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EFFECT OF ESSENTIAL FATTY ACID DEFICIENCY ON ACTIVITY OF LIVER PLASMA MEMBRANE ENZYMES IN THE RAT

S. L. Louis, R. P. Brivio-Haugland, and M. A. Williams

Department of Nutritional Sciences, University of California, Berkeley, California

Liver plasma membranes (LPM) were isolated from rats fed an essential fatty acid-supplemented diet (+EFA) or from rats fed an essential fatty acid-deficient diet (−EFA). The proportions of linoleate and arachidonate in membrane total fatty acids in the −EFA preparations were one-half or less than the values for the +EFA preparations. Basal, F^-, or glucagon-stimulated adenylate cyclase activities were significantly lower in EFA-deficient livers than in nondeficient ones. Addition of GTP significantly enhanced glucagon-stimulated adenylate cyclase in both groups, but extent of stimulation above basal was greater in EFA-deficient livers. Portal vein injection of glucagon in vivo resulted in significantly higher cAMP formation in +EFA livers than in −EFA livers. When glucagon was used in vitro at 1–1,000 nM, stimulation of adenylate cyclase remained lower in EFA-deficient membranes, but extent of stimulation above basal activity was larger in −EFA membranes than in +EFA. Total Na^+, K^+ (Mg^{2+})-ATPase from EFA-depleted LPM exhibited significantly higher values of apparent K_m and V_{max}. 5′-Nucleotidase activity, in contrast, was considerably decreased in EFA-deficient rats. These findings show that, in animals, changes in unsaturated fatty acid composition can affect the properties of membrane-bound enzymes. These alterations could be due to changes in membrane physical properties and/or prostaglandin formation.

INTRODUCTION

The physiological significance of Na^+, K^+ (Mg^{2+})-ATPase[1] in maintaining electrical properties and transport in cell membranes and the key role played by adenylate cyclase in hormone action are widely accepted. Membrane lipids appear to play a major role in the functioning of these plasma membrane-associated enzymes (1–3). Removal of lipids from liver plasma membranes decreased basal activity of adenylate cyclase, with loss of sensitivity to stimulation by hormones, fluoride, and guanylnucleotides (4). Addition of phosphatidylserine partially restored glucagon and fluoride sensitivity. Variations in chain

[1] Abbreviations: EFA, essential fatty acids; LPM, liver plasma membranes; ATPase, adenosinetriphosphatase; ATP, 5′-adenosinetriphosphate; cAMP, 3′, 5′ cyclic-adenosine monophosphate; GTP, 5′-guanosinetriphosphate; BSA, bovine serum albumin.

length and degree of fatty acid unsaturation of membrane phospholipids have been shown to influence directly the activity of Na^+, K^+ (Mg^{2+})-ATPase (5).

Feeding animals a diet varying in essential fatty acids (EFA) will produce large changes in unsaturated fatty acid composition of cell membranes, including plasma membranes (6–9). Such changes might be expected to alter the activity of membrane-associated enzymes requiring lipid for activation. Data in support of these expectations have been reported recently. Counis (10) observed that norepinephrine stimulation of adenylate cyclase in fat cell ghosts was greater in ghosts prepared from rats fed a diet rich in linoleate than with ghosts prepared from rats fed a diet low in linoleate. Basal and fluoride-stimulated adenylate cyclase activities did not differ between the two preparations. Sun and Sun (7) found that Na^+, K^+ (Mg^{2+})-ATPase activity was greater in brain homogenates or synaptosomes from EFA-deficient mice. Reduced affinity of Na^+, K^+ (Mg^{2+})-ATPase for Na^+ and K^+ has been shown in erythrocyte membranes from EFA-deficient rats (8). No changes, however, were reported for the Na^+, K^+ (Mg^{2+})-ATPase activity in liver plasma membranes from EFA-deficient rats (6), although 5′-nucleotidase activity was reduced by 50%. Decreased 5′-nucleotidase activity was also found in brain homogenates from EFA-deficient rats (11).

