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Fatty Acid-Induced Modulation of Ouabain Responsiveness of Rat Na,K-ATPase Isoforms

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Abstract. Membrane phospholipids represent a potential influence on the enzymatic properties of the Na,K-ATPase. Little is known concerning the effects of the fatty acid environment surrounding the enzyme on the kinetic properties of the Na,K-ATPase. We used the most obvious difference among the α isoforms of rat, their affinities for digitalis glycosides, to examine the relationship between the lipid environment and the Na,K-ATPase. Specific membrane environments that differ in their fatty acid composition were produced by drug-induced diabetes, as well as variations in diet. The α1 isoforms in various tissues were then characterized by their resistance to ouabain in Na,K-ATPase-enriched membrane microsomal fractions. The Na,K-ATPase activity in nerves and hearts were altered by diabetes and partially restored in nerves after a fish oil diet. Evaluation of enzyme kinetics (dose-response curves for ouabain) in membrane preparations allowed us to correlate the ouabain affinity of α1 isoform with fatty acid composition. The affinity of the α1 isoform for ouabain was significantly increased with accretions in the total amount of fatty acids of the n-6 series ($P < 0.0001$). Our observations provide a partial explanation for the observed difference in isoform properties among tissues. Moreover, these results underline the interaction between membrane fatty acids and the glycoside binding site of the Na,K-ATPase α1 subunit.

Key words: Na,K-ATPase — Isoenzymes — Rat — Ouabain — Fatty acids — Membrane — MaxEPA

Introduction

Na,K-ATPase is a membrane-spanning enzyme complex consisting of two subunits (α and β) surrounded by a closely associated ring of lipids (Murphy, 1990). Indeed, about 40% of the α subunit is embedded within the membrane (Lingrel & Kuntzweiler, 1994). Both subunits exist in at least three isoforms, α1, α2, α3 and β1, β2, β3 (Schull et al., 1986, Martin-Vasallo et al., 1989, Malik et al., 1996). In rodents (both rat and mouse), the α isoforms exhibit differences in sensitivity to ouabain and other digitalis glycosides, with the α1 exhibiting a relatively low affinity, the α2 a higher affinity, and the α3 a very high affinity (Noël, Fagoo & Godfraind, 1990; Berrebi-Bertrand et al., 1990; Blanco, Berberian & Beangé, 1990; Jamme et al., 1997; Maixent et al., 1998). The relationship, if any, between the kinetic properties of each isoform and the composition of the surrounding membrane is not well established.

Site-directed mutagenesis has provided an important strategy for identifying amino acid residues responsible for ouabain binding and for the affinity of the isoform for the drug. Two residues believed to be within the membrane of the sheep enzyme, Cys-104 and Tyr-108, have been found to influence affinity, despite the fact that digitalis binds to an exterior site on the molecule (Lingrel & Kuntzweiler, 1994). This suggests that the cardiac glycoside may be partially inserted into a hydrophobic membrane binding site(s). Comparisons of drug structure and possible binding sites suggest that the enzyme binds three distinct components of the cardiac glycoside, the steroid skeleton and two differing side chains at C-3 and C-17 (Schönfeld et al., 1985, Maixent, Berrebi-Bertrand & Lelièvre, 1991). In addition, Sweadner (1989) has suggested that the different ourabain affinities observed between different tissues may be attributable in part to variations in membrane environment rather than any structural changes in the Na,K-ATPase itself. Abeywardena and Charnock (1983) have shown a correlation between the degree of saturation within membrane phospholipids and drug affinity. In our previous work, we have shown that modifications in fatty acid composition

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also alter the sensitivity of the α isoforms to ouabain (Gerbi et al., 1993*a*,*b*, 1994).

The aim of the present study was to define further the relation of membrane fatty acid composition with drug affinity. Different membrane environments were obtained by using various tissues known to express predominantly α1: kidney, heart, brain and sciatic nerves. In addition, fatty acid composition was modified further by drug-induced diabetes and lipid diets. The changes in enzyme kinetics with lipid composition that we observed are consistent with an interaction between membrane fatty acids and the glycoside binding site of the Na,K-ATPase.

Materials and Methods

Animals

Four-week-old male Sprague-Dawley ($n = 18$) rats weighing approximately 200 g were randomly divided into three groups of 6. In two groups, diabetes was induced by intravenous injection of streptozotocin (STZ) at 60 mg/kg (STZ, Sigma, L'Isle d'Abeau, Chesne, France) diluted immediately before injection in citric acid buffer (0.01 mol/l, pH 5.5). In the control group only citric acid buffer was injected. One group of diabetic animals (DM) was fed the standard rat chow diet supplemented with (n-3) fatty acid-enriched fish oil concentrate (MaxEPA®, Pierre Fabre Santé, Castres, France) administered during 8 weeks at a daily dose of 0.5 g/kg by gavage. The fatty acid-enriched fish oil concentrate MaxEPA has been determined in Gerbi et al., 1998. This supplement is rich in eicosapentaenoic acid (EPA, C20:5 (n-3)) and docosahexenoic acid (DHA, C22:6 (n-3)). The other group of diabetic animals (DO) was fed the standard rat chow diet supplemented with olive oil using the same dosage regimen. Diabetic rats were not treated with insulin. The nondiabetic control group (CO) was also fed the standard rat chow diet supplemented with olive oil. Olive oil was chosen as the placebo because it contains only traces of (n-3) fatty acids. Water was given ad libitum to all groups. All animal treatments adhered strictly to all institutional and national ethical guidelines. All animals were killed by decapitation after 8 weeks.

