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MODULATION OF CARDIAC GLYCOSIDE INHIBITION OF $(Na^+ + K^+)$-ATPase BY MEMBRANE LIPIDS

DIFFERENCE BETWEEN SPECIES

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The role of lipids in the modulation of the ouabain-sensitivity of membrane $(Na^+ + K^+)$-ATPase from different species has been studied using a reconstitution procedure which promotes lipid exchange during detergent depletion by Sephadex chromatography. Hybrid reconstitution of delipidated $(Na^+ + K^+)$-ATPase preparations from bovine brain into the lipids obtained from crab nerve enzyme preparations significantly reduces the sensitivity of the brain enzyme to inhibition by ouabain. Conversely, reconstitution of crab nerve enzyme into the lipids from bovine brain enzyme preparations increases the sensitivity of the crab enzyme to ouabain inhibition. These opposing effects demonstrate the role of membrane lipids in modulating the enzyme-inhibition relationship in preparations from these different species.

Introduction

Wide differences in the potency of cardiac glycoside inhibition of $(Na^+ + K^+)$-ATPase preparations obtained from different animals and species has been well documented [1–4]. These differences, which frequently correlate with the clinical potencies of these drugs, have often been cited as supporting evidence for the view that the cardiac glycosides exert their clinical action via the inhibition of the sodium pump system at the cellular level [5–7]. Yet detailed study of the composition of either the glycoprotein or the catalytic sub-units of $(Na^+ + K^+)$-ATPase from a variety of sources [8,9] suggests that these moieties have remained remarkably constant throughout evolution [10]. Such observations must focus attention on the role of membrane lipids as possible modulators of the inhibitory action of cardiac glycosides.

While the original evidence demonstrated that cardiac glycosides inhibit $(Na^+ + K^+)$-ATPase by preventing the turnover of a phosphorylated intermediate [12], more recent work has suggested that the lipid components of the membrane matrix, with which the enzyme is strongly associated in situ, play an additional role in determining the sensitivity of $(Na^+ + K^+)$-ATPase to inhibition by these agents [11,13]. For example, the marked difference in inhibitor sensitivity between $(Na^+ + K^+)$-ATPase of homeotherms and poikilotherms has been attributed to changes in the physical properties of the surrounding membrane lipids, rather than to any difference in the enzyme protein [11].

The incorporation of delipidated enzyme proteins into liposomes of defined lipid composition provides an ideal system for the direct study of lipid-protein interactions. Recently we have devel-

oped a lipid-exchange procedure for the reconstitution of $(Na^+ + K^+)$-ATPase into liposomes [15,16] which, by using Sephadex chromatography for the rapid removal of detergent, avoids the denaturant effect of either direct sonication, or the effect of prolonged exposure of enzyme protein to relatively high concentrations of detergent during lengthy dialysis. In addition this procedure yields enzyme preparations of sufficiently high specific activity suitable for the examination of the effects of either exogenous or endogenous lipids on the inhibitory action of ouabain on reconstituted $(Na^+ + K^+)$-ATPase.

Because of the demonstrated difference in sensitivity to inhibition by ouabain of $(Na^+ + K^+)$-ATPase preparations from poikilotherms and homeotherms [11,20], in the present study we have investigated the role of the lipid matrix in modulating the ouabain sensitivity of $(Na^+ + K^+)$-ATPase preparations from bovine brain and crab nerve membranes.

Materials and Methods

Chemicals

Deoxycholic acid (sodium salt), ouabain octahydrate, Sepharose CL-4B were supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sephadex G-50 was purchased from Pharmacia (Canada) Ltd.

Supelco Inc. (Bellafonte, PA, U.S.A.) supplied the following phospholipids: dimyristoyl-, dipalmitoyl-, distearoyl- and dioleoyl-L-α-phosphatidylcholines. 1-Palmitoyl-2-oleoyl-L-α-phosphatidylcholine was obtained from Serdary Research Laboratories (London, Ontario, Canada).

Tris-ATP was prepared by ion-exchange chromatography of Na_2-ATP (Sigma) on a column of Dowex-50W (Bio-Rad Laboratories).

All other chemicals were of analytical grade purity.

