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Diet deficient in alpha-linolenic acid alters fatty acid composition and enzymatic properties of Na^+, K^+-ATPase isoenzymes of brain membranes in the adult rat

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The effects of dietary (n-6)/(n-3) polyunsaturated fatty acid balance on fatty acid composition, ouabain inhibition, and Na^+ dependence of Na^+, K^+-ATPase isoenzymes of whole brain membranes were studied in 60-day-old rats fed over two generations a diet either devoid of α-linolenic acid [18:3(n-3)] (sunflower oil diet) or rich in 18:3(n-3) (soybean oil diet). In the brain membranes, the sunflower oil diet led to a dramatic decrease in docosahexaenoic acid [22:6(n-3)] membrane content. The activities of Na^+, K^+-ATPase isoenzymes were discriminated on the basis of their differential affinities for ouabain and their sensitivity to sodium concentration. The ouabain titration curve of Na^+, K^+-ATPase activity displayed three inhibitory processes with markedly different affinity [i.e., low (α1), high (α2), and very high (α3)] for brain membranes of rats fed the sunflower oil diet, whereas the brain membranes of rats fed the soybean oil diet exhibited only two inhibitory processes, low (α1) and high ($\alpha2' = \alpha2 + \alpha3$). Regardless of the diet, on the basis of the Na^+ dependence of Na^+, K^+-ATPase activity, three isoenzymes were found: α1 form displaying an affinity 1.5- to 2-fold higher that of than α2 and 3-fold higher that of α3. In rats fed the sunflower oil diet, α2 isoenzyme exhibited higher affinity for sodium (Ka = 8.8 mmol/L) than that of rats fed the soybean oil diet (Ka = 11.7 mmol/L). These results suggest that the membrane lipid environment modulates the functional properties of Na^+, K^+-ATPase isoenzymes of high ouabain affinity (α2). (J. Nutr. Biochem. 10:230–236, 1999) *© Elsevier Science Inc. 1999. All rights reserved.*

Keywords: (n-3) polyunsaturated fatty acids; brain; Na, K-ATPase; isoenzyme; rats

Introduction

Na^+, K^+-ATPase is a membrane-bound enzyme [Na/K-activated adenosine triphosphatase (ATP); Mg-dependent, Na, K-activated phosphohydrolase; EC 3.6.1.37] that catalyzes the active transport of Na^+ and K^+. It consists of two types of protein subunits: a catalytic (α) receptor for digitalis and a glycoprotein subunit (β). The α subunit exists in multiple forms (i.e., α1, α2, and α3). It is possible to discriminate these forms according to their affinity to ouabain ($\alpha3 > \alpha2 \gg \alpha1$) using binding experiments or inhibition of enzyme activity.[1–3] The α1 isoform is present in all animal cells, α2 is more specifically represented in muscle and neuronal tissue, and α3 is extensively located in neuronal tissue.[4] These different isoenzymes exhibit specific affinities to various substrates, particularly to sodium[5,6] and potassium ions,[7] and modulation by the hormonal system.[4] These properties could reflect their cellular speci-

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Table 1 Composition of the experimental diets

Ingredients	Diets (g/100 g diet)	
	Soybean	Sunflower
Corn starch	45.94	46.24
Sucrose	23.00	23.00
Casein	22.00	22.00
DL-Methionine	0.16	0.16
Vitamin mixture*	1.00	1.00
Mineral mixture†	4.00	4.00
Cellulose	2.00	2.00
Lipids		
Soybean oil	1.90	
Sunflower oil		1.60

*Composition of vitamin supplement per kilo (tritrated in dextrose) (United States Biochemical, Cleveland, OH USA): dL α-tocopherol (1,000 Iu/mg), 5.0 g; L-ascorbic acid, 45.0 g; choline chloride, 75.0 g; D-calcium pantothenate, 3.0 g; inositol, 5.0 g; menadione, 2.25 g; niacin, 4.5 g; para-aminobenzoic acid, 5.0 g; pyridoxine HCl, 1.0 g; riboflavin, 1.0 g; thiamin HCl, 1.0 g; retinyl acetate, 900,000 Iu; ergoalciferol, 100,000 Iu; biotin, 20 mg; folic acid, 90 mg; vitamin B_{12}, 1.35 mg.
†Composition of the mineral mixture/100 g: $CaHPO_4$, 2 H_2O, 38.0 g; K_2HPO_4, 24.0 g; $CaCO_3$, 18.1 g; NaCl, 7.0 g; MgO, 2.0 g; $MgSO_4$, 7 H_2O, 9.0 g; $FeSO_4$, 7 H_2O, 0.7 g; $ZnSO_4$, H_2O, 0.5 g; $MnSO_4$, H_2O, 0.5 g; $CuSO_4$, 5 H_2O, 0.1 g; NaF, 0.1 g; $Al_2(SO_4)_3$ K_2SO_4, 24 H_2O, 0.02 g; KI, 0.008 g; Na_2SeO_3, H_2O, 0.001 g.