Tissue Preparations

Whole kidneys and brains, hearts (ventricles), and sciatic nerves were rapidly removed, rinsed with ice-cold saline (less than 30 sec), frozen in liquid nitrogen and stored at −80°C until use. The tissue preparation consisted of isolating a highly Na,K-ATPase-enriched membrane fraction according to a previously described procedure (Maixent et al., 1991*b*). Frozen tissues were homogenized in 10 volumes of ice-cold buffer containing 20 mmol/L sodium pyrophosphate, 0,1mmol/L phenylmethanesulfonyl fluoride, 1 mmol/L EDTA, 250 mmol/l sucrose, 80 mmol/L Kcl, and 20 mmol/L imidazol/HCl (pH 7.4 at 25°C) with a polytron PT20 (25 *sec*; setting 7 for sciatic nerves and 15 sec; setting 5 for the other tissues). The homogenates were subfractionated by three sequential differential centrifugations at 120 × *g* for 5 min, 7,000 × *g* for 15 min and 48,000 × *g* for 30 min using JA. 20 rotor (Beckman Instrument France, Gagny, France) in the Beckman J2,21 (Beckman Instrument France, Gagny, France). The pellets were resuspended in 100 mmol/L NaCl, 250 mmol/L sucrose, 30 mmol/L imidazol/HCl (pH 7.4 at 25°C) and stored frozen in liquid nitrogen.

Enzymological Study

Na,K-ATPase activity was determined by coupled assays at 37°C with or without ouabain as previously described (Gerbi et al., 1993*a*). The activity was measured in an ATP-regenerating medium by continuously recording NADH (reduced form of NAD) oxidation using a spectrophotometer. Enzymatic activities were measured at 37°C and were standardized to protein. Each cell contained (final volume, 0.6 ml) 2 mmol/L phosphoenolpyruvate, 10 mmol/L KCl, 100 mmol/L NaCl, 4 mmol/L Mg^{2+}-ATP, 30 mmol/L imidazol/HCl (pH7.4 at 25°C), 0.4 mmol/L NADH, 3.5 units of pyruvate kinase, and 5 units of lactate dehydrogenase. To unmask the «latent» enzyme activity from closed right side out vesicles and the ouabain inhibition from inside out vesicles, treatment with sodium dodecyl sulfate (SDS) was performed to reveal the maximum Na,K-ATPase activity (Jorgensen & Skou, 1971). We performed detergent treatment with 3 SDS concentrations (0.1, 0.2 and 0.3 mg SDS/mg protein or 30 min at 20°C). The most efficient amount of SDS to reveal activity was 0.2 mg SDS/mg protein and this amount was the same in the 4 organs.

The relative proportion of isoenzymes as a function of ouabain affinity was inferred from affinities, as estimated from dose-response curves on permeabilized membranes with the highest Na,K-ATPase activity. Inhibition was calculated by comparing the activities in the presence of various ouabain concentrations or absence of ouabain after correcting for ouabain-insensitive ATPase activity measure in the presence of 2 mmol/L ouabain. Curves were fitted to experimental data by a nonlinear regression model using the cooperative model as described by Berrebi-Bertrand et al. (1990) using MKModel® software (Biosoft, Cambridge, England). The number of saturable and independent sites used to model the data was chosen according to the Schwarz criterion (Schwarz, 1978). Unless otherwise noted, were attributed the activity associated with the highest affinity site to the α3 isoform, the intermediate affinity to α2 and the lowest affinity to α1. Only the α1 isoform from the complex curves was chosen to evaluate correlation since its ouabain binding equilibrium was reached in less than one minute, its discrimination by ouabain was unambiguous and this isoenzyme was present in all the tissues tested in the present study.

Fatty Acid Composition of Various Membrane Fractions

After extraction of free lipids according to the method of Folch, Lees & Sloane-Stanley, (1957), fatty acid methyl esters were prepared according to Hagenfeldt (1966). Fatty acids were analyzed as methyl esters on a Varian model 3300 gas chromatograph equipped with a flame ionization detector (FID) using a spirawax capillary column (25 m × 0.2 mm i.d.). The temperature program was 150 to 210°C at 1.5°C/min. Peak areas from the resulting chromatogram were measured with a Merck model D 2000 integrator. Nonadecanoic acid (C19:0) was added to the mixture before methylation as an internal standard.

Statistical Analyses

Differences between the three groups (CO, DO, DM) were evaluated by one-way ANOVA followed by multiple comparison with Scheffé's tests. Values of $P < 0.05$ were considered statistically significant. Correlations were evaluated by a single linear regression analysis with the Statview Software. Correlation analysis has been done with the average values from each group. All analyses were performed by Statview software.

