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RESPONSE OF RAT HEART MEMBRANES AND ASSOCIATED ION-TRANSPORTING ATPases TO DIETARY LIPID

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The effects of different dietary fat intake on the lipid composition and enzyme behaviour of sarcolemmal $(Na^+ + K^+)$ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase from rat heart were investigated. Rat diets were supplemented with either sunflower seed oil (unsatd./satd. 5.6) or sheep kidney fat (unsatd./satd. 0.8). Significant changes in the phospholipid fatty acid composition were observed in both membranes after 9 weeks dietary lipid treatment. For both membranes, the total saturated/unsaturated fatty acid levels were unaffected by the dietary lipid treatment, however the proportions of the major unsaturated fatty acids were altered. Animals fed the sunflower seed oil diet exhibited an increase in $n-6$ fatty acids, including linoleic (18:2($n-6$)) and arachidonic (20:4($n-6$)) while the sheep kidney fat dietary rats were higher in $n-3$ fatty acids, principally docosahexaenoic (22:6), with the net result being a higher $n-6/n-3$ ratio in the sunflower seed oil group compared to sheep kidney fat dietary animals. Fluorescence polarization indicated that the fluidity of sarcoplasmic reticular membrane was greater than that of sarcolemmal membrane, with a dietary lipid-induced decrease in fluidity being observed in the sarcoplasmic reticular membrane from sheep kidney fat dietary animals. Despite these significant changes in membrane composition and physical properties, neither the specific activity nor the temperature-activity relationship (Arrhenius profile) of the associated ATPases were altered. These results suggest that with regard to the parameters measured in this study, the two ion-transporting ATPases are not modulated by changes which occur in the membrane lipid composition as a result of the diet.

Introduction

Recent experiments in our laboratory have demonstrated that the cardiac performance in the intact rat, determined by the radionuclide angiographic technique [1] and the contractile responses of isolated papillary muscles to pharmacological agents such as calcium and isoprenaline [2,3] can be significantly influenced by manipulation of the dietary lipid intake. We have also shown that the membrane phospholipid fatty acid composition of the rat myocardium undergoes significant changes concurrent with the alterations in the myocardial contractility [4,6]. These alterations in the performance of cardiac muscle may have occurred due to changes in the availability of calcium ions in the myocardial cell [3].

Several membrane systems including sarcolemma and sarcoplasmic reticulum are considered to be involved in regulating calcium in the

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myocardium [8,9,16]. Both sarcolemmal and sarcoplasmic reticular membrane systems contain ion pumps such as $(Na^+ + K^+)$- and Ca^{2+}-ATPase as well as other ion-transporting systems which play a major role in the contraction/relaxation of cardiac muscle [9,10]. Therefore, changes in the properties of these ion pumps could alter the ionic distribution in the cell and affect myocardial contractility. The regulatory role of various membrane systems in cardiac function has been reviewed by Dhalla and co-workers [9,12,14–16], while several other investigators have drawn attention to the possible role which membranes could play in the pathogenesis of ischaemia and the subsequent development of arrythmias [13,18,19]. Failure to maintain proper transmembrane ionic distribution is thought to be central to these effects.

It is well established that the properties of several membrane-bound enzyme systems including Ca^{2+}- and $(Na^+ + K^+)$-ATPase are modulated by lipids of the membrane matrix [5,7,11]. Not only is the presence of lipid essential for enzyme activity, but various characteristics of the membrane phospholipid molecule (e.g., head-group, chain-length, unsaturation) can affect the function of these enzyme systems [5,11,21,22,46].

This paper reports on the effects of different dietary lipid supplements on the lipid composition, membrane fluidity, enzyme activity and temperature-activity relationship of Ca^{2+}- and $(Na^+ + K^+)$-ATPase-enriched membrane fractions isolated from rat heart. We report a lack of response of adenosinetriphosphatases to dietary lipid-induced changes in membrane lipid composition and properties.

Materials and Methods

Methods

Male Hooded Wistar rats aged 60 days and weighing between 225 and 250 g were randomly assigned to three groups (16 per group) and maintained on different diets ad libitum for a further period of 9 weeks. One group of rats received standard laboratory pellets, termed reference (REF.) diet obtained from Milling Industries, Vic. A second group of rats received pellets supplemented with sunflower seed oil (Nuttelex Food Products Ltd, Vic.) – designated Sunflower Seed Oil diet; and a third group of rats were fed standard pellets supplemented with sheep kidney (perirenal) fat (designated Sheep Kidney Fat diet). The preparation of these lipid-supplemented diets has been described [4,27]. The three diets had similar caloric values as determined by combustion calorimetry [4].

Preparation of cardiac membranes

Sarcoplasmic reticulum. Animals were killed by decapitation under light ether anaesthesia and hearts were removed and washed in Krebs-Henseleit buffer. Each heart (with atria removed) was separately chopped and rinsed in ice-cold medium containing 250 mM sucrose/30 mM L-histidine/20 mM Tris (pH 6.8). The tissue was homogenized in 40 ml of the above medium, using a Polytron tissue disintegrator (PT 35; Kinematica GmbH, Switzerland). Two separate bursts of 10 s at setting 3.5 were used to disrupt the tissue and the brei was centrifuged at $1000 \times g$ for 15 min. The resulting pellet (low-speed pellet) was retained for the isolation of sarcolemma as described below, while the supernatant was used to isolate the sarcoplasmic reticular fraction as described for procedure I vesicles by Jones et al. [23]. The washed pellet thus produced was resuspended in 2 ml 250 mM sucrose/20 mM Tris/30 mM histidine (pH 7.4) (buffer I) and stored at −80°C. Pellets from four animals were pooled, diluted with 20 mM Tris/30 mM histidine (pH 7.4) and centrifuged at $48\,000 \times g$ for 45 min. The final pellet was resuspended in buffer I to give a protein concentration of approx. 1 mg/ml and used for Ca^{2+}-ATPase assays and lipid analysis.