Tissues

Fresh bovine (*Bos taurus*) brains were obtained from a local slaughterhouse and transported to the laboratory on crushed ice. After removing large blood vessels, the cerebral cortex was cut into approx. 50-g portions and immediately frozen in liquid N_2.

Axons were isolated from the walking legs of the cold-water crab *Cancer magister*, collected from a depth of 14–17 fathoms (ambient temperature 4–8°C) off Barkley Sound, Vancouver Island. The animals were kept alive in sea water (ambient temperature 6–8°C) at the Bamfield Marine Biology Station for 8–10 days prior to dissection. The nerves were quickly removed and immersed in an ice-cold buffer solution of 20 mM Tris/2 mM EDTA (pH 7.6), containing 0.9% NaCl. The pooled axons (approx. 10 g) from several animals were transferred into small plastic vials and rapidly frozen in an acetone/solid CO_2 mixture and stored at −20°C.

Preparation of membrane fractions

Bovine brain. Untreated membrane preparations of bovine brain were obtained by a procedure of Polytron homogenization followed by differential centrifugation described in full in a previous publication [14].

Crab nerve. The axons (approx. 10 g) were thawed in 250 mM sucrose/30 mM histidine/20 mM Tris/2 mM H_4-EDTA, pH 7.6 (homogenizing buffer). In order to remove the NaCl used in the storage solution, the nerves were washed twice using this sucrose-histidine-Tris medium by resuspension and centrifugation at $5000 \times g$ for 15 min in a Sorvall RC-2B centrifuge (rotor SS-34). The wet weight of the final pellet was noted and the axons were homogenized (20 ml buffer/g tissue) using a Polytron homogenizer (PT 20; Brinkmann Instruments Ltd.). Three separate bursts of 15 s, 10 s and 10 s (with a period of cooling in between) at setting 8 were employed to homogenize the axons. After removing the cellular debris by a low-speed spin ($2000 \times g$ for 15 min), the supernatant was centrifuged at $123\,000 \times g$ for 60 min in a Beckman L3-40 ultracentrifuge fitted with a Ti 60 rotor. The microsomes were washed by resuspension and centrifugation ($120\,000 \times g$ for 60 min) in 20 mM Tris/1 mM EDTA (pH 7.6). The washed pellets were taken up in 20 mM Tris/1 mM EDTA (pH 7.6), containing 250 mM sucrose, prior to extraction with deoxycholate.

Partial delipidation

After substrate protection by incubation of the enzyme with 5 mM ATP, both bovine and crab

membrane preparations were partially delipidated by extraction with 0.1% deoxycholate (in 20 mM Tris/1 mM EDTA, pH 7.6) for 30 min at 30°C. Enzyme preparations were recovered and washed free of excess detergent by centrifugation at 46 000 $\times g$ for 30 min (Sorvall SS-34 rotor) for bovine brain and 123 000 $\times g$ for 60 min (Beckman Ti 60 rotor) for crab nerve enzyme, respectively. Two further washes by resuspension in 20 mM Tris/1 mM EDTA buffer (pH 7.6) and centrifugation as described above were carried out before the final resuspensions of the enzyme preparation in 250 mM sucrose/20 mM Tris/1 mM EDTA (pH 7.6) prior to assay and storage at −20°C.

Lipid extraction and analysis

Membrane enzyme preparations, washed free of sucrose by centrifugation and resuspension in demineralized water, were extracted with chloroform/methanol (2 : 1, v/v) in the presence of 0.01% (w/v) butylated hydroxytoluene [17]. Total fatty acid analysis was carried out by the gas chromatographic procedure described previously [18]. Phospholipids were separaed into their major classes by thin-layer chromatography as described by Fine and Sprecher [30]. The phospholipid spots were visualized with iodine vapour and identified by comparison with purified standards. The spots were scraped from the plates and assayed for phospholipid phosphorous by the method of Bower and King [40].

Preparation of proteoliposomes by lipid exchange

For the reconstitution of $(Na^+ + K^+)$-ATPase, an aliquot of the partially delipidated enzyme preparation was thawed, assayed for ouabain-sensitive ATPase activity [14] and added to a detergent-lipid mixture to give a final protein concentration of 1.0–1.5 mg/ml. The detergent-lipid mixture was obtained from a total lipid fraction of either bovine brain membrane or crab nerve membrane enzyme preparations made by extraction of the enzyme preparations with chloroform/methanol (2 : 1, v/v) according to the method of Bligh and Dyer [17]. Dried lipids were hydrated in 20 mM Tris/1 mM EDTA buffer containing 1% deoxycholate to give a 3 : 1 molar ratio of detergent : phospholipid immediately prior to the addition of the partially delipidated membrane enzyme preparations.