ficity. Moreover, numerous experiments have identified the role of lipids in the modulation of Na^+, K^+-ATPase activity.[8–10] However, the effects of lipid environment on the function of these different isoenzymes remain to be documented. Most of the previous studies on the relationship between membrane structure and isoenzyme function were performed using degradative techniques such as phospholipase digestion.[11,12] The results revealed the importance of the effects of lipids on the Na^+, K^+-ATPase isoenzymes of high ouabain affinity (α2 and/or α3). Using the dietary deficient in α-linolenic acid [18:3(n-3)] in weaned rats, we showed in a previous study[13] that the increase of (n-6)/(n-3) polyunsaturated fatty acids (PUFA) in whole brain membrane influences certain functional properties of Na^+, K^+-ATPase isoenzymes (α2 and α3), such as their affinity to ouabain and their sensitivity to sodium ion.

It is now well demonstrated that the Na^+, K^+-ATPase activity and the expression of the corresponding isoenzymes change during brain development.[14] Moreover, the composition of PUFA as well as that of monounsaturated fatty acids (MUFA) change during brain development.[15,16] The goal of the present work is to describe the Na^+ dependence of Na^+, K^+-ATPase isoenzyme activities in brain membranes of the rat from conception to 60 days postnatal age fed on a diet deficient in 18:3(n-3).

Materials and methods

Diet and animals

Two semi-synthetic diets—the sunflower oil and the soybean oil based diets—were used. They differed mainly by the amount of 18:3(n-3) (*Tables 1 and 2*): The sunflower oil diet was deficient in 18:3(n-3) and the soybean oil diet was rich in 18:3(n-3). They both

Table 2 Fatty acid composition of the diets*

Fatty acids	Diets (g/Kg diet)	
	Soybean	Sunflower
ΣSFA	2.98	1.86
14:0	–	–
16:0	2.09	1.06
17:0	–	–
18:0	0.7	0.64
20:0	0.11	0.05
22:0	0.08	0.11
ΣMUFA	4.44	3.10
16:1(n-9)	–	–
16:1(n-7)	–	0.03
18:1(n-9)	4.0	2.88
18:1(n-7)	0.36	0.16
20:1(n-9)	0.08	0.03
ΣPUFA(n-6)	10.16	11.0
18:2(n-6)	10.16	11.0
20:4(n-6)	–	–
ΣPUFA(n-3)	1.4	0.03
18:3(n-3)	1.4	0.03
(n-6)/(n-3)	7.26	366

*Values represent the average obtained from at least three separate extractions and analyses of the respective diets and are relative amounts, expressed as a percentage of the total identified fatty acids by weight.
SFA–saturated fatty acids. MUFA–monounsaturated fatty acids. PUFA–polyunsaturated fatty acids.

supplied equivalent amounts of linoleic acid [18:2(n-6); (approximately 10 mg/g of diet), which is a prerequisite for essential (n-6) PUFA[17] leading to a ratio of approximately 7 for the soybean oil diet and 366 for the sunflower oil diet (*Table 2*). Twenty-one-day-old Wistar female rats weighing 40 to 50 g (IFFA CREDO, Domaine des Oncins, France) were fed a semi-synthetic standard diet (Extra-labo, Ets. Pietremont, France). Animals were randomly divided into two groups and were given either a sunflower oil or soybean oil diet throughout the experimental period. After 2 weeks, female rats were mated at 10 weeks of age. The male Wistar rats received a semi-synthetic standard diet (Extra-labo). The litters weaned at 21 days were kept on the same diet. Sixty-day-old males were used for this study. Approved experimental protocols met the government guidelines (Ministry of Agriculture, authorization no. 03007, June 4, 1991).