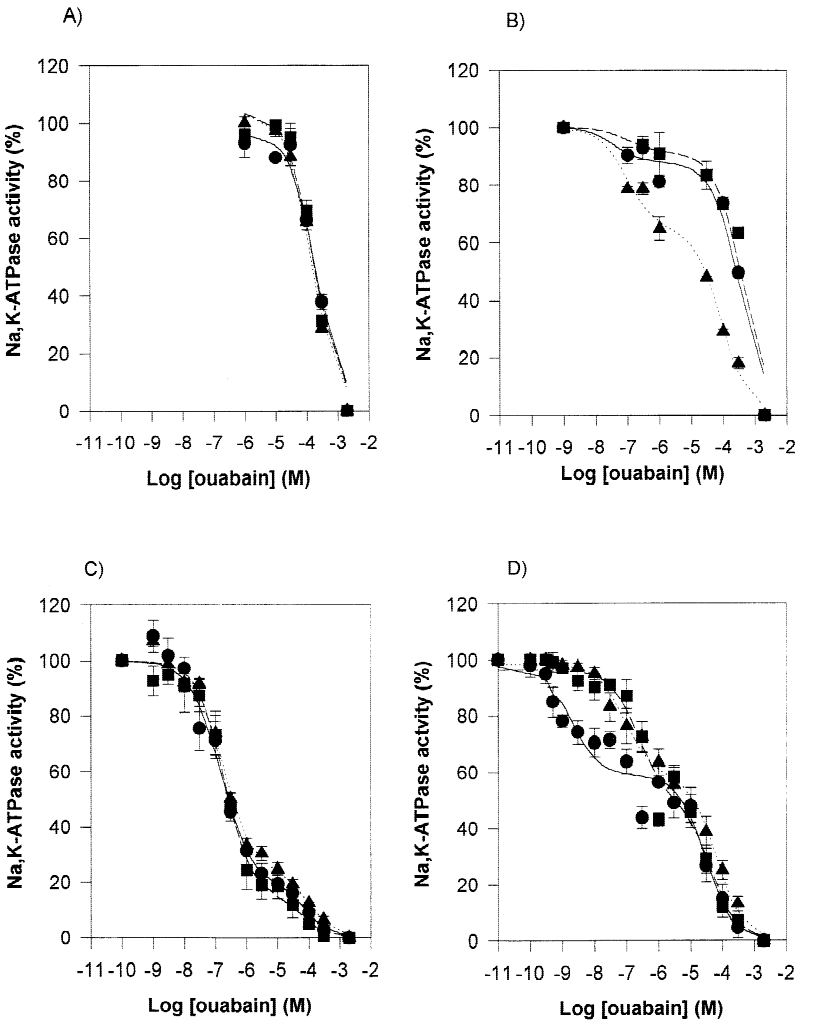

Fig. 1. Effect of diabetes and dietary fish oil supplementation on the ouabain inhibition of the Na,K-ATPase activity in different tissues. (*A*) Kidney, (*B*) Heart, (*C*) Brain, (*D*) Sciatic nerve. Three groups of six animals were analyzed (● control + olive; ■ Diabetic + olive; ▲ Diabetic + fish oil). Values are means ± SEM of experiments done in triplicate. Data were analyzed by a nonlinear regression model. Lines represent the theoretical curves assuming one to three site model fit. The computed affinities are reported in Table 1.

Results

As expected, dose-response curves of ouabain inhibition of Na,K-ATPase activity varied among the different tissues in diabetic and control rats (Fig. 1, Table 1). Only α1 was detectable in membranes from kidney. Cardiac tissue displayed a biphasic dose-response curve with intermediate and low ouabain affinities attributed to α1 and α2, respectively. All three isoforms were observed in sciatic nerve and brain as dose-response curves spanned at least six orders of magnitude and the experimental points were best fitted by nonlinear regression that assumes the existence of a population of three, rather than two independent inhibitory sites (attibuted to α1, α2 and α3 isoenzymes of Na,K-ATPase) with different affinities for the inhibitor according to the likelihood ratio chi-square test (MKModel® software). From the concentration dependence of enzymatic inhibition by ouabain, we assigned the observed Na,K-ATPase activity in each sample to the various isoforms. Presumably, the low affinity site corresponded to α1, and the second site of ouabain inhibition a mixture of α2 and α3 (Fig. 1). Only the relationship between fatty acid content and responsiveness to ouabain was analyzed for α1.

No significant changes were produced by diabetes and variations in lipid diet in kidney and brain membranes. By contrast in heart, diabetes decreases significantly the enzymatic activity of the α1 isoform, fish oil supplementation potentiates this decrease in activity. In sciatic nerve membranes, the α1 enzymatic activity was decreased by diabetes and improved by fish oil supplementation (Table 1).