After mixing by vortex action and incubation at room temperature for 5 min, the enzyme/lipid/detergent mixture was chromatographed on a column of Sephadex G-50 in 100 mM KCl/20 mM Tris/1 mM EDTA (pH 7.6) containing 0.02% (w/v) NaN_3.

Full details of the procedure for the formation of proteoliposomes during the removal of detergent by Sephadex chromatography, the subsequent chromatography of the void volume of this column on Sepharose CL-4B, the determination of the elution profiles of tracer labelled enzyme protein and detergent, and their separation from lipid are given in detail in other publications [15,16,19]. Reconstituted $(Na^+ + K^+)$-ATPase liposomes eluted from the Sepharose column were concentrated by centrifugation at 161 000 $\times g$ for 60 min in the Ti-60 fixed angle rotor of a Beckman L3-40 ultracentrifuge before resuspension of the proteoliposomes in a small volume of 20 mM Tris/1 mM EDTA buffer.

Ouabain dose-response curves

The inhibition of both native and reconstituted $(Na^+ + K^+)$-ATPase by ouabain was determined at not less than fifteen different concentrations of drug between 10^{-9} and 10^{-3} M, using the coupled optical assay procedure described in detail elsewhere [11,14,21]. A particular virtue of this system is the ease with which linear reaction rates can be determined, thus avoiding any potential problems which may arise from different rates of ouabain interaction. For both enzyme preparations, linear rates of reaction (in the presence of ouabain) were achieved within a few minutes. All assays were carried out at 37° ± 0.2°C. The enzyme activity was measured in the presence of 2 mM Mg^{2+}, 20 mM K^+ and 80 mM Na^+, plus varying concentrations of ouabain. The maximum inhibition by ouabain occurred when the ouabain-inhibited enzyme activity was similar to the basal activity observed in the absence of 80 mM Na^+. The ouabain concentration which caused a 50% inhibition of the maximum activity (I_{50}) of $(Na^+ + K^+)$-ATPase activity was calculated by using the linear portion of the log dose-response curve (usually between 30–70% inhibition). The I_{50} value was obtained by computer regression analysis and the difference in I_{50} values between enzyme prepara-

tions were analyzed by means of Student's *t*-test (unpaired) at the 1% level. Protein concentration was assayed according to the method of Peterson [22] with bovine serum albumin as the standard.

Results

Previous studies in our laboratory have shown that the partially delipidated preparations of bovine brain $(Na^+ + K^+)$-ATPase can be reconstituted into either egg phosphatidylcholine or dioleoylphosphatidylcholine liposomes with almost complete recovery of enzyme activity [16,19]. In the present study it was first necessary to determine both the extent of reactivation and the possible effect on ouabain inhibition of reconstitution of $(Na^+ + K^+)$-ATPase into liposomes of their endogenous lipids. The results are summarized in Table I, which demonstrates that, although there is some loss of enzyme activity when either bovine brain or crab nerve $(Na^+ + K^+)$-ATPase are reconstituted into liposomes made from the total lipid extracts of their native membranes, no change occurs in the I_{50} value for ouabain inhibition. Serial substitution of endogenous lipids by repeated exposure of enzyme to the lipid/detergent/Sephadex G-50 chromatography procedure (three cycles) leads to a further loss in enzyme activity, but does not influence the I_{50} value for ouabain inhibition. Thus, in confirmation of our earlier findings with egg phosphatidylcholine, the process of reconstitution by lipid exchange is without effect upon the sensitivity of the enzyme preparation to inhibition by ouabain. In addition, there is more than a 500-fold difference in the concentration of ouabain necessary to half-maximally inhibit the $(Na^+ + K^+)$-ATPase enzyme of reconstituted preparations of crab nerve compared to the reconstituted enzyme obtained from bovine brain, exactly parallel to the difference in ouabain sensitivity seen in native enzyme preparations [11].