Sarcolemma. The low-speed pellets from four animals were combined, resuspended in 20 mM Tris/1 mM EDTA (pH 7.6) (buffer II) and centrifuged at $10\,000 \times g$ for 15 min. The pellet was resuspended in 100 ml 1 M KCl (in buffer I) and an equal volume of 2 M NaI (in 20 mM Tris/1 mM EDTA, pH 8.0), was added. After stirring for 60 min on ice, this suspension was centrifuged at $27\,250 \times g$ for 20 min. The pellet was resuspended in buffer II, filtered through cheesecloth and centrifuged as described above. The resulting pellet was resuspended in 8 ml buffer II containing 250 mM sucrose and stored overnight at −80°C. After thawing, the pellet was

extracted at 30°C with 0.05% (w/v) deoxycholate at a detergent/protein ratio of 1 : 1 (w/w), in the presence of KCl (250 mM) and Na_2-ATP (5 mM). After 30 min, the mix was centrifuged at 12 000 × *g* for 15 min and the supernatant was centrifuged at 48 000 × *g* for 60 min to obtain a membrane fraction highly enriched in $(Na^+ + K^+)$-ATPase activity. The pellet was washed twice by centrifugation in buffer II and the final pellet was resuspended in 250 mM sucrose/20 mM Tris/1 mM EDTA (pH 7.6) to yield a protein concentration of approx. 1 mg/ml and used for the assay of $(Na^+ + K^+)$-ATPase and lipid analysis. Protein was determined by the method of Peterson [26].

Enzyme assays

ATPase activity was measured spectrophotometrically using the coupled assay method described previously [28,29]. The oxidation of NADH at 340 nm was monitored continuously using a Gilford spectrophotometer (model 250) equipped with an automatic cuvette positioner (model 2451-A) and fitted with a Gilford Thermoset Controller. Enzyme assays were performed under maximum velocity conditions and an optimum pH was maintained throughout the entire temperature range [53–55].

$(Na^+ + K^+)$-ATPase. The assay medium contained 3.14 mM phospho*enol*pyruvate, 22 U/ml pyruvate kinase, 13.5 U/ml lactate dehydrogenase, 0.28 mM NADH, 80 mM Na^+, 20 mM K^+, 2 mM Mg^{2+}, 0.2 mM EDTA in 100 mM glycylglycine buffer (pH 7.6) which contained 250 mM sucrose. After adding the enzyme protein (10–20 μg), this mixture was incubated in a 1-ml cuvette at the desired temperature for 2 min before the reaction was initiated by the addition of Tris-ATP to a final concentration of 1.5 mM. The final assay volume was 525 μl. The ATPase activity which was sensitive to inhibition by 2 mM ouabain was taken as the $(Na^+ + K^+)$-ATPase activity. More than 88% of total ATPase activity was inhibited by this concentration of ouabain. The ouabain concentration which caused 50% inhibition (I_{50}) of enzyme activity was obtained as described previously [20].

Ca^{2+}-ATPase. The assay mix was similar to that used above for $(Na^+ + K^+)$-ATPase except Na^+ was replaced with 50 μM Ca^{2+}, K^+ was increased to 100 mM and EDTA was omitted. The Ca^{2+}-ATPase was taken as that activity sensitive to inhibition by 2 mM EDTA. More than 85% of the total ATPase activity was inhibited by this concentration of EDTA.

Extraction and analysis of lipids

The method for the extraction of lipids, the preparation of phospholipid fatty acid methyl esters and their identification, have all been described previously [4]. In brief, lipids were extracted by the method of Bligh and Dyer [24] using chloroform/methanol (2 : 1, v/v) containing 0.01% (w/v) butylated hydroxytoluene. The phospholipids were separated from the total lipids by thin-layer chromatography on Kieselgel 60 H and methyl esters were prepared by heating (60°C, 30 min) in methanol containing 14% (w/v) borontrifluoride. The methyl esters were analysed by gas-liquid chromatography using a Hewlett-Packard gas chromatograph (HP 5710A). Columns were packed with 5% SP-2310 on 100/120 chromosorb WAW (Supelco Inc., Bellafonte, PA).

Fluorescence polarization measurements

The fluidity of sarcolemmal and sarcoplasmic reticular membrane fractions was measured by the fluorescence polarization technique using two types of fluorescent probes, namely, diphenylhexatriene and a series of *n*-(9-anthroyloxy) fatty acid probes. A stock solution (0.46 mg/ml) of diphenylhexatriene was prepared in tetrahydrofuran; 25 μl of the stock solution was dispersed in buffer (50 ml of Tris (20 mM)-imidazole (50 mM), pH 7.6) and the dispersion mixed 1 : 1 with the membrane suspension. Final concentrations were 0.5 μM for diphenylhexatriene and 30 μg membrane protein/ml.

For measurement with the anthroyloxy fatty acid probes, membrane fractions were diluted with Tris-imidazole buffer (pH 7.6) to yield a final protein concentration of 30 μg/ml and the probes were added in small volumes of methanol (10 μl) to give a final probe concentration of 3.3 μM. Membrane suspensions were left in the dark for 1 h at room temperature to allow infusion of the probe into the membrane lipids. Polarization measurements were made at 37 ± 0.2°C as previously described [25,30]. All measurements were cor-

rected for the scattering of exciting light using a cuvette containing membrane protein (30 μg/ml) but no fluorescent probe. The fluorescence polarization (p) is given by,

$$p = \frac{I_v - I_H}{I_v + I_H}$$

where I_v and I_H are the intensities of vertical and horizontal components of the emitted fluorescence, respectively.