Preparation of whole brain membranes

The 60-day-old rats were euthanized by decapitation without anesthesia to avoid membrane perturbation. Brain membranes were prepared according to the method of Sweadner[18] as modified by Berrebi-Bertrand et al.[1]: The whole brains were rapidly dissected and individually homogenized with a glass homogenizer in 10 volumes of 0.32 mol/L sucrose containing 0.1 mmol/L EDTA, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 30 mmol/L imidazole/HCl buffered to pH 7.2. The homogenate was centrifuged at 850 × g for 20 minutes (Sorvall, rotor, SS34), and then the supernatant was centrifuged at 8,500 × g for 20 minutes (Sorvall, rotor, SS34) and at 130,000 × g for 30 minutes (Kontron, rotor, TFT 65-38). The final pellet, which was membrane fraction enriched in Na^+, K^+ ATPase,[18] was resuspended in the same buffer and stored at −80°C until analysis (storage did not exceed 3 weeks). The protein concentration was determined according to Lowry et al.[19]

Lipid extraction

Thawed membrane suspensions were extracted according to the method of Folch et al.[20] The fatty acid composition was determined after methylation, according to the technique of Morisson and Smith.[21] The fatty acid methyl esters were analyzed on a Carlo Erba gas liquid chromatography that was equipped with an automatic injector on column, a flame ionization detector, and a C.P WAX 52 C.CB. bonding fused silica capillary column (50 m $\times$ 0.3 mm internal diameter). The assays were carried out with a programmed oven temperature rise of 3°C/min from 54° to 220°C. The pressure of hydrogen carrier gas was 0.8 bar.

Na^+, K^+-ATPase assay

To avoid the alteration of membrane protein environment,[22] the enzymatic assay was carried out with native membrane (without detergent treatment). The Na^+, K^+-ATPase activity was determined using the coupled assay method.[23] The activity was measured in ATP regeneration medium by continuously recording nicotinamide adenine dinucleotide (NADH) oxidation at 37°C using an spectrophotometer Uvikon 930. Each cell contained (final volume 0.6 mL) 2 mmol/L phosphoenolpyruvate (Sigma, L'Isle D'abeau Chesnes, France), 10 mmol/L KCl, 100 mmol/L NaCl, 4 mmol/L ATP-Mg^{2+}, 0.4 mmol/L NADH, 3.5 units of pyruvate kinase, and 5 units of lactate dehydrogenase (Sigma) in 30 mmol/L imdazole/HCl buffered to pH 7.2. Mg^{2+}-ATPase activity was determined in the presence of 3×10^{-4} mol/L digitoxigenin (Sigma). The enzymatic reaction was initiated by the addition of membranes (1–3 μg protein). NADH oxidation was continuously monitored for 20 minutes and the results were taken when the time course of hydrolysis was linear. The specific activities recorded were in the range of 26 to 29 μmol of inorganic phosphate per hour per milligram of protein.

The proportion of each isoenzyme as a function of ouabain affinity was obtained from dose-response curves (difference activities in the presence and in the absence of various ouabain concentrations). The best fit of the curves was analyzed as the sum of two or three saturable and independant sites by non-linear regression using the cooperative model.[1] The number of independent sites to fit the data for ouabain inhibition was chosen according to the Schwarz criterion[24] and the likelihood ratio chi-square test.

Na^+, K^+-ATPase dependence on sodium ion

The activity of Na^+, K^+-ATPase as a function of variable concentrations of sodium was evaluated as described above with various amounts of sodium chloride (NaCl; up to 100 mM). To maintain a constant ionic strength, choline chloride was included in the assay so that NaCl + choline chloride equalled 100 mmol/L. Enzymatic activity measured with 100 mmol/L of sodium and 10 mmol/L of potassium was used as reference (100%) with change of ouabain concentration according to the experiment. The Na^+ dependence of each Na^+, K^+-ATPase isoenzyme expressed in whole brain membranes of rats fed the different diets was determined in the presence of different concentrations of ouabain. The concentration of Na^+ in the Na^+ free medium was measured by flame spectrophotometry. It was found to be 60 μmol/L, corresponding to an error of 15% at the lowest Na^+ concentration (0.4 mmol/L) used in the experiments.

Data analysis

All Na^+ curves fitted to the experimental data were obtained using the following equation:

$$A = \sum_{j=1}^{k} Rj$$

where A = activity (%), k = number of reactivities R_j, and R_j = 0 if $[Na^+]$ is less than a threshold T_j; otherwise:

$$R_j = \frac{A_j}{1 + \left(\dfrac{Ka_j - T_j}{[Na^+] - T_j}\right)^{n_j}}$$

where A_j = the maximum activity; Ka_j = the concentration for half-maximal activity; n_j = the Hill coefficient; and T_j = the threshold of stimulation.