Earlier studies from this laboratory led to the suggestion that the sensitivity of $(Na^+ + K^+)$-ATPase to ouabain inhibition resides at least in part in the lipid component of the membrane-bound enzyme system [18,23–25]. If these speculations are correct, then one would expect to find a difference in the membrane lipid composition between bovine brain and crab nerve $(Na^+ + K^+)$-ATPase preparations. The distribution of the phospholipid classes of the partially purified $(Na^+ + K^+)$-ATPase membrane preparations is given in Table II. It is clear that after enzyme activation by partial delipidation with 0.1% deoxycholate in the presence of ATP (see under Methods), the enzyme preparations from bovine brain and crab nerve have remarkably similar phospholipid compositions. Phosphatidylethanolamine and phosphatidylcholine occur in nearly equal proportions in

TABLE I

SPECIFIC ACTIVITY AND SENSITIVITY TO INHIBITION BY OUABAIN OF RECONSTITUTED ATPase IN LIPOSOMES OF ENDOGENOUS LIPID

Values given are means ± S.E. The specific activity is expressed in μmol ATP hydrolysed per mg protein per h at 37°C. n.s., not significant at 1% level.

Enzyme	Conditions	*n*	Specific activity		I_{50} (M)	*P*
			Mg^{2+}-ATPase	$(Na^+ + K^+)$-ATPase		
Bovine	Control	4	13.0 ± 4.6	95.4 ± 7.2	$(3.1 \pm 0.4) \cdot 10^{-7}$	n.s.
Bovine	Single substitution	4	6.7 ± 1.1	63.1 ± 8.7	$(4.0 \pm 0.4) \cdot 10^{-7}$	
Bovine	Control	3	17.7 ± 2.4	145.0 ± 14.7	$(3.2 \pm 0.4) \cdot 10^{-7}$	n.s.
Bovine	Serial substitution	3	3.8 ± 1.0	37.2 ± 10.7	$(2.6 \pm 0.1) \cdot 10^{-7}$	
Crab	Control	3	2.6 ± 1.0	17.6 ± 2.6	$(1.5 \pm 0.1) \cdot 10^{-4}$	n.s.
Crab	Single substitution	3	0.6 ± 0.1	13.0 ± 0.8	$(1.8 \pm 0.2) \cdot 10^{-4}$	

TABLE II

THE PHOSPHOLIPID COMPOSITION OF PARTIALLY PURIFIED MEMBRANE ATPase PREPARATIONS FROM BOVINE BRAIN AND CRAB NERVE

Values given are mol% total phospholipid (mean ± S.E.; $n = 6$). PE, phosphatidylethanolamine; PC, phosphatidylcholine; Sph, sphingomyelin; PS. phosphatidylserine; PI, phosphatidylinostol.

Enzyme	PE	PC	Sph	PS	PI
Bovine	36.8 ± 0.8	36.0 ± 1.6	11.0 ± 0.9	13.3 ± 1.1	2.8 ± 0.6
Crab	33.3 ± 2.1	31.7 ± 2.4	19.2 ± 1.4	10.2 ± 1.0	5.5 ± 0.5

both preparations, and amount to at least 65% of the total phospholipids. Phosphatidylserine and phosphatidylinositol are also similar in both preparations accounting for 15% of the total phospholipids. The sphingomyelin composition of the crab $(Na^+ + K^+)$-ATPase membrane preparation (19.2 mol%) was significantly greater ($P < 0.01$) than that of the bovine brain enzyme preparation (11.0 mol%). In addition, dramatic differences in the membrane lipid fatty acid composition were also found in these two enzyme preparations. Table III summarizes the total fatty acid profile of both beef brain and crab nerve membrane enzyme preparations. It is clear that crab nerve enzyme preparations contain less saturated fatty acids and more unsaturated fatty acids than do bovine brain enzyme preparations. In particular the long chain unsaturated acids $C_{20:1}$ and $C_{20:5}$, which are characteristic of the lipids of many marine species, amount to more than 25% of the total fatty acids of crab nerve enzyme preparations, but occur in only small amounts in the membrane enzyme preparations from bovine brains. It is also clear from this data (Table III) that in bovine brain enzyme only 2% of the total saturates contributed towards 'long' chain (C > 20) fatty acids. In contrast, 27% of the total saturates of lipids of $(Na^+ + K^+)$-ATPase from crab nerve contained fatty acids with 'longer' chain lengths. Among the unsaturates, 56% of the total unsaturated fatty acids of bovine brain enzyme consisted of 'long' chain lengths, while in the crab enzyme this value had increased to 71%.