In spite of the attention given to the role of phospholipids in regulation of adenylate cyclase and Na^+, K^+ (Mg^{2+})-ATPase activity in liver plasma membranes (LPM), there is almost no information on whether activity of these enzymes is affected by variations in unsaturated fatty acid composition of the LPM phospholipids. The only study, to our knowledge, is that of Chandrasekhara and Ananth-Narayan (6), who found no difference in Na^+, K^+ (Mg^{2+})-ATPase activity between EFA-deficient rats and rats fed a chow diet. The interpretation that EFA deficiency had no effect on the total ATPase activity is made questionable, however, by the fact that the "control" rats were fed a chow diet rather than the purified EFA-deficient diet plus a supplement of EFA. The latter diet is the only suitable control for this type of experiment. It is possible that Na^+, K^+ (Mg^{2+})-ATPase activity with the EFA-supplemented purified diet might have differed significantly from that of the chow diet. Differences in enzyme activities between rats fed a complete purified diet and those fed a chow diet are well established (12). Without this information, no conclusions can be made on the effect of EFA deficiency on liver Na^+, K^+ (Mg^{2+})-ATPase activity.

We have begun experiments to determine whether variations in dietary EFA intake affect adenylate cyclase and Na^+, K^+ (Mg^{2+})-ATPase of rat LPM. EFA-deficient animals might serve as test systems for investigation of the role of EFA in functioning of membrane-bound enzymes and in hormone action in the liver.

METHODS

Diet and Experimental Animals

The purified diet (13) contained 20% casein and sucrose, and 5% fat, which was hydrogenated coconut oil in the EFA-deficient diet and corn oil in the non-EFA-deficient control diet. Male weanling Sprague-Dawley rats were fed such diets for at least 2 mo before sacrifice.

Liver Plasma Membrane (LPM) Preparation

Pairs of EFA-deficient and control rats were killed by decapitation. Livers were perfused immediately with cold saline, removed, and processed as described by Ray (14).

Cell membranes were purified by the two-phase polymer system described by Lesko et al. (15). Contamination by other membranes of our LPM preparation was checked by measuring activities of succinic-cytochrome c reductase (16) and glucose-6-phosphatase (17). The degree of purification was based on the increase in specific activity of 5′-nucleotidase, alkaline phosphatase, and Na^+, K^+ (Mg^{2+})-ATPase as plasma membrane marker enzymes.

Adenylate Cyclase

The standard assay was based on the methods of Oka et al. (18) and Maguire and Gilman (19). The assay system contained in a total volume of 200 μl—30 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 0.2 mM 1-methyl-3-isobutyl xanthine, 1 mM EDTA, 1.25 mM ATP, 10 μg bovine serum albumin, 10 mM phosphocreatine, and 7.5 U of creatine phosphokinase. Reactions were started by addition of 40 μg membrane protein and stopped after 15 min at 34°C by adding sodium acetate-acetic acid buffer, pH 4.5, to a final concentration of 50 mM. Samples were frozen at –20°C until assay of cAMP. Measurement of cAMP (20, 21) was carried out in a final volume of 120 μl containing 50 mM sodium acetate-acetic acid buffer, 1.25 pmoles [^{3}H] cAMP, 9 μg rabbit muscle protein kinase, 0.2 mg/ml calf thymus histone, and 10–30 μl of sample. The rest of the assay was according to Gilman (20). Cyclic AMP standard curves were made with 1–20 pmoles of cAMP.

5′-Nucleotidase

5′-Nucleotidase was assayed at 30°C as described by Solyom and Trams (22).

Total Na^+, K^+ (Mg^{2+})-ATPase

For general purposes, ATPase activity was measured at 30°C as reported by Solyom and Trams (22). For kinetic studies, total Na^+, K^+ (Mg^{2+})-ATPase was assayed at 37°C by the method of Barnett (23), in which ADP formation is correlated to oxidation of NADH. By providing an ATP-regenerating system, this method is less sensitive to the action of other phosphatases, which are fairly abundant in LPM preparations (24). In both assays, the incubation medium contained 3 mM ATP, 3 mM $MgCl_2$, 80 mM NaCl, 10 mM KCl, and 50 mM Tris-HCl buffer, pH 7.5.

Glucose-6 Phosphatase

Glucose-6 phosphatase was assayed at 30°C according to Hübscher and West (17).

Alkaline Phosphatase

Alkaline phosphatase was measured by hydrolysis of p-nitrophenylphosphate at pH 9 (15).

Succinic-Cytochrome c Reductase

Succinic-cytochrome c reductase was used as marker of mitochondrial membranes and the degree of contamination was calculated according to Newkirk and Waite (25).