That the onset of diabetes and lipid diet produced profound differences in lipid composition is demonstrated in Table 2. The main result displayed by the Table 2 is the differential fatty acid composition observed in the different tissues. Heart and kidney membranes are altered similarly by diabetes. Indeed, in these two tissues, the C18:2(n-6) (linoleic acid) is significantly increased and the C20:4(n-6) (arachidonic acid) is de-

Table 1. Effects of STZ-induced diabetes and MaxEPA supplementation on affinities for ouabain (IC_{50}) and isoenzymes activities of the different isoenzymes expressed in kidney, heart, sciatic nerve and brain tissues

Tissues and groups	Low ouabian affinity (α1)		High ouabain affinity (α2)		Very high ouabain affinity (α3)	
	Activity μmol Pi/h/mg Prot	IC_{50} (M)	Activity μmol Pi/h/mg Prot	IC_{50}(M)	Activity μmol Pi/h/mg Prot	IC_{50}(M)
Kidney						
CO	35.6 ± 11.3	$1.9 \pm 0.8\ 10^{-4a}$				
DO	30.3 ± 10.3	$1.9 \pm 0.8\ 10^{-4a}$				
DM	27.7 ± 12.5	$1.6 \pm 0.9\ 10^{-4a}$				
Heart						
CO	28.0 ± 2.9	$3.6 \pm 0.2\ 10^{-4a}$	4.0 ± 0.6	$4.0 \pm 1.0\ 10^{-8a}$		
DO	20.7 ± 2.3	$4.4 \pm 0.3\ 10^{-4a}$	2.3 ± 0.7	$10.0 \pm 0.4\ 10^{-8b}$		
DM	9.3 ± 0.7	$7.7 \pm 0.2\ 10^{-5b}$	4.7 ± 0.7	$8.1 \pm 0.3\ 10^{-8b}$		
Sciatic nerve						
CO	2.49 ± 0.23	$4.2 \pm 0.8\ 10^{-5a}$	1.11 ± 0.14	$5.9 \pm 1.2\ 10^{-8a}$	1.18 ± 0.19	$1.3 \pm 0.9\ 10^{-10a}$
DO	1.09 ± 0.07	$4.4 \pm 0.7\ 10^{-5a}$	0.97 ± 0.10	$2.0 \pm 0.9\ 10^{-7b}$	0.11 ± 0.03	$3.1 \pm 1.9\ 10^{-10a}$
DM	1.72 ± 0.18	$1.0 \pm 0.6\ 10^{-4b}$	0.87 ± 0.14	$1.7 \pm 0.5\ 10^{-6c}$	0.97 ± 0.10	$2.7 \pm 0.6\ 10^{-8b}$
Brain						
CO	7.1 ± 2.2	$7.9 \pm 0.3\ 10^{-5}$	19.4 ± 1.5	$2.0 \pm 0.1\ 10^{-7}$	8.7 ± 1.7	$4.3 \pm 0.1\ 10^{-8}$
	Two site model		Mixed of the high and very high ouabain affinity (α2 + α3)			
CO	7.8 ± 1.4	$6.4 \pm 0.5\ 10^{-5a}$	27.4 ± 1.4		$1.3 \pm 0.8\ 10^{-7a}$	
DO	5.4 ± 1.1	$6.1 \pm 0.4\ 10^{-5a}$	28.8 ± 1.3		$1.8 \pm 0.9\ 10^{-7a}$	
DM	8.4 ± 0.8	$9.9 \pm 0.6\ 10^{-5a}$	25.2 ± 1.1		$1.7 \pm 0.9\ 10^{-7a}$	

Values are means ± SE from 6 animals, experiments were done in triplicate. CO, control + olive oil supplementation; DO, diabetic + olive oil supplementation; DM, diabetic + fish oil supplementation. Data were analyzed by nonlinear regression model (Materials and Methods). IC_{50} (mol/L) is the ouabain concentration at which there is 50% maximal inhibition. Significance was determined by ANOVA. IC_{50} values in the same row not bearing the same superscript letters were significantly different at $P < 0.05$.

creased. In these two tissues, the lipid treatment increased the incorporation of the eicosapentaenoic acid (C20:5(n-3)). In brain, where the fatty acid composition is resistant to diet manipulation, we observed no effect of the treatment. In contrast, diabetes induced an increase in linoleic acid and no effect on the arachidonic proportion. For sciatic nerve membranes, diabetes increased the relative proportion of (n-3) fatty acids. Concerning the (n-6) fatty acids incorporation, diabetes increased the incorporation of C18:2(n-6), C20:2(n-6) and C22:5(n-6) and decreased the incorporation of C20:3(n-6). Fish oil supplementation induced a restoration of (n-6) fatty acid incorporation to the level of control.

Relation of Fatty Acid Membrane Composition to Ouabain Affinity

Given these changes in enzymology (Fig. 2, Table 1) and membrane fatty acid composition (Fig. 3, Table 2), we have looked for relations between α1 ouabain affinity and fatty acid content. Changes in glycoside affinity of the α1 isoform was negatively and significantly correlated with changes in total amount of n-6 fatty acids ($P < 0.0001$) (Fig. 4). These data suggest that the enzymatic properties of α1 isoforms are influenced by the fatty acid composition of the surrounding membranes. Table 3 displayed other correlations obtained with a coefficient of correlation higher than 0.8 between total fatty acid and the two others ouabain binding sites. Some of these correlation appears significant for α2 but not for α3 ($P > 0.15$) but we felt that these correlations have been obtained with too few data points and from two organs.

