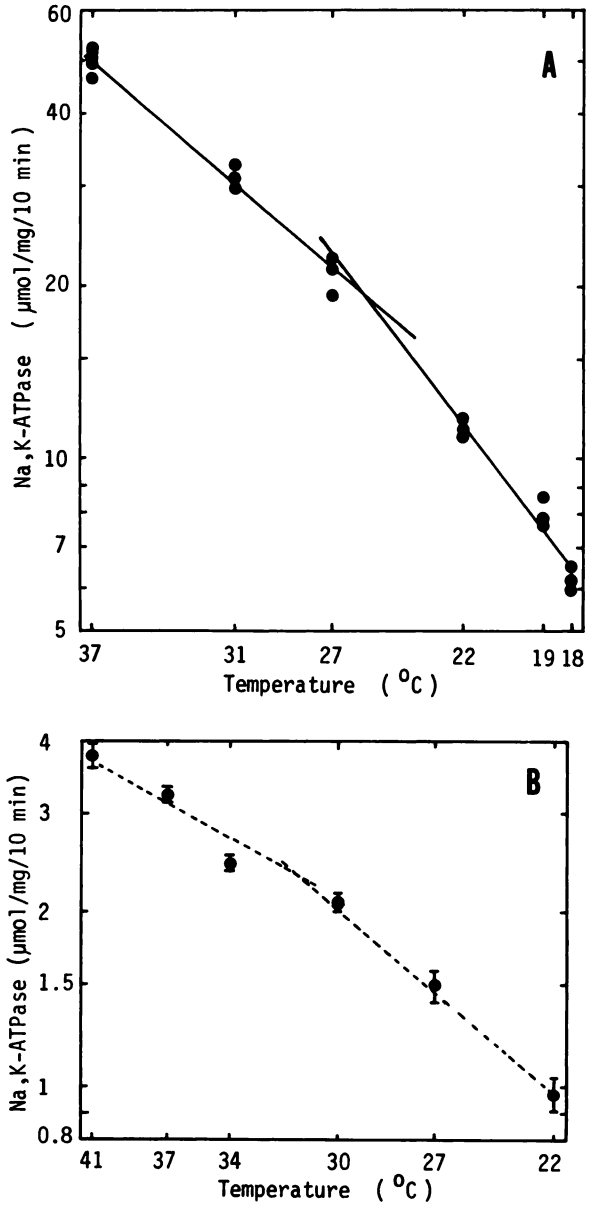

Fig. 4. Arrhenius plots of Na^+,K^+-ATPase activity. Enzyme preparations were obtained from rat brain (A) or rat heart (B) and their activities were assayed at the indicated temperature in the presence of 100 mM NaCl, 15 mM KCl, 5 mM $MgCl_2$, 5 mM Tris-ATP, and 50 mM Tris-HCl buffer (pH 7.5). Mg^{2+}-ATPase activity assayed in the absence of NaCl and KCl was subtracted to calculate Na^+,K^+-ATPase activity. *Numbers* along the *abscissa* are converted from $1/T$ (reciprocal of absolute temperature) for Arrhenius plots. *B*. Each point represents the mean of three to seven experiments. *Vertical lines* indicate standard error.

Discussion

The aims of this study were to examine (a) whether there are differences in physical properties of the membrane lipids which may be related to low affinity of rat heart Na^+,K^+-ATPase for ouabain; (b) whether ouabain is capable of inducing changes in the membrane lipids; and (c) whether such an effect of ouabain, if observed, is related to the specific interaction of the glycoside with Na^+,K^+-ATPase. Alterations of membrane lipids, resulting from detergent treatments that are required to obtain highly purified enzymes, may modify their physical properties or interaction with Na^+,K^+-ATPase. For example, excessive detergent or glycerol treatment has been shown to alter the characteristics of the glycoside-binding sites or the membrane lipids closely associated with the binding site on Na^+,K^+-ATPase (7). Therefore, relatively crude enzyme preparations presumably having relatively unaltered membrane lipids were used in the present studies.

The potential problem that spin probe studies yield information on bulk lipids, whereas membrane lipids associated with Na^+,K^+-ATPase may have characteristics different from those of the bulk lipids, was assessed from the comparison of ESR data with results obtained from the Na^+,K^+-ATPase assay. The discontinuity in Arrhenius plots of the enzyme activity is attributed to the interaction of the enzyme protein with tightly associated annular lipids (24–26). The Arrhenius plots obtained with Na^+,K^+-ATPase activity of the rat heart enzyme suggest a transition point at approximately 32°. This value is close to the transition temperature of 31.6° obtained with ESR studies of the same enzyme preparations. The transition temperature for rat brain enzyme activity was about 26°. This value is higher than the transition temperature of 20° obtained with the present spin probe studies in the presence of Na^+, K^+, Mg^{2+}, and ATP, i.e., under the ligand condition used in Na^+,K^+-ATPase assay. The latter value, however, is close to the reported value of approximately 19° for Na^+,K^+-ATPase preparations obtained from various sources (27, 28). The precise reason for this discrepancy is unknown; however, there would appear to be uncertainties in the present study in estimating Na^+,K^+-ATPase activity at low temperatures. This problem is confounded by the fact that confidence limits cannot be estimated for the transition temperature. It may be concluded, however, that the results obtained with the two methods are generally in good agreement with respect to the finding that lipid phase transition temperatures are lower in the rat brain than in the rat heart enzyme preparations.