As the membrane sphingomyelin and fatty acyl composition of beef brain and crab nerve preparations differ significantly, the role of lipids in modulating the ouabain-sensitivity of $(Na^+ + K^+)$-ATPase was investigated using a 'cross-over' or

TABLE III

FATTY ACID COMPOSITION OF BOVINE BRAIN AND CRAB NERVE MEMBRANE PREPARATIONS OF ATPase

The methods for preparation of the total lipid extracts and analysis of the total fatty acid profile of bovine brain and crab nerve membrane preparations are described in the text. The values shown are the average of triplicate determinations that agreed within 0.3% for an individual component, and within 2.5% for the total fatty acid composition. Trace is less than 0.3%.

Fatty acid	% Total lipid	
	Bovine brain	Crab nerve
14:0	1.8	trace
iso 16:0	3.6	1.0
16:0	21.7	10.4
18:0	21.1	11.0
20:0	trace	0.8
22:0	trace	6.0
24:0	1.1	1.5
Σ Sat	49.3	30.7
16:1	trace	1.5
18:1	20.6	16.3
18:2 (ω-6)	0.8	0.9
18:3 (ω-3)	trace	1.0
20:1	1.2	5.2
20:2 (ω-6)	0.4	1.4
20:3 (ω-3)	0.5	0.9
20:4 (ω-6)	6.2	3.0
20:5 (ω-3)	trace	21.2
22:1	1.2	trace
22:4 (ω-6)	4.2	1.6
22:5 (ω-3)	1.1	trace
22:6 (ω-3)	12.7	15.6
Σ Unsat	48.9	68.6
Σ (ω-3)	14.3	38.7
Σ (ω-6)	11.6	6.9
Σ (ω-3)/(ω-6)	1.2	5.6

hybrid reconstitution process. In these experiments, hybrid preparations of $(Na^+ + K^+)$-ATPase were obtained with the enzyme protein from one species being reconstituted into the membrane lipids from the other species. The results of such substitution experiments are given in Table IV. Unlike the reconstitution of bovine brain $(Na^+ + K^+)$-ATPase into its endogenous lipids, reconstitution of the bovine enzyme into lipids extracted from crab nerve membranes had a significant effect upon the I_{50} value for ouabain. Thus, the concentration of ouabain necessary to cause a one-half maximal inhibition of $(Na^+ + K^+)$-ATPase was significantly increased ($P < 0.01$) from $(3.1 \pm 0.4) \cdot 10^{-7}$ M to $(1.4 \pm 0.2) \cdot 10^{-6}$ M. This decrease in sensitivity to ouabain became even more apparent after further substitution which involved three successive cycles of the detergent/lipid/Sephadex G-50 chromatography procedure, and resulted in an I_{50} of $(2.6 \pm 0.2) \cdot 10^{-6}$ M. Possibly even greater shifts in I_{50} might be obtained by further lipid exchange, but the decrease in enzyme activity that would occur (see Table I) makes impractical the determination of accurate dose-response curves for ouabain inhibition after more than three cycles of lipid exchange.

Similar results were seen when preparations of crab nerve $(Na^+ + K^+)$-ATPase were reconstitution in liposomes of beef membrane lipids. Here the sensitivity of the crab enzyme preparations to inhibition by ouabain was increased significantly ($P < 0.01$) by a single exposure to bovine brain membrane lipids (Table IV). The value for one-half maximal inhibition of crab nerve $(Na^+ + K^+)$-ATPase was decreased from $(1.5 \pm 0.1) \cdot 10^{-4}$ M in the control preparation, to $(5.4 \pm 0.9) \cdot 10^{-5}$ M in the preparations reconstituted with bovine brain membrane lipids. In contrast to the results of the bovine enzyme/crab lipid reconstituted system, no further change in the I_{50} value for ouabain occurred by serial (three cycles) substitution with bovine brain membrane lipids.