Chemicals

Glycyclglycine (free base), ouabain octahydrate, deoxycholate (sodium salt), phospho*enol*pyruvate (trimonocyclohexylammonium salt), NADH (cyclohexylamine salt), pyruvate kinase (Type II) and lactate dehydrogenase were all supplied by Sigma Chemical Co., St. Louis, MO. Sodium iodide (AR) was obtained from BDH Chemicals (U.K.). Tris-ATP was prepared by ion-exchange chromatography of Na_2-ATP (Sigma) on a column of Dowex-50W (Bio-Rad Laboratories). *n*-(9-Anthroyloxy) fatty acids, namely, 2-(9-anthroyloxy)palmitic acid (2-AP) and 6-, 9-, and 12-(9-anthroyloxy)stearic acids (6-AS, 9-AS, 12-AS) were synthesized as described elsewhere [25]. Diphenylhexatriene was from the Aldrich Chemical Co., Milwaukee, U.S.A.

Results

The fatty acid composition of the reference and the various lipid-supplemented diets are shown in Table I. The sunflower seed oil diet contained reduced proportions of the saturated fatty acids myristic (14 : 0), palmitic (16 : 0) and stearic (18 : 0) compared to both reference and sheep kidney fat diets. Conversely, the highest proportion of the unsaturated fatty acid linoleic (18 : 2(n − 6)) was found in the sunflower seed oil diet, where this fatty acid alone accounted for more than 50% of the total fatty acids present in the diet. All three diets contained a considerable amount of oleic (18 : 1(n − 9)) acid. Comparable levels of this monounsaturated fatty acid were found in both reference and sunflower seed oil diets, while the sheep kidney fat diet contained the highest proportion. The two lipid-supplemented diets (sunflower seed oil and sheep kidney fat) differ markedly with regard to the levels of total saturates, and total unsaturates and hence in the ratio of unsatd./satd. fatty acids. The 18 : 2(n − 6) (linoleic acid) content of all diets provided more than 1% of the total dietary energy, which exceeds the reported minimum requirement of this fatty acid for rats [31].

TABLE I

MAJOR FATTY ACIDS OF STANDARD AND LIPID-SUPPLEMENTED RAT DIETS

Values given are the means of at least eight determinations. n.d., not detected. Diets: REF., reference diet, standard laboratory show; SSO, sunflower seed oil; and SKF, sheep kidney fat supplemented diets.

Fatty acid	Percentage (w/w)		
	REF.	SSO	SKF
14 : 0	1.2	0.3	5.0
16 : 0	20.2	9.6	24.6
16 : 1	1.8	n.d.	2.0
18 : 0	5.8	5.1	22.1
18 : 1(n − 9)	22.5	23.4	33.3
18 : 2(n − 6)	38.9	58.5	6.3
18 : 3(n − 3)	2.8	1.2	1.2
20 : 1	1.2	0.4	0.4
22 : 1	1.5	0.3	n.d.
22 : 6(n − 3)	1.6	n.d.	n.d.
Σ Satd.	27.2	15.0	51.7
Σ Unsatd.	70.3	83.8	43.2
Unsatd./satd.	2.6	5.6	0.8

The fatty acid composition of the total phospholipids of cardiac sarcolemmal ($Na^+ + K^+$)-ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase preparations from rats fed standard diet (reference diet) are shown in Table II. It is clear that the relative proportions of total saturated and total unsaturated fatty acids between the two membrane systems are similar. Both membrane fractions contained similar amounts of palmitic (16 : 0) and stearic (18 : 0) acids. However, the proportion of oleic (18 : 1(n − 9)) acid was significantly higher in the sarcoplasmic reticular membrane in comparison to that found in the sarcolemma. The ($Na^+ + K^+$)-ATPase enriched fraction (sarcolemmal) contained significantly higher proportions of both linoleic (18 : 2(n − 6)) and docosahexaenoic (22 : 6(n − 3)) acids compared to the sarcoplasmic reticular membrane system. Both membrane systems contained equal proportions of

TABLE II

FATTY ACID COMPOSITION OF TOTAL PHOSPHOLIPIDS OF CARDIAC MEMBRANE FRACTIONS ENRICHED IN $(Na^+ + K^+)$- AND Ca^{2+}-ATPase FROM RATS FED THE STANDARD (REFERENCE) DIET

Values shown are mean ± S.E. The composition of standard (reference) diet is given in Table I. Significance was determined by Student's *t*-test. n.s., not significant ($P > 0.01$). DMA, dimethylacetal derivative.

Fatty acid	Percentage (w/w)		*P*
	Ca^{2+}-ATPase (sarcoplasmic reticulum, $n = 8$)	$(Na^+ + K^+)$-ATPase (sarcolemma, $n = 9$)	
DMA 16:0	1.5 ± 0.2	1.6 ± 0.2	n.s.
16:0	11.7 ± 0.5	10.3 ± 0.4	n.s.
DMA 18:0	0.5 ± 0.1	0.6 ± 0.1	n.s.
18:0	23.9 ± 0.6	23.3 ± 0.5	n.s.
18:1($n-9$)	10.7 ± 0.8	8.0 ± 0.3	< 0.01
18:2($n-6$)	19.6 ± 0.6	22.1 ± 0.4	< 0.01
20:4($n-6$)	16.3 ± 0.4	16.2 ± 0.2	n.s.
22:4($n-6$)	0.6 ± 0.1	0.4 ± 0.1	n.s.
24:0	0.3 ± 0.1	0.3 ± 0.1	n.s.
22:5($n-3$)	1.3 ± 0.1	1.6 ± 0.1	n.s.
22:6($n-3$)	11.5 ± 0.5	14.8 ± 0.1	< 0.001
Σ satd.	37.9	36.1	
Σ unsatd.	60.0	63.1	
Σ($n-6$)	36.5	38.7	
Σ($n-3$)	12.8	16.4	
$n-6/n-3$	2.8	2.3	