Protein

Protein was determined by the method of Lowry (26).

Fatty Acid Analysis

LPM were extracted with chloroform-methanol (2:1) (27), methylated, and analyzed by gas liquid chromatography on 15% diethyleneglycol succinate coated on

60/80 mesh Chromosorb W in a 0.3/150-cm column with a Varian Aerograph 204B (28).

Liver cAMP

Liver content of cAMP was determined before and after injection of glucagon (100 μg/100 g body weight). The glucagon injection technique was based on the methods of Klain and Weiser (29), and De Robertis et al. (30). At 0, 1, 5, and 10 min after glucagon injection a liver lobe was ligated and quickly frozen by the freeze-clamping technique. Controls were injected with saline. cAMP levels did not differ between lobes and were not altered by saline injection.

RESULTS

Isolation of LPM

The preparations of LPM from both EFA-deficient and control rats exhibited a similar degree of purification (Table I). This degree of purification and the extent of

TABLE I. Enzymatic Activity of Rat LPM Preparations

		+ EFA diet		– EFA diet		
Enzyme	Fraction	Specific activity	Purification (times)	Specific activity	Purification (times)	Differences in activities
5′-Nucleotidase	Hom.	2.7 ± 0.5		2.2 ± 0.8		NS
	LPM	45.1 ± 6.3	17	31.2 ± 6.2	14	< 0.005
Na^+, K^+ (Mg^{2+})-ATPase	Hom.	5.9 ± 1.2		7.6 ± 1.3		< 0.05
	LPM	57.8 ± 13.8	10	67.2 ± 22.5	9	NS
Alkaline phosphatase	Hom.	0.14 ± 0.03		0.23 ± 0.08		< 0.01
	LPM	1.4 ± 0.70	11	2.6 ± 0.9	11	< 0.025
			Contamination %		Contamination %	
Glucose-6-phosphatase	LPM	3.7 ± 1.3	3.7	5.2 ± 1.9	4.4	
Succinic-cytochrome reductase	LPM	0.015 ± 0.015	3.1	0.012 ± 0.005	5.7	

$\bar{X}$ ± SD; N = 6.

Rat LPM preparations were made by the two-phase polymer method of Lesko et al. (15). Values given are means and standard deviations for six pairs of EFA-deficient and control rats. Specific activities are expressed as μmoles Pi per mg protein per hr.

mitochondrial and microsomal contamination is in the range of values reported by others for preparations made either with the two-phase polymer or sucrose gradient methods (15, 31). 5′-Nucleotidase was significantly lower in the membrane preparations from the EFA-deficient rats. The Na^+, K^+ (Mg^{2+})-ATPase activity in the EFA-depleted membranes was higher than in the controls, but the difference in these values was not statistically significant when the activity was measured by assaying the inorganic phosphate released during the reaction. This method, however, does not differentiate real ATPase activity from the action of less specific phosphatases which contaminate or are part of plasma

membranes (24), since proper controls are difficult to run. However, when assayed by the enzymatic method with a system-regenerating ATP (pyruvate kinase) (23), the values for the EFA-deficient rats were significantly higher. As expected, LPM from EFA-deficient rats showed a large decrease in the relative proportion of linoleate (1.6% vs. 10.5%), and arachidonate (7.0% vs. 26.0%), with a corresponding increase in 5, 8, 11-eicosatrienoate (15.7%) in the EFA-depleted preparations.

Adenylate Cyclase

Activities of basal, fluoride, and glucagon-stimulated adenylate cyclase are given in Table II. EFA-deficient and control rats were paired according to their daily food intakes

TABLE II. Effect of Essential Fatty Acid (EFA) Deficiency on Adenylate Cyclase of Rat Liver Plasma Membranes

	n moles cAMP/g protein/min		Significance of mean differences
	+ EFA diet	−EFA diet	
Basal	30 ± 0.9	13 ± 2	$P < 0.001$
NaF, 10 mM	92 ± 7	46 ± 5	$P < 0.001$
Glucagon, 10^{-6} M	71 ± 4	40 ± 6	$P < 0.01$

$\bar{X}$ ± SE; N = 6.
Assay conditions as described in Methods.