Discussion

By examining the differences in kinetics among the α1 isoforms induced by changes in membrane fatty acids composition, it should be possible to define any potential relationships between particular fatty acids and functional properties of the α1 isoforms. Our observations have focused on the specific role of fatty acids as modulators of glycoside inhibition. By exploiting the natural differences in membrane composition among four tissues from rat, as well as introducing additional variation by use of a fish oil diet and drug-induced diabetes (Holman et al., 1983; Horobin, 1988), we were able to analyze the properties of Na,K-ATPase in up to 12 different fatty acid environments.

Rather than a structural or molecular change in the Na,K-ATPase itself, the differences in ouabain inhibition that we observed seem likely to originate in the altered fatty acid composition of the surrounding membranes.

Table 2. Effects of STZ-induced diabetes and MaxEPA supplementation on total phospholipid fatty acid composition of kidney, cardiac, brain and sciatic nerve membranes

Fatty acids	Kidney			Heart			Brain			Sciatic nerve		
	CO	DO	DM	CO	DO	DM	CO	DO	DM	CO	DO	DM
C16	24.9 ± 0.2	26.82 ± 0.18	29.45 ± 0.11	13.10 ± 0.09	10.5 ± 0.2	11.20 ± 0.06	4.84 ± 0.04	3.73 ± 10.10	3.91 ± 0.20	26.56 ± 0.20	25.37 ± 0.10	25.52 ± 0.20
C16:1(n-7)	0.27 ± 0.01	0.08 ± 0.01	0.17 ± 0.01	trace	trace	trace	trace	trace	trace	1.65 ± 0.01	1.47 ± 0.01	1.83 ± 0.02
C18	26.84 ± 0.27	23.81 ± 0.04	22.85 ± 0.01	25.13 ± 0.08	22.2 ± 0.7	23.0 ± 0.2	16.7 ± 0.1	16.73 ± 0.30	17.25 ± 0.40	9.12 ± 0.09	9.29 ± 0.08	8.96 ± 0.07
C16:1(n-9)	trace	trace	trace	trace	trace	trace	25.6 ± 0.2	24.51 ± 0.60	23.48 ± 0.70	trace	trace	trace
C18:1(n-9)	6.04 ± 0.48	8.01 ± 0.01	7.53 ± 0.16	2.34 ± 0.03	3.5 ± 0.3	3.38 ± 0.05	17.0 ± 0.1	15.31 ± 0.10	15.77 ± 0.60	46.43 ± 0.40	37.47 ± 0.30	42.77 ± 0.42
C18:1(n-7)	1.13 ± 0.22	0.98 ± 0.02	1.23 ± 0.08	2.67 ± 0.01	1.30 ± 0.15	1.71 ± 0.03	trace	trace	trace	trace	trace	trace
C18:2(n-6)	15.00 ± 0.08	20.83 ± 0.14	21.67 ± 0.13	27.6 ± 0.3	45.8 ± 1.4	43.67 ± 0.02	1.03 ± 0.01	2.75 ± 0.06	3.18 ± 0.09	3.32 ± 0.01	5.57 ± 0.03	4.40 ± 0.04