TABLE III

TOTAL PHOSPHOLIPID FATTY ACID COMPOSITION OF HEART SARCOLEMMAL $(Na^+ + K^+)$-ATPase FROM RATS FED DIFFERENT LIPID-SUPPLEMENTED DIETS

Values given are mean ± S.E. Significance was determined by Student's *t*-test. Total number of animals was 32 per each dietary group: ** $P < 0.01$; *** $P < 0.001$; n.s. not significant ($P > 0.01$). The composition of lipid-supplemented sunflower seed oil (SSO) and sheep kidney fat (SKF) diets is given in Table I. DMA, dimethylacetal derivative formed during acid methylation.

Fatty acid	Percentage (w/w)		*P*
	SKF ($n = 8$)	SSO ($n = 8$)	
DMA 16:0	1.3 ± 0.2	1.3 ± 0.2	n.s.
16:0	8.6 ± 0.3	6.9 ± 0.3	**
DMA 18:0	1.8 ± 0.2	1.7 ± 0.2	n.s.
18:0	25.5 ± 0.2	27.5 ± 0.2	***
18:1($n-9$)	8.6 ± 0.4	5.9 ± 0.4	***
18:2($n-6$)	13.9 ± 0.3	18.2 ± 0.6	***
20:4($n-6$)	18.5 ± 0.2	21.9 ± 0.1	***
22:4($n-6$)	0.2 ± 0.1	1.1 ± 0.1	***
24:0	0.2 ± 0.1	2.0 ± 0.1	***
22:5($n-3$)	2.0 ± 0.2	0.9 ± 0.1	***
22:6($n-3$)	18.1 ± 0.4	12.0 ± 0.3	***
Σ satd.	37.4	39.4	
Σ unsatd.	61.3	60.0	
Σ($n-6$)	32.6	41.2	
Σ($n-3$)	20.1	12.9	
$n-6/n-3$	1.6	3.2	

arachidonic (20 : 4($n-6$)) acid, despite the significantly different levels of its precursor, linoleic acid (18 : 2,$n-6$)). As a result of the changes in the relative proportions of these unsaturated fatty acids, the sarcoplasmic reticulum had a $n-6/(n-3)$ value of 2.8 compared to that of 2.3 for the sarcolemmal membrane system.

The fatty acid composition of heart sarcolemmal fraction enriched in $(Na^+ + K^+)$-ATPase from rats fed sheep kidney fat and sunflower seed oil diets for 9 weeks is given in Table III. A significant difference in the relative proportions of the major fatty acids between the two dietary groups was apparent. Only the proportions of dimethylacetal 16 : 0 and 18 : 0 which are produced from the plasmalogen phospholipids during the acid-catalyzed methylation process [32], remained unchanged. The sheep kidney fat fed animals had a higher proportion of palmitic (16 : 0) and a lower level of stearic (18 : 0) acid compared to the sunflower seed oil dietary group with there being a higher level of total saturated fatty acid in the sunflower seed oil fed animals. Among the unsaturated fatty acids, a lower level of oleic (18 : 1($n-9$)) acid and elevated levels of the $n-6$ fatty acids linoleic (18 : 2), arachidonic (20 : 4) and docosatetraenoic (22 : 4) were apparent in the sunflower seed oil dietary animals compared to sheep kidney fat supplemented rats. In addition, a considerable difference was observed in the proportion of $n-3$ unsaturated docosahexaenoic (22 : 6) acid between the two dietary manipulated animal groups. These changes in the relative proportions of $n-6$ and $n-3$ unsaturated fatty acids resulted in a value of 1.6 for the $n-6/n-3$ ratio in the sheep kidney fat dietary group while in the sunflower seed oil dietary animals, this value increased to 3.2. However, in spite of these changes

in the proportions of the individual fatty acids, the overall proportion of total unsaturated fatty acids (Σ unsatd.) in the membrane, was similar between the two groups.

Similar changes to that described above for the sarcolemma were also seen in the lipids of the membrane fraction enriched in Ca^{2+}-ATPase after dietary lipid supplementation. The effects of the sheep kidney fat and sunflower seed oil diets on the phospholipid fatty acid composition of the sarcoplasmic reticular membrane system is shown in Table IV. Here, the two major saturated fatty acids, palmitic and stearic showed no difference, while significant changes were again observed in the type of unsaturated fatty acids present in the two dietary groups. Although the sunflower seed oil dietary animals had significantly higher ($P < 0.001$) proportions of linoleic ($18:2(n-6)$) and arachidonic ($20:4(n-6)$) acids, the relative proportions of oleic ($18:1(n-9)$), docosapentaenoic ($22:5(n-3)$) and docosahexaenoic ($22:6(n-3)$) were low in comparison to the proportions found in sheep kidney fat dietary group. As a result of these changes, a clear difference was seen in the ratio of $n-6/n-3$ unsaturated fatty acids between the two lipid-supplemented groups with the sheep kidney fat dietary animals exhibiting a value of 1.7 and the sunflower seed oil dietary rats exhibiting a value of 3.8.