for the week preceding sacrifice to minimize possible effects due to differences in food intake. Basal enzyme activity was significantly lower ($P < 0.001$) in the EFA-depleted preparations. The adenylate cyclase in the EFA-depleted membranes did not lose sensitivity to fluoride and glucagon since the relative activation by these agents was the same with both EFA-depleted and control preparations. However, specific activity in the ($P < 0.001$ for fluoride; $P < 0.01$ for glucagon) presence of these activators remained lower in comparison with the control values. Dose-response curves to glucagon (Fig. 1) showed that the enzyme from the EFA-deficient animals was activated at all concentrations of glucagon used, but the specific activity remained consistently lower than in the controls. At concentrations of glucagon greater than 10^{-6} M, glucagon caused no additional stimulation of adenylate cyclase with either preparation. GTP addition enhanced the glucagon-stimulated activity of both EFA-deficient and control groups (Table III). The percentage of enhancement by GTP, however, was twice as great ($P < 0.05$) in the deficient group.

Liver cAMP after Glucagon Injection

Glucagon injection into the portal vein produced a large increase in cAMP within 1 min in both the EFA-deficient and control rats (Fig. 2). However, the increase in the controls was significantly greater ($P < 0.001$) than in the deficient rats. In both deficient and control animals, the cAMP values at 5 and 10 min declined from the 1-min values, but at both time points the values for the control rats were significantly higher ($P < 0.05$) than for the deficient animals. The preinjection values for cAMP did not differ significantly between the deficient and control groups.

Total Na^+, K^+ (Mg^{2+})-ATPase

The values for apparent K_m and V_{max} of total Na^+, K^+ (Mg^{2+})-ATPase from six

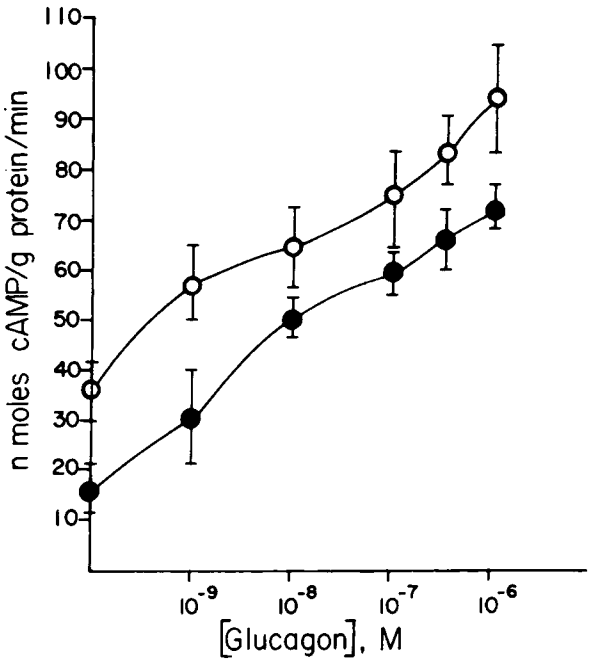

Fig. 1. Effect of glucagon on adenylate cyclase of liver plasma membranes of rats fed +EFA (○——○) and −EFA (●——●) diets. The assay conditions are described in Table II except that glucagon was added at the concentrations indicated. N = four rats.

TABLE III. Enhancement of Glucagon-Stimulated Activity by Addition of GTP

	Glucagon	Glucagon + GTP	Increase in activity due to GTP	Ratios[1]
+ EFA diet	70 ± 8	111 ± 12	40 ± 6	.59 ± 0.06[2]
− EFA diet	30 ± 5	57 ± 7	28 ± 4	1.09 ± 0.19[2]

$\bar{X}$ ± SE; N = 6.

Assay conditions as described in Methods: glucagon, 10^{-6} M; GTP, 10^{-4} M.