Thus, opposing effects were obtained with hybrid enzyme preparations from homeotherms and poikilothermic species. This is better illustrated by the mean dose-response curves shown in Fig. 1, where the effect of serial substitution by exogenous lipids on both bovine brain and crab nerve membrane $(Na^+ + K^+)$-ATPase is given. It can be seen from this figure that the differences in mean I_{50} are also reflected in the concentrations of ouabain that are necessary to produce maximal inhibition. There is little difference between the overall shape of the dose-response curves obtained with hybrid reconstituted enzyme preparations, from those obtained after reconstitution in their endogenous lipid controls.

There is now much evidence to indicate that the physical properties of membrane lipids are vitally important for the optimum function of $(Na^+ +$

TABLE IV

SPECIFIC ACTIVITY AND SENSITIVITY TO OUABAIN OF RECONSTITUTED CONTROL AND HYBRID ATPase ENZYMES IN LIPOSOMES OF ENDOGENOUS AND EXOGENOUS LIPIDS

Values given are means ± S.E. The specific activity is expressed in μmol ATP hydrolysed per mg protein per h at 37°C.

Enzyme	Lipid	n	Specific activity		I_{50} (M)	P
			Mg^{2+}-ATPase	$(Na^+ + K^+)$-ATPase		
Bovine	Bovine (single)	4	17.4 ± 3.4	131.6 ± 14.9	$(3.1 \pm 0.4) \cdot 10^{-7}$	–
Bovine	Crab (single)	4	3.5 ± 0.7	47.9 ± 1.1	$(1.4 \pm 0.2) \cdot 10^{-6}$	[a]
Bovine	Crab (serial)	5	2.4 ± 0.4	36.3 ± 4.9	$(2.6 \pm 0.2) \cdot 10^{-6}$	[a]
Crab	Crab (single)	4	1.7 ± 0.1	13.9 ± 0.9	$(1.5 \pm 0.1) \cdot 10^{-4}$	–
Crab	Bovine (single)	5	0.9 ± 0.1	5.9 ± 0.8	$(5.4 \pm 0.9) \cdot 10^{-5}$	[a]
Crab	Bovine (serial)	4	1.4 ± 0.3	6.2 ± 1.4	$(5.1 \pm 0.3) \cdot 10^{-5}$	[a]

[a] I_{50} value is significantly different from the respective control at the 1% level.

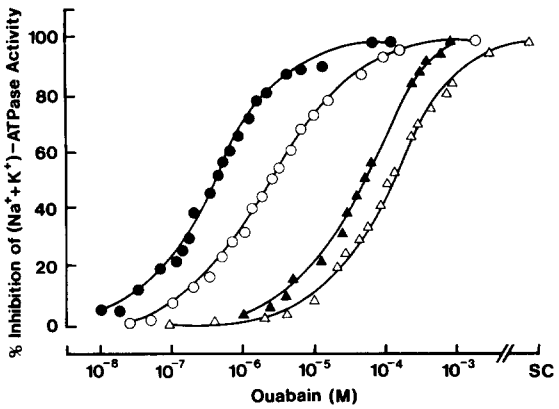

Fig. 1. Dose-response curves of inhibition of reconstituted $(Na^+ + K^+)$-ATPase by ouabain. SC is the saturation concentration of ouabain in the assay medium described previously [11,14] and is at least $1.5 \cdot 10^{-2}$ M. All assays (37°C) were carried out in duplicate or more. The points shown are the means of at least four separate experiments. $I_{(max)}$ is that concentration of ouabain which inhibited the activity of $(Na^+ + K^+)$-ATPase to the level of basal activity determined in the absence of 80 mM Na^+. The values for $I_{(max)}$ are: ●———●, bovine enzyme in bovine lipid, $6 \cdot 10^{-5}$ M; ○———○, bovine enzyme in crab lipid, $9 \cdot 10^{-4}$ M; ▲———▲, crab enzyme in bovine lipid, $2 \cdot 10^{-3}$ M; △———△, crab enzyme in crab lipid, $1.5 \cdot 10^{-2}$ M.