The temperature-activity relationships (Arrhenius plots) of ($Na^+ + K^+$)- and Ca^{2+}-ATPase associated with sarcolemmal and sarcoplasmic reticular membrane systems, are presented graphically in Fig. 1a and b, respectively. The specific activity, critical temperature (T_c^0) and activation energy of both enzymes are summarised in Table V. In Fig. 1a, the normalized Arrhenius plots of cardiac ($Na^+ + K^+$)-ATPase from rats fed the reference, the sunflower seed oil and sheep kidney fat diets are shown. The activity of this enzyme from all three groups exhibited a discontinuity (T_c^0) at

TABLE IV

THE PHOSPHOLIPID FATTY ACID COMPOSITION OF Ca^{2+}-ATPase-ENRICHED SARCOPLASMIC RETICULUM FRACTION FROM HEARTS OF RATS FED LIPID-SUPPLEMENTED DIETS FOR 9 WEEKS

All values given are mean ± S.E. P is probability by Student's t-test. Total number of animals was 32 per each dietary group: $^{**}P < 0.01$; $^{***}P < 0.001$; n.s. not significant ($P > 0.01$). The composition of the sunflower seed oil (SSO) and sheep kidney fat (SKF) diets is given in Table I. DMA, dimethylacetal derivative.

Fatty acid	Percentage (w/w)		P
	SKF $n=10$	SSO $n=9$	
DMA 16:0	1.6±0.2	1.7±0.2	n.s.
16:0	10.4±0.2	9.9±0.3	n.s.
DMA 18:0	1.4±0.2	1.0±0.3	n.s.
18:0	26.9±0.9	28.4±1.0	n.s.
$18:1(n-9)$	12.4±0.8	8.6±0.6	**
$18:2(n-6)$	9.6±0.3	13.3±0.5	***
$20:4(n-6)$	17.6±1.0	22.1±0.7	***
$22:4(n-6)$	0.6±0.1	1.2±0.2	n.s.
24:0	0.6±0.1	1.3±0.2	**
$22:5(n-3)$	1.9±0.2	0.6±0.1	***
$22:6(n-3)$	13.9±0.3	9.1±0.3	***
Σ satd.	40.9	42.3	
Σ unsatd.	56.0	54.9	
$\Sigma(n-6)$	27.8	36.6	
$\Sigma(n-3)$	15.8	9.7	
$n-6/n-3$	1.7	3.8	

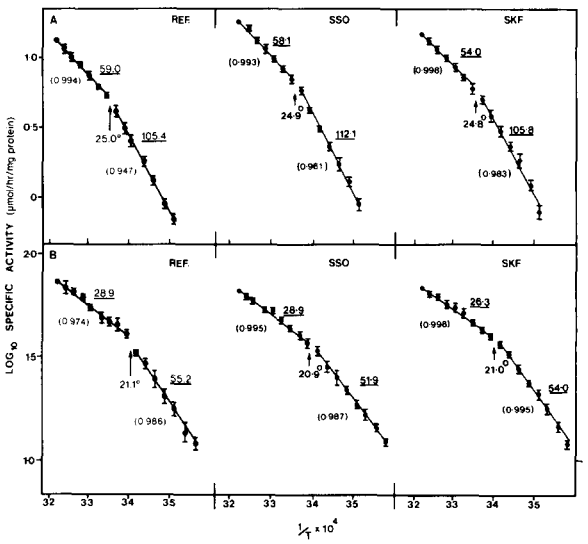

Fig. 1. Arrhenius plots of heart ($Na^+ + K^+$)- and Ca^{2+}-ATPase from rats fed the reference (REF.) and lipid-supplemented diets. (A) ($Na^+ + K^+$)-ATPase. (B) Ca^{2+}-ATPase. Data are presented as the mean ± S.E. from four normalized Arrhenius plots. The critical temperature (T_c^0) for each plot was determined by linear regression analysis [6]. The values underlined are the Arrhenius activation energy (E_a, kJ·mol^{-1}) and numbers in parentheses are the coefficient of determination for the respective linear portions of the plot. The composition of REF., sunflower seed oil (SSO) and sheep kidney fat (SKF) supplemented diets is shown in Table I.

TABLE V

EFFECTS OF LIPID SUPPLEMENTATION FOR 9 WEEKS ON THE RAT-HEART $(Na^+ + K^+)$- AND Ca^{2+}-ATPases

All values are the means ± S.E. for four separate experiments. Specific activity: μmol/h per mg protein at 37°C. The determination of enzyme activities is described under Materials and Methods. T_c^0 and E_a were calculated by linear regression analysis [6]. Mean I_{50} values for ouabain inhibition were calculated from respective dose-response curves [20]. Specific activities were not significantly different ($P > 0.01$) in all dietary groups for each particular enzyme as determined by Student's *t*-test. n.a., not applicable. REF., reference diet; SKF, sheep kidney fat supplemented diet; SSO, sunflower seed oil supplemented diet.

	I_{50} ((M) ouabain)	Specific activity	T_c^0	kJ · mol^{-1}	
				E_a (I)	E_a (II)
$(Na^+ + K^+)$-ATPase					
REF.	$8.5 \cdot 10^{-5}$	13.1 ± 2.2	25.0	59.0	105.4
SKF	$8.0 \cdot 10^{-5}$	15.0 ± 2.7	24.8	54.0	105.8
SSO	$9.0 \cdot 10^{-5}$	18.6 ± 1.4	24.9	58.1	112.1
Ca^{2+}-ATPase					
REF.	n.a.	72.8 ± 6.4	21.1	28.9	55.2
SKF	n.a.	67.9 ± 2.9	21.0	26.3	54.0
SSO	n.a.	63.2 ± 0.8	20.9	28.9	51.9

about 25°C. In the reference group, the Arrhenius activation energy (E_a) increased from 59.0 (E_a(I)) to 105.4 (E_a(II)) kJ · mol^{-1} as the assay temperature was reduced from 38 to 12°C (Table V). Comparable increases in the activation energies of $(Na^+ + K^+)$-ATPase over similar temperature regions were also observed in the two lipid-supplemented groups. The specific activity of sarcolemmal $(Na^+ + K^+)$-ATPase at 37°C from the three animal groups was not significantly altered by lipid supplementation of the diet. Since the ouabain sensitivity of $(Na^+ + K^+)$-ATPase is reported to be strongly modulated by the lipids of the membrane matrix [20], the sensitivity to ouabain inhibition of $(Na^+ + K^+)$-ATPase from normal and dietary manipulated rats was also determined. The concentration of ouabain which caused a 50% inhibition (I_{50}) of enzyme activity was calculated from the respective dose-response curves and is also shown in Table V. No significant change in the I_{50} value occurred after supplementation of the reference diet with different fats.