C22	0.8 ± 0.2	1.40 ± 0.11	1.21 ± 0.08	0.69 ± 0.08	trace	0.9 ± 0.1	trace	trace	trace	trace	trace	trace
C18:3(n-3)	0.10 ± 0.01	0.15 ± 0.01	0.14 ± 0.01	0.18 ± 0.01	0.2 ± 0.1	0.14 ± 0.01	trace	trace	1.50 ± 0.07	trace	trace	trace
C20:2(n-6)	0.43 ± 0.18	0.35 ± 0.01	0.33 ± 0.01	0.26 ± 0.03	0.1 ± 0.1	0.25 ± 0.02	1.44 ± 0.01	1.86 ± 0.02	0.56 ± 0.01	0.99 ± 0.01	2.35 ± 0.23	0.73 ± 0.07
C20:3(n-6)	0.97 ± 0.02	1.11 ± 0.01	0.89 ± 0.01	0.40 ± 0.01	1.00 ± 0.05	0.75 ± 0.05	trace	0.82 ± 0.04	0.32 ± 0.04	1.92 ± 0.02	0.89 ± 0.09	2.59 ± 0.03
C20:4(n-6)	19.9 ± 0.1	12.09 ± 0.16	10.55 ± 0.11	21.2 ± 0.2	10.0 ± 0.1	8.75 ± 0.02	12.00 ± 0.1	11.49 ± 0.20	11.94 ± 0.10	5.30 ± 0.05	4.69 ± 0.04	4.12 ± 0.04
C20:5(n-3)	0.36 ± 0.22	0.4 ± 0.1	1.01 ± 0.04	0.29 ± 0.08	0.27 ± 0.01	0.57 ± 0.03	trace	trace	trace	1.19 ± 0.01	2.40 ± 0.02	2.71 ± 0.03
C22:4(n-6)	0.45 ± 0.01	0.40 ± 0.02	0.27 ± 0.04	0.5 ± 0.1	0.47 ± 0.07	0.36 ± 0.04	0.96 ± 0.01	0.77 ± 0.03	0.87 ± 0.09	2.04 ± 0.02	5.55 ± 0.05	3.75 ± 0.10
C22:5(n-6)	trace	trace	trace	0.27 ± 0.02	0.1 ± 0.1	0.25 ± 0.01	2.90 ± 0.02	2.74 ± 0.05	3.05 ± 0.02	0.73 ± 0.07	2.73 ± 0.09	0.58 ± 0.07
C22:6(n-3)	0.28 ± 0.01	0.27 ± 0.02	0.38 ± 0.01	1.9 ± 0.3	1.94 ± 0.06	1.7 ± 0.7	17.6 ± 0.1	19.29 ± 0.20	18.17 ± 0.10	0.74 ± 0.01	2.22 ± 0.12	2.03 ± 0.10
Σ PUFA	37.7 ± 0.4	36.4 ± 0.5	35.4 ± 0.4	52.9 ± 1.1	60.2 ± 2.5	56.6 ± 0.9	35.9 ± 0.3	39.72 ± 0.4	39.59 ± 0.30	16.23 ± 0.10	26.40 ± 0.20	20.91 ± 0.20
Σ MUFA	7.53 ± 0.07	9.27 ± 0.09	9.05 ± 0.09	5.30 ± 0.09	4.9 ± 0.7	5.5 ± 0.2	42.6 ± 0.2	39.82 ± 0.30	39.29 ± 0.20	48.08 ± 0.20	38.94 ± 0.30	44.6 ± 0.12
Σ SFA	52.8 ± 0.2	52.35 ± 0.50	53.8 ± 0.5	39.5 ± 0.3	32.9 ± 1.0	35.3 ± 0.3	21.5 ± 0.1	20.46 ± 0.20	21.46 ± 0.20	35.68 ± 0.30	34.66 ± 0.30	34.48 ± 0.20
Σ (n-6)	36.8 ± 0.4	34.83 ± 0.30	33.7 ± 0.3	50.3 ± 0.7	57.5 ± 2.1	54.0 ± 0.1	18.3 ± 0.2	20.43 ± 0.20	21.42 ± 0.20	14.30 ± 0.10	21.79 ± 0.09	16.17 ± 0.09
Σ (n-3)	0.74 ± 0.01	0.82 ± 0.01	1.52 ± 0.01	2.40 ± 0.4	2.4 ± 0.2	2.4 ± 0.8	17.59 ± 0.05	19.29 ± 0.09	18.17 ± 0.09	1.93 ± 0.02	4.62 ± 0.10	4.74 ± 0.09
(n-6)/(n-3)	49.75 ± 0.40	42.5 ± 0.4	31.2 ± 0.3	20.9 ± 1.9	23.4 ± 2.5	22.1 ± 2.2	1.04 ± 0.01	1.06 ± 0.02	1.18 ± 0.01	7.40 ± 0.20	4.71 ± 0.09	3.41 ± 0.15