$K^+)$-ATPase enzyme system [16,26–28]. Maximum enzyme activity has frequently been observed when the membrane lipids are in a fluid state [16,26,27,29]. Previous studies with a fluorescent membrane probe [11] and the present total lipid fatty acid analysis data (Table III) suggest a different degree of 'fluidity' between the bovine brain and crab nerve enzyme preparations. Since changes in lipid 'fluidity' alter the function of $(Na^+ + K^+)$-ATPase [16,27,29], it seems possible that the interaction of specific inhibitors (such as ouabain) with this enzyme system is influenced by the physical state of membrane lipids. Table V summarizes the results of a comparative experiment where the effect of changes in the fluidity of the membrane lipid phase on the ouabain sensitivity of bovine brain membrane $(Na^+ + K^+)$-ATPase was investigated. Although our earlier results with a series of synthetic phosphatidylcholines revealed a good correlation between $(Na^+ + K^+)$-ATPase activity on the one hand, and the thermal transition temperature of the phospholipids on the other [16], the results shown in Table V do not support the idea that membrane lipid fluidity alone can modulate the ouabain sensitivity of $(Na^+ + K^+)$-ATPase. Rather, a relationship between enzyme activity and the chain length of the constituent membrane fatty acids seems possible. This suggestion is reinforced by the finding that the phospholipid class distribution was very similar between the two different membrane enzyme preparations studied.

TABLE V

EFFECT OF MEMBRANE LIPID FLUIDITY ON THE INHIBITION OF BOVINE BRAIN $(Na^+ + K^+)$-ATPase BY OUABAIN

Reported phase transition temperatures T_c of synthetic phosphatidylcholines are taken from the literature which is cited in full in a previous paper [16]. The specific activity of these reconstituted preparations, the method of reconstitution into various phosphatidylcholine liposomes and the determination of the I_{50} values for ouabain inhibition at 37°C are all described in the text and in Ref. 16. DOPC, dioleoyl-PC; DMPC, dimyristoyl-PC; DPPC, dipalmitoyl-PC; DSPC, distearoyl-PC; 1-P-2-O-PC, 1-palmitoyl-2-oleoyl-PC. The I_{50} values shown are means ± S.E. n.s., the differences from the control value is not significant at the 1% level.

	T_c(°C)	n	I_{50} (M)($\times 10^7$)	P
E/bovine lipid	–	5	4.1 ± 0.4	–
E/DOPC	−22	4	3.5 ± 0.5	n.s
E/1-P-2-O-PC	−2.6	4	5.1 ± 0.6	n.s
E/DMPC	+23	4	4.0 ± 0.5	n.s
E/DPPC	+41	3	6.4 ± 0.5	n.s
E/DSPC	+58	4	7.2 ± 1.2	n.s

Discussion

Although there is a considerable body of evidence in the literature to indicate that the lipids of the membrane matrix are able to influence the activity of $(Na^+ + K^+)$-ATPase [5,26,27,29], direct evidence for their role in modulating the ouabain-sensitivity of this enzyme system has been lacking. However, more recent studies which compared the enzyme from homeotherms and poikilotherms [11], or the seasonal variations which occur in hibernating animals [23,24] indicate that despite the apparent consistency of the distribution of phospholipid classes in the various membrane preparations studied [25], the differences found in the cardiac-glycoside receptor properties of $(Na^+ + K^+)$-ATPase are more likely to be due

to alterations in the fatty acyl components of the supporting membranes, rather than to any structural or molecular changes in the $(Na^+ + K^+)$-ATPase protein itself [23,24]. In support of these previous reports, in the present study we have observed that the ouabain sensitivity of reconstituted $(Na^+ + K^+)$-ATPase is capable of significant modulation by the lipid composition of the supporting liposomes.

No changes in the cardiac glycoside inhibitor sensitivity of $(Na^+ + K^+)$-ATPase are seen following reconstitution of the enzyme into liposomes of endogenous membrane lipids. However, significant shifts in the dose-response curves were observed when the enzyme was reconstituted into exogenous membrane lipids of similar phospholipid class distribution, but different amounts of long-chain saturated and unsaturated fatty acids. It seems reasonable to interpret these shifts in the dose-response curves as changes in the apparent affinity constant of the $(Na^+ + K^+)$-ATPase 'receptor complex' for the ligand ouabain. These shifts result in either an increase or a decrease in drug sensitivity, depending upon the source of the exogenous lipid. Thus, this action is probably due to a direct lipid-protein interaction, and not to either some differences in the structure of the enzyme protein or be caused by the process of reconstitution itself. In addition, preliminary experiments have revealed that the protein sub-units of both beef brain membrane and crab nerve membrane enzyme preparations are remarkably similar, as judged by their electrophoretic mobilities in polyacrylamide gels [19].