In preliminary experiments, Ca^{2+} ionophore A23187 (Calbiochem) was included in the assay medium when sarcoplasmic reticular fractions were assayed. However, as no change in activity was observed, in all three dietary groups, A23187 was later omitted from the assay mix.

Fig. 1b shows the normalized Arrhenius plots of sarcoplasmic reticulum Ca^{2+}-ATPase from the reference and dietary manipulated animals. This enzyme system from all three dietary groups exhibited a critical temperature (T_c^0) value of about 21°C which is 4°C lower than that observed for the $(Na^+ + K^+)$-ATPase enzyme system of sarcolemmal membrane. Animals maintained on the reference diet displayed mean values of 28.9 and 55.2 kJ · mol^{-1} for the activation energy above (E_a(I)) and below (E_a(II)) the T_c^0, respectively. No significant change in the activation energies was found in response to changes in the dietary fat intake (Table V). These two adenosinetriphosphatases exhibited clearly different Arrhenius profiles with the sarcolemmal $(Na^+ + K^+)$-ATPase displaying values for both E_a(I) and E_a(II) which were almost twice the values of those observed with the Ca^{2+}-ATPase activity. As previously seen with the $(Na^+ + K^+)$-ATPase enzyme system, the specific activity of sarcoplasmic reticulum Ca^{2+}-ATPase remained unchanged ($P > 0.01$) after lipid supplementation (Table V).

The effect of membrane lipid composition on several rat heart membrane associated enzyme systems is shown in Fig. 2. Here, the Arrhenius critical temperature (T_c^0) of each enzyme system is plotted against the ratio of $n-6/n-3$ unsaturated fatty acids for the corresponding membrane system. The results of succinate-cytochrome *c* reductase and F_1-ATPase, both present in the mitochondrial membrane, are taken from our pre-

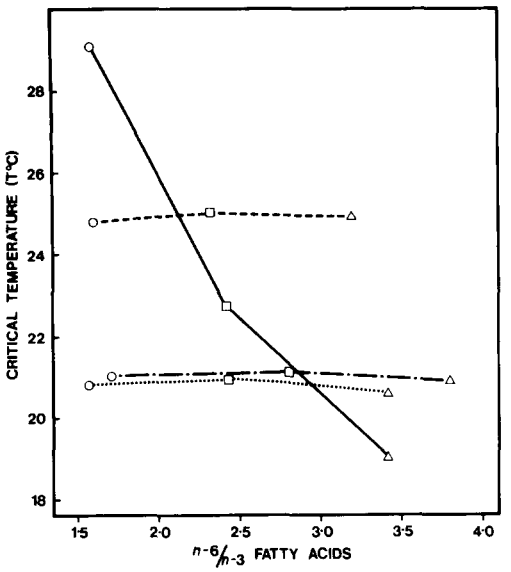

Fig. 2. Effect of membrane lipid composition on the Arrhenius critical temperature (T_c^0) of several membrane-bound enzymes from rat heart. (———) succinate-cytochrome *c* reductase; (- - - - -) (Na^+/K^+)-ATPase; (–·–) Ca^{2+}-ATP; (····) F_1-ATPase. Enzyme fractions were prepared from rats fed reference (□), sunflower seed oil (△) and sheep kidney fat (○) supplemented diets and the T_c^0 of each enzyme system was determined by linear regression analysis of the respective Arrhenius plot (Fig. 1A and B and Ref. 6). The phospholipid fatty acid data (Tables II, III and Ref. 6) of the corresponding membrane system was used to calculate the ratio of $n-6/n-3$ unsaturated fatty acids.

vious studies [6,33]. It is clear that only the T_c^0 of succinate-cytochrome *c* reductase is affected by changes in the membrane lipid composition.

The fluidity of cardiac sarcoplasmic reticular and sarcolemmal membrane fractions from rats fed normal and lipid-supplemented diets for 9 weeks was determined by using both diphenylhexatriene and a series of *n*-(9-anthroyloxy) fatty acid fluorescent probes. The results of these fluorescent-polarization experiments are shown in Table VI and Fig. 3, respectively.

The polarization values obtained for both sarcolemma and sarcoplasmic reticulum with the *n*-(9-anthroyloxy) fatty acid probes are presented graphically in Fig. 3. These results indicate that sarcoplasmic reticular membrane fraction enriched in Ca^{2+}-ATPase had lower polarization (p) values compared to sarcolemmal ($Na^+ + K^+$)-ATPase ($P < 0.001$). Since p provides information on the molecular ordering in the membrane lipids, which is related to the lipid microviscosity [25,30], these results can be interpreted as evidence that the fluidity of sarcoplasmic reticular membranes is greater than that of sarcolemma. As the position of fluorophore to the acyl chain is moved from 2 to 9, there is a linear decrease in polarization in both membrane systems. Thereafter, a steeper decrease in p was reported by 12-AS for the sarcoplasmic reticular membrane system than for the sarcolemma. However, this series of fatty acid probes reported that the overall membrane fluidity of both the sarcoplasmic reticular and sarcolemmal membrane systems was not altered in response to the different dietary lipid supplements, although these dietary treatments resulted in significant changes in the phospholipid fatty acid composition of the membranes (Tables II and III). Further experiments using the fluorescent probe, diphenylhexatriene, (Table VI) have reinforced the findings with the anthroyloxy fatty acid probes that sarcoplasmic reticular and sarcolemmal mem-