Estimation of Aj, Kaj, nj, and Tj (j = 1 to K) were obtained using MKMODEL® Software (Biosoft, Cambridge, UK) with a model that had been written according to Garner et al.[25] without weighing because the data (means) were observed with variances approximately equal for low and high Na^+ concentrations. The number of independent sites to fit the data for Na^+ dependence of Na^+, K^+-ATPase was chosen according to the Schwarz criterion[24] and the likelihood ratio chi-square test.

Statistical analysis

Membranes from rats fed the soybean oil and the sunflower oil diets, which were prepared on the same day and analyzed at the same time, were evaluated by Student's *t*-test. $P < 0.05$ was considered statistically different. For ouabain and Na^+ dose response curves, statistical analysis was performed using a two-way analysis of variance (ANOVA) procedure with Tukey test for multiple comparisons.

Results

Effects of the diet deficiency in 18:3(n-3) on fatty acid composition of brain membranes

Compared with the fatty acid composition of brain membranes of rats fed the soybean oil diet, the membranes of rats fed the sunflower oil diet showed higher concentrations of docosatetraenoic acid [22:4(n-6)] and docosapentaenoic acid [22:5(n-6)] and lower concentrations of docosahexaenoic acid [22:6(n-3)], resulting in a marked increase of (n-6)/(n-3) molar ratio (5.16 versus 1.08) (*Table 3*). The total levels of saturated fatty acids, MUFA, and PUFA were not altered.

Sensitivity of brain membranes Na^+, K^+-ATPase activity to ouabain

In the soybean and sunflower oil groups, the dose-response curve of Na^+, K^+-ATPase activity to ouabain (*Figure 1*) spanned over almost five to six orders of magnitude of ouabain concentrations, indicating the presence of more than one population of catalytic subunits. The data obtained from the membrane fractions of rats fed the sunflower oil diet (*Figure 1*, ○), were best fitted by an equation according to the Schwarz criterion[24] and the likelihood ratio chi-square test ($P < 0.001$) that assumed the existence of three rather than two forms. The computed IC_{50} values (concentration of drug leading to 50% inhibition of total activity) were 58×10^{-6} mol/L, 5.4×10^{-6} mol/L, and 0.032×10^{-6} mol/L for low ouabain affinity ($\alpha 1$), high ouabain affinity ($\alpha 2$), and very high ouabain affinity ($\alpha 3$), respectively. Their estimated proportions were calculated to be

Table 3 Total phospholipids fatty acid composition of brain membrane fractions enriched for Na^+, K^+-ATPase from rats fed soybean and sunflower diets*,†

Fatty acids	Diets (g/100 g fatty acids)	
	Soybean	Sunflower
16:0	20.4 ± 0.2	21.3 ± 0.3
16:1(n-9)	0.7 ± 0.3	0.7 ± 0.2
16:1(n-7)	0.7 ± 0.2	1.1 ± 0.2
18:0	22.0 ± 0.2	22.3 ± 0.3
18:1(n-9)	15.0 ± 0.1	13.6 ± 0.2[a]
18:1(n-7)	4.0 ± 0.2	4.4 ± 0.2
18:2(n-6)	0.4 ± 0.1	0.5 ± 0.1
20:0	0.6 ± 0.1	0.5 ± 0.1
20:1(n-9)	1.4 ± 0.1	1.1 ± 0.2
20:1(n-7)	0.5 ± 0.1	0.4 ± 0.1
20:3(n-6)	0.3 ± 0.1	0.3 ± 0.1
20:4(n-6)	9.9 ± 0.1	10.7 ± 0.2
22:0	0.5 ± 0.1	0.4 ± 0.1
22:4(n-6)	2.8 ± 0.1	4.1 ± 0.3[b]
22:5(n-6)	2.4 ± 0.5	10.6 ± 0.4[b]
24:0	0.9 ± 0.1	0.7 ± 0.1
22:6(n-3)	14.8 ± 0.6	5.1 ± 0.3[b]
24:1(n-9)	1.1 ± 0.1	0.8 ± 0.2
24:4(n-6)	0.4 ± 0.1	0.3 ± 0.1
ΣSFA	44.4 ± 0.3	45.4 ± 0.3
ΣMUFA	23.4 ± 0.3	22.1 ± 0.1
Σ(n-6)	16.2 ± 0.4	26.5 ± 0.3[b]
Σ(n-3)	14.8 ± 0.6	5.1 ± 0.3[b]
ΣPUFA	31.0 ± 1.0	31.6 ± 0.3
(n-6)/(n-3)	1.08 ± 0.10	5.16 ± 0.40

*Values are means ± SEM, $n = 4$.
†Significance was determined by paired Student's t-test. [a]$P < 0.005$; [b]$P < 0.001$ compared with a soybean group.