Enzyme activity can be estimated only in the combined presence of Na^+, K^+, Mg^{2+}, and ATP. Therefore, ligand-induced changes in the properties of membrane lipids were examined using the order parameter obtained from 5DS spectra. The order parameter represents the deviation of the observed ESR signal from that of a uniformly oriented solid. This value is equal to unity for a rigid environment, and approaches zero for a highly fluid environment. Because the spin label is located at the 5-position of the stearic acid, the order parameter is sensitive to the physical state of the hydrophobic interior of the membrane (17). Generally, changes in the order parameter greater than 2% are regarded as significant with respect to the physical structure of membrane lipids (29).

As expected, the order parameter indicated a decreased membrane lipid order (increased fluidity) at higher temperatures. At 37°, the lipids in brain and heart enzyme preparations are

in a relatively disordered state. At corresponding temperatures, rat brain enzyme preparations had higher order parameters compared to rat heart enzyme preparations (Table 2). This difference, however, is tissue specific rather than related to the affinity of these enzymes for ouabain. Dog heart enzyme preparations, which have a high affinity for ouabain, had a lower order parameter similar to that of rat heart enzyme preparations. These results do not support the hypothesis that a difference in physical properties of the membrane lipids is the primary determinant of the affinity of Na^+,K^+-ATPase for the cardiac glycoside, although some modifications of glycoside affinity by changes in the membrane lipids have been reported (10, 11, 13).

In the presence of Mg^{2+}, Na^+, and ATP, i.e., under the conditions which favor phosphoenzyme formation (21), a transition in the temperature dependence of the order parameter was observed at about 30° in rat brain enzyme preparations. The addition of K^+ to this preparation, which causes dephosphorylation of the phosphoenzyme and a conformational change in enzyme protein (21), caused a marked decrease in the transition temperature. This effect of K^+ observed in rat brain enzyme preparations was reduced by 1 μM ouabain. With rat heart enzyme preparations, however, the comparable effect of ouabain could not be observed. The transition temperatures were generally higher in rat heart enzyme preparations and the effect of K^+ to reduce the transition temperature was smaller. Moreover, the 100 μM ouabain required to cause a complete inhibition of rat heart Na^+,K^+-ATPase activity abolished the transition. This effect of 100 μM ouabain is apparently unrelated to enzyme inhibition. In rat brain, dog brain, or dog heart enzyme preparations, 1 μM ouabain failed to cause similar effects (present results) although this concentration has been shown to be sufficient to inhibit Na^+,K^+-ATPase activity in these preparations (15, 30). A high concentration (100 μM) of ouabain, similar to that required for rat heart enzyme preparations, was necessary to abolish the transition in these enzyme preparations.

Kimelberg and Papahadjopoulos (31) suggested that cholesterol may interfere with Na^+,K^+-ATPase activity through general disordering of membrane lipids. Subsequently, Davis *et al.* (32) reported that the synthetic estrogen, ethynyl estradiol, inhibits hepatic Na^+,K^+-ATPase activity, presumably by a similar mechanism. Therefore, elimination of the apparent transition observed in the presence of a high concentration of ouabain probably results from the general disruptive properties that various steroids seem to have on the membrane lipid order. It should be pointed out that concentrations of ouabain higher than 100 μM are frequently used to ensure a complete inhibition of the sodium pump. The above results, however, indicate that effects of such high concentrations of ouabain are not limited to the specific inhibition of Na^+,K^+-ATPase or the sodium pump, and, therefore, results of Na^+,K^+-ATPase or sodium pump studies obtained by subtracting values observed with high concentrations of ouabain should be interpreted cautiously.

Barnett and Palazzotto (33) observed no abrupt change in temperature dependence of the [^{3}H]ouabain binding observed in the presence of Mg^{2+}, Na^+, and ATP. Under similar ligand conditions, the present results indicate that brain and heart enzyme preparations obtained from the rat or dog show a transition in the temperature dependence of the order parameter at 30–35°. The transition at this relatively high temperature may be difficult to detect in the above study (33) because of the limited temperature range examined. Alternatively, the temperature and amplitude of the structural transition may be dependent on the source of the enzyme in addition to the ligand conditions. Ahrens (34) reported that the inflection temperature of the Arrhenius plots of Na^+,K^+-ATPase activity depends on the total concentration, but not on the specific properties, of added monovalent ions. The present results, however, indicate that the transition temperature observed with spin probe studies is specifically affected by the monovalent cation added.

The only action of ouabain observed at a low concentration which corresponds to enzyme inhibition observed with rat brain enzyme preparations was the elimination of the K^+-induced lowering of the transition temperature. Whether this action of ouabain is related to a ouabain-induced "conformational change" in Na^+,K^+-ATPase, proposed to be the mechanism of the positive inotropic action of the glycoside (22, 23), has yet to be examined. Unfortunately, it was not possible to observe similar effects of ouabain in the rat heart enzyme preparation because K^+ did not cause a marked effect on the transition temperature and because a high concentration of ouabain, which is required to cause an enzyme inhibition in this preparation, abolished the transition. It should also be pointed out that if the changes caused by glycoside binding to Na^+,K^+-ATPase are limited to those in annular lipids, the changes may not be detected by the spin probe studies.

In conclusion, there are significant differences in physical properties of the membrane lipids associated with rat brain and rat heart Na^+,K^+-ATPase preparations, as detected by an ESR probe, 5DS. The influence of differences in the physical state of the membrane lipids on the glycoside sensitivity of the Na^+,K^+-ATPase appears to be relatively minor. The binding of ouabain to the glycoside-binding sites on Na^+,K^+-ATPase seems to have a minimal effect on the physical state of the bound lipids. In high concentrations, such as those required to cause a significant inhibition of the rat heart enzyme, ouabain appears to cause a nonspecific change in the membrane lipids.

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