TABLE VI

EFFECT OF LIPID SUPPLEMENTATION ON THE STEADY-STATE FLUORESCENCE POLARIZATION (p) OF DIPHENYLHEXATRIENE IN RAT-HEART ($Na^+ + K^+$)- AND Ca^{2+}-ATPases

Fluorescence polarization values shown are the mean ± S.E. for duplicate determinations of three separate experiments. All measurements were carried out at 37°C as described in the text. The composition of reference and lipid-supplemented diets is given in Table I. Statistical significance was determined by Bonferroni's method [52]; (($Na^+ + K^+$)-ATPase REF. vs. Ca^{2+}-ATPase REF., $P < 0.001$; Ca^{2+}-ATPase REF. and SSO vs. SKF, $P < 0.001$). REF., reference diet; SSO, sunflower seed oil supplemented diet; SKF, sheep kidney fat supplemented diet.

Membrane preparation	Lipid supplement		
	REF.	SSO	SKF
($Na^+ + K^+$)-ATPase (sarcolemma)	0.154 ± 0.001	0.153 ± 0.002	0.154 ± 0.002
Ca^{2+}-ATPase (sarcoplasmic reticulum)	0.133 ± 0.002	0.135 ± 0.002	0.154 ± 0.002

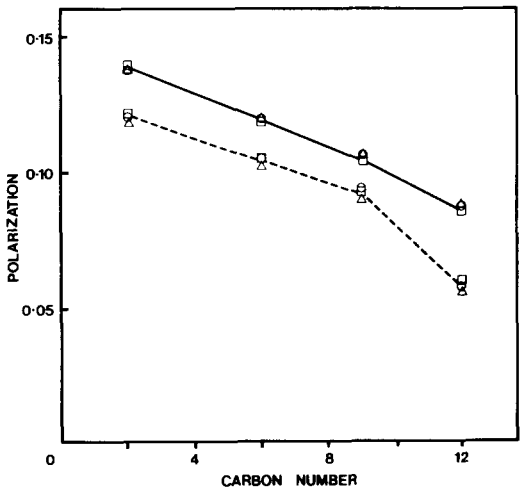

Fig. 3. Effect of 9 weeks dietary lipid supplementation on the polarization gradients for the *n*-(9-anthroyloxy) fatty acid probes in rat-heart membrane preparations enriched in (———) $(Na^+ + K^+)$- and (- - - - - -) Ca^{2+}-ATPase. (□) Reference; (△) sunflower seed oil and (○) sheep kidney fat fed animals. Data are presented as the mean values for duplicate determinations of three separate experiments. The standard error of the mean for each treatment did not exceed the diameter of the respective symbol. The measurements were performed at 37°C as described in Materials and Methods. Statistical analysis by Bonferroni's method [52] indicated that polarization values for the two membrane systems were significantly different ($P < 0.001$).

brane systems have different levels of lipid fluidity. However, in contrast to the observation with the fatty acid probes, the sarcoplasmic reticular membrane fraction enriched in Ca^{2+}-ATPase from rats fed the sheep kidney fat diet now displayed a significantly higher ($P < 0.001$) polarization value when compared to either reference or sunflower seed oil diet fed animals.

Discussion

The rat heart sarcoplasmic reticular and sarcolemmal membranes contained significantly different proportions of oleic ($18:1(n-9)$), linoleic ($18:2(n-6)$) and docosahexaenoic ($22:6(n-3)$) acids. This indicates that the preparations enriched in Ca^{2+}- and $(Na^+ + K^+)$-ATPases employed in this study have originated predominantly from different membrane systems in the myocardial cell. The differences in the lipid composition, particularly with regard to the increased proportions of 18:2 and 22:6 in the sarcolemma compared to the sarcoplasmic reticulum may reflect the different roles played by these two membrane systems in normal cardiac function. While sarcoplasmic reticulum contains various enzymes and transport proteins and is mainly involved in the regulation of intracellular Ca^{2+} levels within cardiac cells [9], the sarcolemmal membrane system plays host to various ion channels, carriers, enzyme systems, receptors, and binds and transports Ca^{2+} in addition to being involved in the propagation of electrical depolarization in the myocardium [9,10,17].

The proportion of the major saturated fatty acids in the sarcolemma was changed after dietary manipulation but this was not the case for sarcoplasmic reticulum. Although the sheep kidney fat diet was relatively rich in 18:0 compared to the sunflower seed oil diet, sarcolemma from the sunflower seed oil rats contained a higher proportion of this fatty acid than did the sheep kidney fat dietary rats. A similar observation has been made in the composition of rat atrium and ventricle phospholipid fatty acids after identical dietary treatment for 12 months [4]. However, the major dietary lipid-induced changes were in the proportions of unsaturated fatty acids of the sarcoplasmic reticular and sarcolemmal membranes. Although the extent of these changes in the respective membranes were different, they occurred in the same direction in both membrane systems.