As the effect of crab lipids on bovine enzyme is increased by serial substitution of the exogenous lipids, it seems likely that only partial substitution of endogenous lipids occurs at the first exposure to crab lipids, and that increased substitution can be achieved by repeated detergent solubilization in the presence of exogenous lipid followed by chromatography. However, the resistance of the crab preparations to further change after the initial substitution with exogenous lipids suggests that either the process is 'complete' after one cycle of substitution – that is that maximum exchange has occurred – or that the remaining endogenous lipids in crab nerve preparations are too tightly bound for further exchange to occur via this process.

Although the inherently much lower specific activity of the crab nerve membrane enzyme makes resolution of these hypotheses very difficult, our recent findings [16] indicate that the reconstitution technique employed in this study – detergent depletion using column chromatography – results in about 70% and 90% substitution of exogenous lipid after the single and serial (three cycles) substitution processes respectively, irrespective of the fluidity of the exogenous lipid employed. However, the difficulty of 'activating' crab nerve $(Na^+ + K^+)$-ATPase by exposure to detergents [19], and the diminished shift in the dose-response curve for crab enzyme preparations exposed to lipids from bovine brain membrane enzyme, supports the view that crab nerve preparations contain a small but very 'tightly bound' residual lipid fraction resistant to complete exchange.

While it is tempting to interpret these latter results in terms of an immobilized layer of lipid immediately adjacent to the macromolecular transmembrane crab enzyme protein molecule, analogous to the boundary layer of lipid described by others [31–34], it should be recalled that recent evidence obtained from nuclear magnetic resonance studies of membranes does not support the concept of long-lived boundary or annular layers of lipid which regulate enzyme activity [35]. Although the phospholipid class distribution was very similar in both enzyme preparations, the changes in membrane lipid fatty acid composition between the crab and bovine preparations support our earlier view, derived from a comparison of the mobility of fluorescent probes in these systems [11], which suggested that the 'fluidity' of crab nerve enzyme preparations exceeds that of bovine brain preparations at any given temperature in the physiological range. However, the limited number of experiments with synthetic phosphatidylcholines reported here (Table V) do not support the idea that changes in the 'bulk' lipid fluidity alone can modulate the ouabain-sensitivity of $(Na^+ + K^+)$-ATPase.

Conversely, the very significant difference in the percentage composition of the long chain fatty acids ($C > 20$) that was found in these two enzyme preparations, whether saturated or unsaturated, may contribute significantly to the difference in properties between bovine brain and crab nerve

enzyme preparations. In this regard a recent study by Metcalfe and his colleagues has suggested that the thickness of the membrane bilayer, which is a function of the length of the fatty acyl chains of the membrane phospholipids, will influence the activity of $(Na^+ + K^+)$-ATPase [37]. In addition, the regulation of both enzyme activity and sensitivity to inhibitors by the chain length and the degree of unsaturation of phospholipid acyl chains has been reported for at least one other membrane-bound ATPase enzyme system, namely that of the mitochondrial membrane [38,39]. Thus it can be speculated that the chain length and the degree of unsaturation of phospholipid acyl chains of the lipid micro-environment of $(Na^+ + K^+)$-ATPase play a major role in the modulation of its cardiac-glycoside receptor properties, at least in the two species studied here.

However, the recently reported hybrid lipid exchange study of Hegyvary et al. [36], which employed a centrifugation procedure for enzyme reactivation rather than the column chromatography technique used here, failed to demonstrate any change in the ouabain-sensitivity of cardiac $(Na^+ + K^+)$-ATPase after reactivation in hybrid lipids from the dog or the rat. Whether this reflects some difference in the extent of exchange between exogenous and endogenous lipids between these different experimental procedures, or some unique property of cardiac membrane $(Na^+ + K^+)$-ATPase such as the presence of cardiolipin, which is not shared by membrane enzyme preparations from bovine brain or crab nerve, cannot be determined until cardiac $(Na^+ + K^+)$-ATPase preparations have been subjected to the type of lipid exchange utilized in the experiments reported in this paper.

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