Values represent the relative amount, expressed as a percentage of the total identified fatty acids by weight. Values are means ± SE from 6 animals. CO, control + olive oil supplementation; DO diabetic + olive oil supplementation; DM, diabetic + fish oil supplementation. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

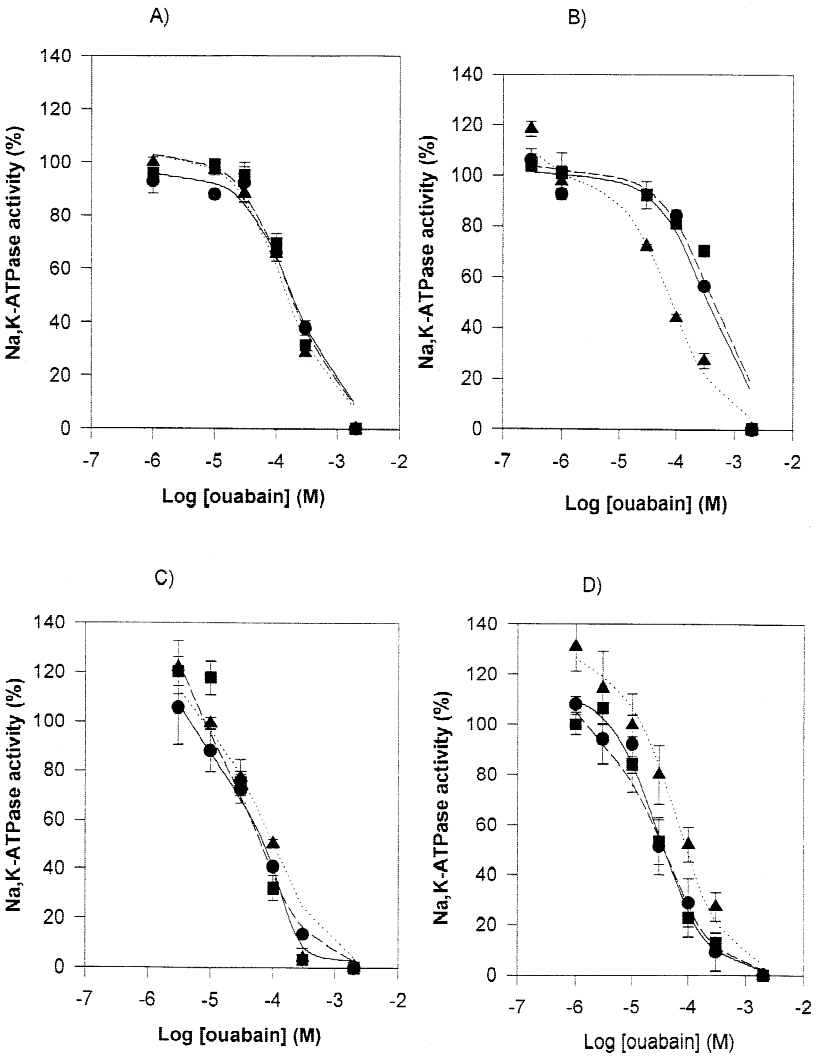

Fig. 2. Effect of diabetes and dietary fish oil supplementation on the ouabain inhibition of the α1 subunit Na,K-ATPase activity in different tissues. (*A*) Kidney, (*B*) Heart, (*C*) Brain, (*D*) Sciatic nerve. Three groups of six animals were analyzed (● control + olive; ■ Diabetic + olive; ▲ Diabetic + fish oil). Values are means ± SEM of experiments done in triplicate. Data were analyzed by a nonlinear regression model. Lines represent the theoretical curves assuming one site model fit. The computed affinities are reported in Table 1.

We consider the possibility that the SDS treatment used to permeabilize the membrane for ions and ATP and ouabain affects the IC_{50} for ouabain. That seems unlikely since in our experience (*data not shown*) we previously compared the ouabain reactivity in native vesicles and after detergent treatments and we did not see changes in IC_{50} values after SDS treatments. The detergent treatment appears not to alter the lipid bilayer surrounding the membrane bound Na,K-ATPase during the experiments reported here, but in some conditions where an excess of SDS was added we observed an inactivation of the enzyme activity presumably by affecting the surrounding phospholipids but in this condition it was impossible to measure a ouabain inhibition. We also consider the possibility that the change in affinity may have been the result of α2 or α3 rather than α1. This cannot be the case because all changes in the α1 sensitivity were less than one Log unit whereas the affinities for the α2 and α3 isoforms differed by a 1,000-fold factor. We have chosen kidney, heart, central and peripheral nervous tissues for their well-defined composition of α-isoforms of Na,K-ATPase. We attribute any differences in enzyme kinetics that we observe to the changes in fatty acid environment. These tissues are also known to differ in their expression of the β isoforms (Martin-Vasallo et al., 1989). An alternative explanation for our results might be diabetes or diet-induced changes in β isoform expression (Martin-Vasallo et al., 1989; Maixent et al., 1991*b*). We considered this possibility, since analysis of the kinetic properties of different complexes showed equivalent ouabain sensitivities (Blanco et al., 1995).

Two amino acids in the H1 transmembrane domain of the α subunit have been found to alter the affinity of the Na,K-ATPase for ouabain. It seems likely that lipids are displaced during the conformation transition that accompanies catalysis, and the resistance of the membrane to this displacement could be modified by its lipid constituents. We hypothesized that these same lipid constituents could modulate ouabain affinity by association with the H1 transmembrane domain of the α subunit.