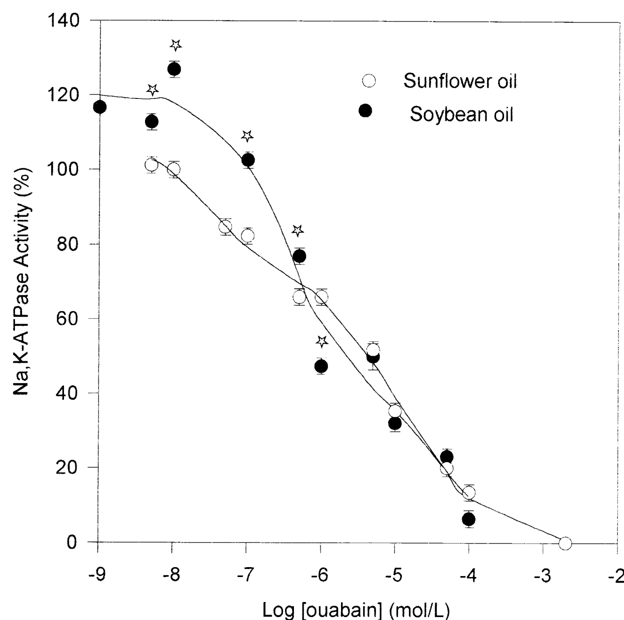

Figure 1 Ouabain inhibition of membrane fraction Na^+, K^+-ATPase from rats fed sunflower and soybean diets. Values are means ±SEM, $n = 4$; experiments were done in triplicate. Data were analyzed by a non-linear regression model (see Materials and methods). Lines (○) represent the theoretical curves assuming a three-site model rather than a two-site model ($P < 0.001$). Lines (●) represent the theoretical curves assuming a two-site model rather than a three-site model ($P < 0.001$). These data were analyzed by two-way analysis of variance (ANOVA). Post-ANOVA comparisons (Tukey test) showed that some Na, K-ATPase activity was significantly different between sunflower and soybean diets. *$P < 0.05$. ○, sunflower diet; ●, soybean diet.

28%, 36%, and 36%, respectively (*Table 4*). In marked contrast (*Figure 1*, ●), for the membrane fraction from rats fed the soybean oil diet, the best fit of the experimental points was obtained using only a two-site model rather than a three-site model ($P < 0.001$; *Figure 2*). The computed IC_{50} was 45 × 10^{-6} mol/L for low ouabain affinity (α1) and 0.37 × 10^{-6} mol/L for high ouabain affinity (α2′). Their calculated proportions were 32.5% and 67.5%, respectively (*Table 4*).

Na^+ dependence of Na^+, K^+-ATPase isoenzymes according to their ouabain sensitivity

As a result of the data presented *Figure 1*, we were able to choose two ouabain concentrations that were suitable for discriminating between isoenzymes. We found that 10 × 10^{-6} mol/L and 0.1 × 10^{-6} mol/L ouabain were suitable to discriminate between isoenzyme reactivities for rats fed soybean oil and 30 × 10^{-6} mol/L and 1 × 10^{-6} mol/L ouabain to discriminate isoenzyme forms for rats fed sunflower oil.

Comparison of the Na^+ dependence of the low ouabain affinity isoforms. In 10^{-5} M ouabain in membrane fractions from rats fed the soybean oil diet, α2′ isoenzyme activity was completely inhibited (*Figure 1*). Under these conditions, the best fit of the experimental points (*Figure 2*, ●) corresponded to two reactivities saturated at 8 mmol/L sodium a with Ka_1 of 0.7 mmol/L and a Ka_2 of 6.0 mmol/L for α1 isoenzyme. The relative proportions of α1 isoenzyme activities were estimated to be 32% and 68%, respectively.

On the basis of the ouabain dose-response curves (*Figure 1*) an appropriate ouabain concentration in sunflower oil fed rats can be chosen (30 × 10^{-6} mol/L of ouabain) to selectively inhibit α2 and α3 isoenzyme activities. Under these conditions, the experimental points (Na^+ dependence of α1 isoenzyme), were best fitted by two reactivities (*Figure 2*, ○) that saturated at 12 mmol/L sodium with a Ka_1 of 0.4 mmol/L (34.8%) and a Ka_2 of 6.0 mmol/L (65.2%).