[1] Ratios were calculated as: $\dfrac{\text{sp act (+ gluc. + GTP)} - \text{sp act (+ gluc.)}}{\text{spec act (+ gluc.)}}$

[2] $P < 0.05$ between these means.

different paired preparations of LPM of EFA-depleted and control membranes are given in Table IV. The values for both K_m and V_{max} for the EFA-depleted preparations showed a moderate but consistent increase in comparison with the controls. The increase in K_m was significant at $P < 0.005$. The increase in V_{max} was significant at $P < 0.05$. When statistical analysis was done on the increase in value of the ATPase K_m and V_{max} in the EFA-depleted membranes in comparison with the corresponding paired control, the difference in V_{max} also became significant ($P < 0.005$), in spite of the variability among the different LPM preparations. Lineweaver-Burk plots of the data are shown in Fig. 3. Here, for each

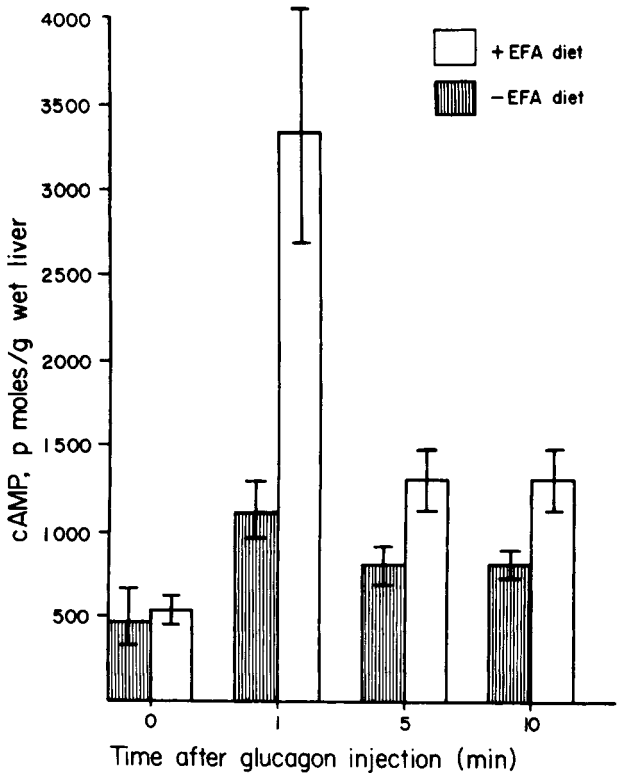

Fig. 2. Effect of glucagon injected into portal vein on liver cAMP levels of rats fed +EFA and −EFA diets. The surgical procedure and the cAMP determination are described in the text. N = 6.

TABLE IV. Values for Apparent K_m and V_{max} of Total Na^+, K^+ (Mg^{2+})-ATPase from Six Different Paired Preparations of LPM

Pair	+ EFA diet		− EFA diet	
	K_m mM ATP	V_{max} μmoles ADP / mg protein × min	K_m mM ATP	V_{max} μmoles ADP / mg protein × min
1	0.37	0.85	0.67	1.36
2	0.32	1.25	0.69	1.86
3	0.37	1.36	0.48	1.68
4	0.36	1.46	0.46	1.81
5	0.45	1.80	0.55	2.50
6	0.36	1.08	0.55	2.50
Mean	0.37	1.30	0.57[1]	1.95[2]
± SE	0.018	0.33	0.038	0.45

[1] $P < 0.005$. [2] $P < 0.05$.

The values for K_m (mM ATP) and V_{max} (μmoles ADP/mg/min) for total Na^+, K^+, (Mg^{2+})–ATPase activity of six paired preparations of LPM from control and EFA-deficient rats. Conditions for assay are as described under Methods.