Changes in the ratio of the $n-6/n-3$ unsaturated fatty acids were observed after dietary lipid supplementation. The sunflower seed oil diet which was relatively rich in linoleic acid ($18:2(n-6)$) caused an increase in arachidonic ($20:4(n-6)$) acid, whilst the sheep kidney fat fed rats showed an elevation of docosahexaenoic ($22:6(n-3)$) acid. This latter long-chain $n-3$ polyunsaturated fatty acid presumably originated from conversion of dietary linolenic ($18:3(n-3)$) acid although both diets contained relatively low levels of this fatty acid. The increase in unsaturated fatty acids of the $n-6$ series was always accompanied by a decrease in the $n-3$ series and vice versa, indicating the operation of some form of regulatory mechanism in the lipid metabolism of the rat in response to different dietary lipid intakes, as suggested by Castor et al. [34]. The relatively high level of oleic ($18:1(n-9)$) acid in the sheep kid-

ney fat diet (in comparison to the sunflower seed oil diet) was also reflected in the cardiac membrane fractions as both the sarcolemma and sarcoplasmic reticulum from sheep kidney fat fed rats exhibited a higher proportion of this monounsaturated fatty acid in comparison to membranes isolated from the sunflower seed oil dietary animals.

Nonlinearity of the Arrhenius profile for certain membrane-bound enzymes is well documented [27,44,46]. A similar observation has been made for both enzyme systems examined in this study. Such discontinuities in the Arrhenius plot of enzyme activity have previously been attributed to changes in the physical state of lipid molecules associated with the enzyme [44,45], although several other explanations have also been suggested [56,57]. However, information gathered by using a number of biophysical and biochemical techniques which include delipidation/reconstitution experiments indicate that the nonlinearity of the activity-profile of several ATPases is linked to a phase change or phase transition of the lipids surrounding the enzyme protein [21,22,38,58–66]. Therefore, the lack of change in the Arrhenius profiles of both ATPases after lipid supplementation reported in this study may be due to one or more of the following reasons. Firstly, the breaks in the Arrhenius plots may be due to some intrinsic property common to the two ATPase proteins and thus is independent of the lipid environment. Indeed, this has been suggested for Ca^{2+}-ATPase from skeletal muscle [35,36] as a nonlinear Arrhenius plot with a break near 20°C has been observed after almost complete substitution of membrane phospholipid by the detergent, dodecyl octa-ethylene glycol monoether ($C_{12}E_{18}$) [36]. Secondly, it is possible that in spite of the significant changes in phospholipid fatty acids, the 'fluidity' of bulk membrane was unaffected. Another possible explanation is that although a change in the molecular order or fluidity of the bulk membrane lipids had occurred, the enzyme maintained its integrity, as it is insulated from an effect by a layer of annular or boundary lipid [37–40,67], thus maintaining a constant lipid microenvironment adjacent to the ATPase protein. Although the current status of the existence of such a lipid annulus around intrinsic proteins is debatable [41,42], the observations in this study may be explained by assuming the presence of such a layer of insulating lipid.

The possibility of changes in membrane fluidity induced by changes in lipid composition can be explored experimentally by fluorescent probe techniques. Steady-state fluorescence polarization is affected by the type and rate of the depolarizing rotations of probe molecules, as well as by the restriction of probe motion in the anisotropic environment of the membrane [47,48]. Moreover, certain fluorescence probes vary in their preference for laterally segregated or phase-separated areas of membrane lipid. Diphenylhexatriene partitions almost equally between fluid and crystalline areas, whereas the anthroyloxy probes prefer the fluid environment [49,50]. Thus, two chemically and physically distinct probes may respond differently (with respect to fluorescence polarization) to changes in membrane lipid composition [48,50,51]. Indeed, in the present study, the anthroyloxy fatty acid probes do not detect differences in fluidity arising from compositional changes induced by diet, whereas diphenylhexatriene detects such effects in the sarcoplasmic reticular membrane system. Similarly, results from the anthroyloxy probes indicate that the fluidity of sarcoplasmic reticular membranes is higher than the sarcolemmal membranes regardless of the depth at which the fluorophore resides in the bilayer, whereas for diphenylhexatriene, the differences between sarcoplasmic reticular and sarcolemmal membranes depend on the dietary treatment.

The findings of the present study are consistent with our earlier observation [6,33] that only succinate cytochrome *c* reductase, but not F_1-ATPase (both of which are associated with the mitochondrial inner membrane), was responsive to this type of dietary treatment. Conversely, the ATPase systems which completely span the membrane and require a fixed orientation to carry out their function, seem to be protected from or insensitive to, changes in the bulk lipid matrix (Fig. 3). This is in contrast to certain other enzyme or receptor proteins which may undergo lateral diffusion within the plane of the membrane bilayer in the course of their normal function [6,43]. Such membrane proteins could be expected to be more readily affected by changes in the molecular order-

ing of the bulk membrane lipids.

The differences in cardiac contractility induced by changes in dietary lipids may be due to changes in the transmembrane distribution of ions, particularly Ca^{2+}, Na^+ and K^+ [1–3]. Nevertheless, in this study, we did not observe any dietary lipid effect on the activity of Ca^{2+} and/or ($Na^+ + K^+$)-ATPase. However, it should be stressed that the present studies on ATPases were carried out only on the ATP-hydrolyzing mode of the enzymes and not on their function as ion pumps. Therefore, the possibility remains that the ion-transport property of these two ATPases may be altered in response to changes in the membrane lipid composition. However, dietary lipid-induced changes in membrane lipid composition may affect cardiac function by other mechanisms. For example, changes in fatty acid composition could alter the physical properties of lipid and affect the permeability characteristics of the membrane and consequently myocardial contractility. Furthermore, changes in membrane lipid composition may alter the availability of substrates for the synthesis of biologically active compounds such as the prostaglandins and thromboxanes. In this regard, it is worth noting that cardiac membranes of sunflower seed oil dietary animals contained higher proportions of both arachidonic acid ($20:4(n-6)$) and its precursor linoleic acid ($18:2(n-6)$) in comparison to rats fed sheep kidney fat diet which was rich in saturated fats.

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