If the concentration for half activity (Ka_1 and Ka_2) and the contribution of the two stimulatory processes were comparable (*Table 5*), some data differ significantly (*Figure 2*).

Comparison of the Na^+ dependence of the high ouabain affinity isoforms. Based on the hypothesis that the α2′ isoenzyme activity may represent the activity of high and very high ouabain affinity sites not detected by mathematical analysis (because the affinity of this α2′-isoenzyme is intermediate between α2 and α3 isoenzyme), 0.1 × 10^{-6} mol/L of ouabain was used to discriminate between them in the soybean oil fed rats (*Figure 1*). The best fit of the experimental points (*Figure 3*, ●) corresponded effectively to three reactivities; the two higher sensitive reactivities for sodium accounted for 43% of the total activity and reflected the reactivities observed at 10 × 10^{-6} mol/L ouabain. The

Table 4 Computed affinities for ouabain (IC_{50}, mol/L) and contribution (%) of the very high (α3), high (α2), and low (α1) affinity Na^+, K^+-ATPase isoenzymes in whole brain from rats fed soybean and sunflower diets*,†

Diets	Low affinity (α1)		High affinity (α2)		Very high affinity (α3)	
	IC_{50} (mol/L)	Contribution (%}	IC_{50} (mol/L)	Contribution (%)	IC_{50} (mol/L)	Contribution (%)
Soybean	$4.5 \pm 0.5\ 10^{-5}$	32.5 ± 0.8	$3.7 \pm 0.8\ 10^{-7}$	67.5 ± 0.8	ND	ND
Sunflower	$5.8 \pm 0.5\ 10^{-5}$	28.0 ± 0.8	$5.4 \pm 0.4^a\ 10^{-6}$	36.0 ± 1.9^a	$3.2 \pm 0.6\ 10^{-8}$	36.0 ± 2.5

*Data were analyzed by non-linear regression model (see Materials and methods). Values are means ± SEM, $n = 4$; experiments were done in triplicate.
†Significance of differences was determined by paired Student's t-test.
[a]$P < 0.001$ compared with a soybean group.
ND–not detected.

third class of reactivity, with a Ka_3 of 11.7 mmol/L (57%), was indicative of a high ouabain affinity isoenzyme (α2).

At 1×10^{-6} mol/L ouabain, α3 isoenzyme was specifically inhibited in sunflower oil fed rats. The best fit of the experimental points (*Figure 3*, ○) was consistent, with a three-component response to Na^+ stimulation. The two higher sensitive reactivities, which accounted for 65% of the total activity, reflected the reactivities observed at 30×10^{-6} mol/L ouabain whereas the third reactivity, with a Ka_3 of 8.8 mmol/L and 35% of the total activity, corresponded to the Na^+ dependence of the high ouabain affinity α2 isoenzymes.

The Na^+ affinity of the high ouabain affinity site (α2) as expressed in *Table 5* differs significantly between the diets (see *Figure 3*).

Comparison of the Na^+ dependence of the very high ouabain affinity isoforms. The activity measured in the absence and in the presence of 0.1×10^{-6} mol/L ouabain in soybean oil fed rats allowed us to visualize a fourth reactivity with a Ka_4 of 16 mmol/L, which saturated at 23.5 mmol/L Na^+ (*Table 5*), corresponding to the α3 isoenzyme.

The difference between the activity in the absence and in the presence of 1×10^{-6} mol/L ouabain in sunflower oil fed rats yielded a fourth reactivity with a Ka_4 of 17 mmol/L, which saturated at 23 mM sodium (*Table 5*), which corresponded to α3 isoenzyme.

The Na^+ dependence of the α3 isoenzyme activity in the two experimental groups showed no dependence on the diet.

Discussion

According to Galli et al.,[26] and consistent with our previous studies,[16,27] a sunflower oil diet in the rat leads to a decrease in docosahexanoic acid (DHA) 22:6(n-3), which is compensated for by an increase in 22:5(n-6) in purified brain