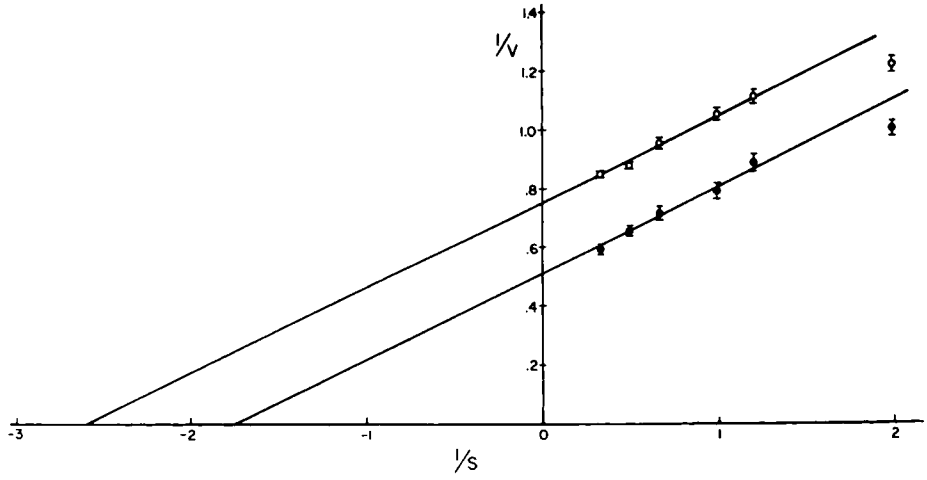

Fig. 3. Illustrates the kinetics of six different preparations of LPM Na^+, K^+ (Mg^{2+})-ATPase from control and EFA-deficient rats. V_{max} is expressed as μmoles ADP per mg protein per min, and K_m as mM ATP. The data points for V_{max} have been normalized multiplying them by observed V_{max}. This procedure was used to overcome the variation in V_{max} among the different preparations (Table IV). $\frac{\text{mean } V_{max}}{\text{mean } V_{max}}$ ○——○ +EFA diet; ●——●, −EFA diet.

preparation, the values of the data points of the reaction velocity at any ATP concentration were normalized by multiplying them by the factor: calculated V_{max}/mean V_{max} (Table IV). This procedure was used to minimize the variability in V_{max} values from the different LPM preparations and to show the similarity in the kinetic behavior of the different enzyme preparations from either EFA-deficient or control rats. K_m and V_{max} values were also calculated by an unweighted nonlinear regression method (32). The standard error of the mean for each value was either less than or close to 10%, which shows good fit to the Michaelis-Menten equation (33). These values did not appear essentially different from those presented in Table III, which are derived by the standard Lineweaver-Burk plot.

DISCUSSION

The results show clearly that a lack of dietary EFA can affect the properties of liver plasma membrane-associated enzymes. These changes could result from altered fatty acid composition of membrane lipids and/or from altered prostaglandin (PG) formation. Unsaturated fatty acids could affect enzyme activity through changing the properties of membrane phospholipids necessary for maintenance of functional conformation of membrane enzymes, i.e. enzyme-substrate or activator-receptor interactions and/or signal transmission (1–3). Changes in PG formation might be expected to alter cell responses to stimulation by hormones whose effects in target cells can be modified by PG (34).

The increase in apparent K_m for ATP, which we observed in measuring the total Na^+, K^+ (Mg^{2+})-ATPase activity of EFA-depleted LPM, could indicate a structural change affecting enzyme-substrate affinity. Since the ouabain-sensitive ATPase of LPM is very

low relative to the Mg^{2+}-ATPase (14, 15), the total Na^+, K^+ (Mg^{2+})-ATPase activity of LPM represents chiefly Mg^{2+}-ATPase. We have also obtained the same values in apparent K_m for ATP with LPM from EFA-deficient and control rats when ATPase activity was determined in the presence of ouabain. The increase in apparent K_m for ATP, which we observed in measuring total Na^+, K^+ (Mg^{2+})-ATPase activity of EFA-depleted LPM, could indicate a structural change affecting membrane-substrate affinity. The kinetic differences in ATPase observed in vitro in isolated membrane fragments might reflect larger differences in the stretched, intact membrane in vivo. However, the kinetics of the ATPase enzyme could also be influenced by the physical location of the enzyme on the LPM fragments, since the depletion of EFA in the membrane could favor formation of a specific sidedness in the membrane vesicles, opposite to that of the control.

The decreased adenylate cyclase activity observed in the EFA-depleted LPM could indicate decreased responsiveness of the cyclase system to activating agents because of changes (a) in the fatty acid composition of lipids necessary for maintenance of adenylate cyclase in a functional conformation, (b) in binding of activators, and/or (c) in transmission of signals from activating agents to adenylate cyclase. The decreased adenylate cyclase activity in the EFA-depleted membranes may also indicate that these membranes have less enzyme protein. This might result from decreased hormonal stimulation of protein synthesis as a result of decreased PG formation. Further work is needed to resolve these questions.

The occurrence of differences in enzyme activity in vitro might not necessarily reflect comparable functional differences in vivo. However, the decreased liver cAMP response to glucagon injection in EFA-deficient rats is an altered in vivo function which correlates with the in vitro changes. Our in vivo results thus support the use of LPM from EFA-depleted rats as a system for studying effects of EFA in plasma membrane function.

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