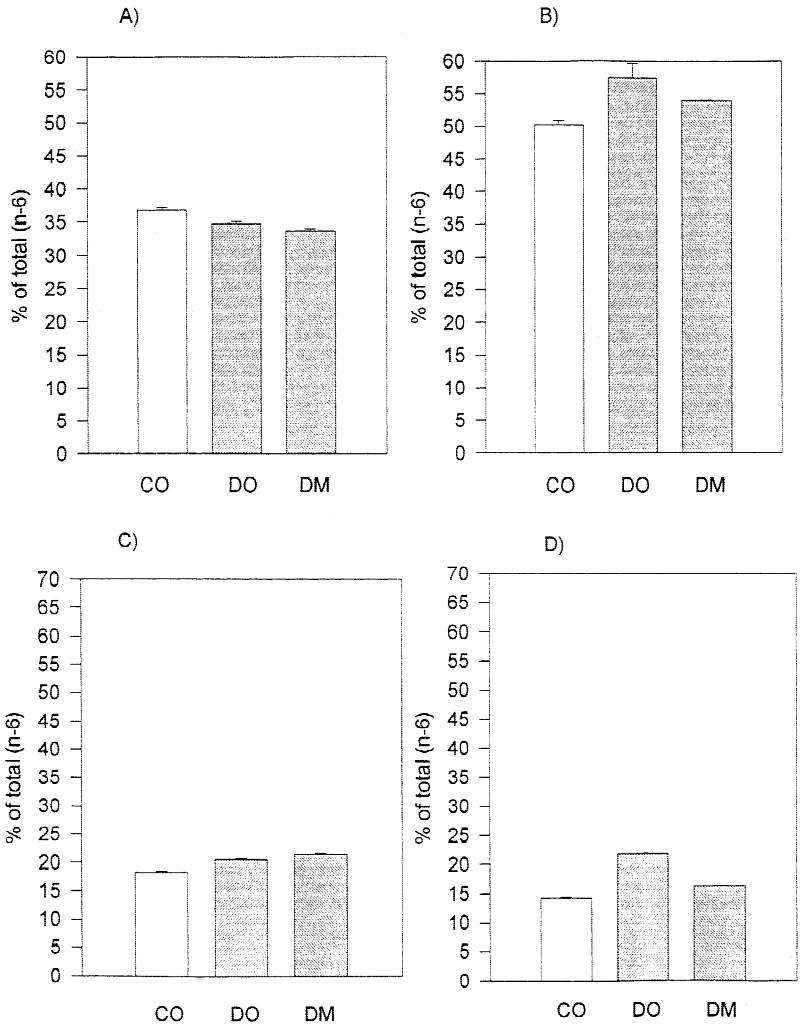

Fig. 3. Effect of diabetes and dietary fish oil supplementation on the total amount of fatty acids of the (n-6) series in the different tissues. (*A*) Kidney, (*B*) Heart, (*C*) Brain, (*D*) Sciatic nerve. Values are means ± SEM of experiments done in triplicate.

Such a dependence of ouabain affinity on overall phospholipid composition was previously observed by Abeywardena & Charnock, (1983), who explained their results by a direct lipid protein interaction. When the fluid mosaic model for cell membrane was formulated, investigators considered the influence of lipids on function of membrane proteins, such as those involved in the ion transport system (Spector & Yoreck, 1985). Membrane thickness, which is dependent on fatty acyl chain length and degree of saturation may be a significant factor and have been implicated in at least one other ATPase enzyme system, that of the mitochodrial membrane (Bruni, Van Dijek & Degier, 1975; Pitotti, Babbeni-Sala & Bruni, 1980).

Although an influence of lipid composition on glycoside affinity might have been anticipated, it was not clear if there were differences among the α1 isoforms. We have found a specific dependence of α1 on the total amount of n-6 fatty acids. Since these lipids can be altered by diet, we can speculate that changes in dietary

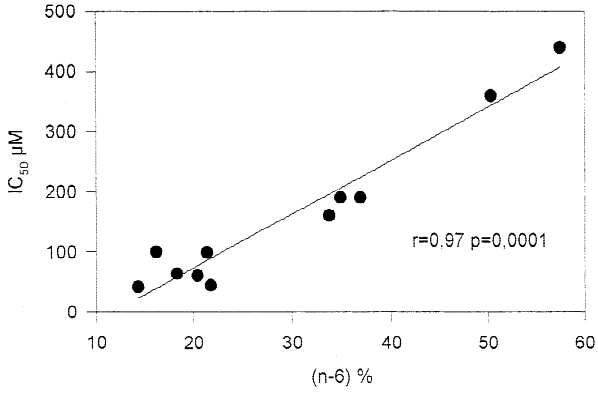

Fig. 4. Correlation of cell membrane (n-6) fatty acid content and ouabain affinity (IC_{50}) of α1 isoenzymes. Correlations were evaluated by a single linear regression analysis. Correlation analysis has been with average values from each group except the cardiac DM value ($r = 0.97$, $P = 0.0009$). A significant correlation was found with the 12 values including the cardiac DM value, ($r = 0.76$, $P = 0.0038$).

Table 3. Relationship between membrane total phospholipid acid and isoenzyme ouabain affinities

Fatty acids	Low ouabain affinity (α1)		High ouabain affinity (α2)		Very high ouabain affinity (α3)	
	r	*P*	*r*	*P*	*r*	*P*
C18:3(n-3)	0.85	0.0009				
Σ(n-6)	0.97	0.0001				
C20:2(n-6)			0.80	0.0572		
C22:5(n-6)			0.92	0.009		
ΣSFA			−0.99	0.0007	−0.83	0.1718
Σ(n-3)			0.94	0.0179	0.85	0.1529
C16					0.81	0.1873
C18					0.82	0.1838
C22:6(n-3)					0.82	0.1815
(n-6)/(n-3)					0.85	0.1529

SFA, monounsaturated fatty acids, *r*, coefficient of correlation, value of *P*, statistical analyses. Correlations were evaluated by single linear regression analysis.

habits could have unexpected effects on Na,K-ATPase kinetics.

From these results, we propose that specific fatty acid within the lipid microenvironment of Na,K-ATPase, such as the (n-6) series, play a major role in the modulation of cardiac glycoside binding of the α1 subunit. It follows that divergence in the amino acid sequence of the isoforms is not the sole factor influencing digitalis affinity. Of particular interest is the effect of these fatty acids on enzymatic conformation, which might enhance or reduce the accessibility of the binding site (Brenner & Reluffo, 1966).

In conclusion, we have shown a significant correlation between the fatty acid membrane composition and kinetic properties of the Na, K-ATPase α1 isoform highly resistant to ouabain.

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