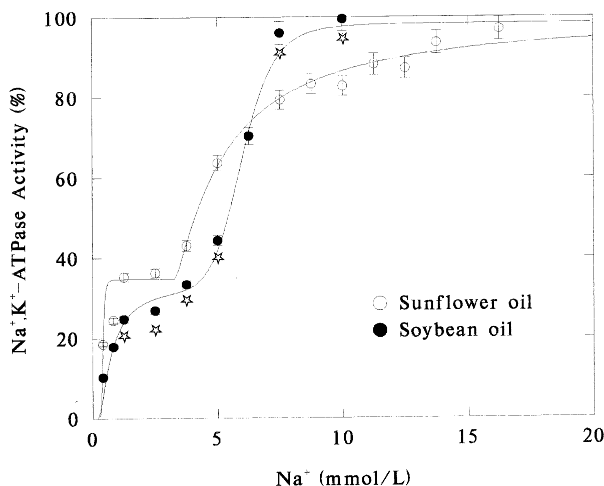

Figure 2 Na^+ dependence of low ouabain affinity isoenzyme in membrane fraction from rats fed the sunflower and soybean diets. Values are means ± SEM, $n = 4$; experiments were done in triplicate. Data were analyzed by a non-linear regression model (see Materials and methods). ○, Na^+ dependence of low ouabain affinity isoenzyme at 3×10^{-5} mol/L ouabain in sunflower diet. ●, Na^+ dependence of low ouabain affinity isoenzyme at 10^{-5} mol/L ouabain in soybean diet. These data were analyzed by two-way analysis of variance (ANOVA). Post-ANOVA comparisons (Tukey test) showed that some Na, K-ATPase activity was significantly different between sunflower and soybean diets. *$P < 0.05$.

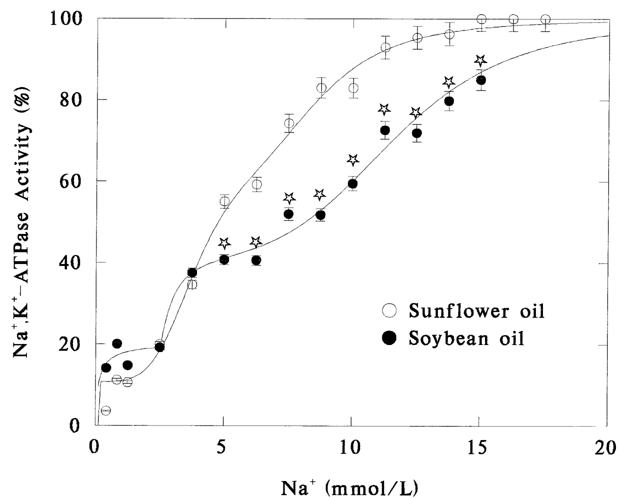

Figure 3 Na^+ dependence of low and high ouabain affinity isoenzymes in membrane fraction from rats fed the sunflower and soybean diets. Values are means ± SEM, $n = 4$; experiments were done in triplicate. Data were analyzed by a non-linear regression model (see Materials and methods). ○, Na^+ dependence of low and high ouabain affinity isoenzymes at 10^{-6} mol/L ouabain in sunflower diet. ●, Na^+ dependence of low and high ouabain affinity isoenzymes at 10^{-7} mol/L ouabain in soybean diet. These data were analyzed by two-way analysis of variance (ANOVA). Post-ANOVA comparisons (Tukey test) showed that some Na, K-ATPase activity was significantly different between sunflower and soybean diets. *$P < 0.05$.

Table 5 Computed sodium sensitivity (Ka, mmol/L) of the very high (α3), high (α2), and low (α1) affinity Na^+, K^+-ATPase isoenzymes activities from rats fed soybean and sunflower diets*[†,‡]

Diets	Low affinity (α1) Ka (mmol/L)		High affinity (α2) Ka (mmol/L)		Very high affinity (α3) Ka (mmol/L)	
	R1	R2	R1	R2	R1	R2
Soybean	0.70 ± 0.03	6.0 ± 0.05	11.7 ± 1.0	ND	16.0 ± 1.5	ND
Sunflower	0.40 ± 0.02	6.0 ± 1.1	8.8 ± 1.2[a]	ND	17.0 ± 2.0	ND

*Data were analyzed by non-linear regression model (see Materials and methods). Values are means ± SEM, $n = 4$; experiments were done in triplicate.
†Significance of differences was determined by paired Student's *t*-test.
[a]$P < 0.05$ compared with a soybean group.
‡When two components were observed, R1 corresponds to the first reactivity and R2 corresponds to the second reactivity. Only one reactivity corresponds to R1.

membranes. Although total PUFA were not different in brain membranes in rats fed either sunflower oil or soybean oil diet, clear differences were seen in the properties of Na^+, K^+-ATPase isoenzymes with respect to their ouabain sensitivity and their sodium affinity. These results suggest that the functional importance of lipid protein interaction is modulated by the fatty acid composition of the membranes in determining the enzymatic properties of Na, K-ATPase isoenzymes.

The brain membranes of rats fed the soybean oil diet were consistent with a two-site model of ouabain inhibition involving two different isoenzymes (i.e., low and high ouabain affinity). This result is in agreement with several studies that, based on their cardiac glycoside sensitivity, electrophoretic mobility, and immunoblotting results,[4,28] demonstrated the existence of two isoenzymes in brain, termed α and α(+). Na, K-ATPase activity in brain membranes in rats fed the sunflower oil diet fitted with a three-site model of ouabain inhibition, suggesting the existence of three different isoenzymes [i.e., low (α1), high (α2), and very high ouabain affinity (α3)]. These results agree with our previous studies[6,13] and with data from other groups[1,2] and confirm the presence of three isoenzymes in the brain, as already demonstrated by cDNA study[29] and by RNA blot and proteolytic fingerprinting analyses.[30] The discrepancy between studies that found two sites and those that found three sites of ouabain inhibition may be due to the difference between the isoenzymes activities or to a close affinity for ouabain between the α2 and the α3 isoforms;[31] the source of the enzyme[4] and/or the procedure used for the enzyme purification also may explain the discrepancy.[22] Nevertheless, it is well known that brain is a tissue in which the three isoenzymes of the α subunit are expressed and are functional in the adult rat.[4] In addition, the present investigation documents the effect of diet on Na^+, K^+-ATPase isoenzyme affinities for ouabain. Moreover, this study shows that there is a difference between the ouabain sensitivity of the different isoenzymes in relation to the fatty acid composition of the membrane lipid bilayer. A low ouabain affinity isoenzyme (α1) was not altered whereas a high ouabain affinity (α2 and/or α3) was sensitive to such fatty acid modification of membrane phospholipids.[32] This finding may support the idea that the modification of phospholipid fatty acids as reflected by n-6 PUFA and DHA concentrations was of sufficient magnitude to alter the lipidic microenvironment around the α2 and/or α3 isoenzyme and hence affect their activities. These results document the importance of the effects of fatty acid composition of membrane phospholipids on the activity of Na^+, K^+-ATPase isoenzymes of high ouabain affinity.[11–13]

Brain membrane isoenzyme affinities for sodium are still debated. Jewell and Lingrel[33] reported that α3 isoenzyme exhibited a lower sodium affinity than α1 and α2, which had the same affinity. Brodsky and Guidotti[5] showed that α1 and α2 or α3 expressed in synaptosomes differ in their sodium affinity: α2 or α3 exhibited a lower affinity for sodium than the α1 isoenzyme.

Whatever the diet, on the basis of the ouabain dose response, the mathematical model used made it possible to demonstrate the existence of three isoenzymes on the basis of the Na^+ dependence of their activity. Their sodium affinity—$\alpha 1 > \alpha 2 > \alpha 3$—corresponds to what we showed in weaned rats.[13] Furthermore, α2 isoenzyme (high ouabain affinity) of the brain membrane of rats fed the sunflower oil diet exhibited a higher affinity for sodium (Ka = 8.8 mmol/L) than that of rats fed the soybean oil (Ka = 11.7 mmol/L). This result might be explained by a different membrane fatty acid composition on the surrounding α2 isoenzyme, as suggested by Brodsky and Guidotti.[5] The biophysical properties of the membrane as induced by the high level of (n-6) PUFA or deficiency of DHA[16,34,35] may influence the functional properties of the Na^+, K^+-ATPase and account for these differences.

The sensitivities of isoenzymes (α2 and α3) to ouabain and to sodium observed in the present study, as well as those measured in weaned rats,[13] clearly show a difference in rats receiving different dietary (n-6)/(n-3) PUFA balance. These results stress the importance of the membrane fatty acid composition (PUFA + MUFA) on the functional properties of the Na^+, K^+-ATPase. Because, as has been previously shown, the cholesterol/phospholipid ratio alters Na, K-ATPase activity by altering insertion of one the Na, K-ATPase subunits into the membrane,[36] one must be careful not to mistake the fatty acid composition as the sole possible factor.

Moreover, the heterogeneous pattern found with the α1 isoenzyme in the Na^+ dependence of Na^+, K^+-ATPase activity may explain the diversity of this isoenzyme, as hypothezed in studies related to cardiac tissue[37] and brain.[6]

This could imply the existence of more than three isoenzymes in whole rat brain membranes.[38,39] These results suggest that the sodium ion can be a better criterion to distinguish the heterogeneity between Na^+, K^+-ATPase isoenzyme than the ouabain does and that nutrition can modify some functional properties of brain enzymes.

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