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Thyroid hormones control lipid composition and membrane fluidity of skeletal muscle sarcolemma

Maria Pilarska [1], Antoni Wrzosek [1], Sławomir Pikuła [1] and Konrad S. Famulski [2]

[1] *Department of Muscle Biochemistry, Nencki Institute of Experimental Biology, Warsaw (Poland) and* [2] *Department of Cellular Biochemistry, Nencki Institute of Experimental Biology, Warsaw (Poland)*

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Sarcolemma membrane lipid phase of skeletal muscles of hyperthyroid animals was compared to that of control (euthyroid) ones. Hyperthyroidism caused 15% decrease in cholesterol and 70% increase in the phospholipid content of the membrane. This was accompanied by the alterations in proportions between individual phospholipid classes, and was followed by changes in the composition of phospholipid fatty acids. The calculated fatty acid unsaturation index was higher for membrane lipid phase of hyperthyroid animals than of euthyroid ones. Thyroxine-induced alterations in the lipid composition of sarcolemma caused changes in the membrane fluidity and the activity of calmodulin-stimulated (Ca^{2+}-Mg^{2+})-ATPase. Measurements of the steady-state fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene indicated that the lipid phase transition of membrane vesicles occurred at 25.9°C and at 28.9°C for preparations isolated from hyperthyroid and euthyroid rabbits, respectively. Arrhenius plot break-point temperature for CaM-stimulated (Ca^{2+}-Mg^{2+})-ATPase activity was lower in membrane preparations isolated from hyperthyroid (26.9°C) than from euthyroid ones (30.0°C). Thus, the increase of the membrane fluidity presumably caused that the enzyme was characterized by the lower activation energy value. This phenomenon may be viewed as a supplementary mechanism for activation of the enzyme by thyroid hormones to previously reported elevation of the amount of (Ca^{2+}-Mg^{2+})-ATPase protein exerted by hyperthyroidism (Famulski et al. (1988) Eur. J. Biochem., 171, 363–368; Famulski and Wrzosek (1988) in The Ion Pumps-Structure, Function and Regulation (Stein, W.D., ed.), pp. 355–360, Alan R. Liss, New York).

Introduction

Biological membranes contain a number of enzymes that are thyroid-sensitive [1,2]. Muscle tissue, which is one of the major targets for thyroid hormones action, responds with changes in contractile properties, activation of calcium and monovalent cations fluxes proportionally to the changes in blood levels of these hormones [3–8]. We have recently shown [9–11] that heart and skeletal muscle CaM-stimulated (Ca^{2+}-Mg^{2+})-ATPase activity of SL membranes is higher in animals of the hyperthyroid status. This could be due to enhanced gene expression, leading to elevated amount of relevant proteins, and/or altered lipid–protein interactions. For the latter, lipid [12] and cholesterol [13] to protein ratios, as well as phospholipid composition [14], and the degree of fatty acid unsaturation [15] are the most important factors.

The ability of thyroid hormones to influence lipid composition of a number of cellular organelles in different tissues is well known [16–26]. Therefore, it is generally postulated that thyroid hormones effects on membrane-bound proteins may also be mediated

Abbreviations: SL, sarcolemma; SR, sarcoplasmic reticulum; T_4, L-thyroxine; T_3, triiodothyronine; HR, hyperthyroidism; EU, euthyroidism; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DPH, 1,6-diphenyl-1,3,5-hexatriene; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; GLC, gas liquid chromatography; CaM, calmodulin; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Correspondence: K.S. Famulski, Department of Cellular Biochemistry, Nencki Institute of Experimental Biology, Pasteura 3, 02-093 Warszawa, Poland.

through changes in membrane lipids. It has to be underlined that these changes may in turn influence the membrane fluidity [16]. As was previously shown, hypothyroidism resulted in alterations of sarcoplasmic reticulum membrane lipid composition [25]. This, was thought responsible for modified enzymatic properties of (Ca^{2+}-Mg^{2+})-ATPase of SR preparation [5].

In the present work we compared the lipid composition and membrane fluidity of SL preparations isolated from L-thyroxine-treated and control rabbits. Analysis of the Arrhenius plots for CaM-stimulated (Ca^{2+}-Mg^{2+})-ATPase activity allowed us to conclude that changes in lipid composition and corresponding changes in membrane fluidity caused by thyroxine administration result in the decrease of the activation energy for the ATPase-catalysed reaction.

Materials and Methods

Animals and their treatment

Male rabbits weighing 2800–3000 g and kept on the standard diet were used throughout this study. Hyperthyroid state of animals was evoked by subcutaneous injections of 500 μg of L-thyroxine (Sigma, St. Louis, MO, U.S.A.) per kg of body weight per day during a period of seven days, as described previously [9]. Levels of triiodothyronine and T_4 were measured using a radioimmunoassay and amounted to 68 ng T_3 and 6.2 μg T_4 in euthyroid animals, and 340 ng T_3 and 70 μg T_4 in hyperthyroid ones in 100 ml of blood serum [9].

Sarcolemma membrane preparation

SL vesicles were isolated from back and hind legs fast-twitch skeletal muscles according to the procedure of Seiler and Fleischer [27] with modifications introduced by Michalak et al. [28]. A proteinase inhibitor, 0.2 mM $PhMeSO_2F$ (Fluka, Ronkohoma, NY, U.S.A.), was added to the homogenization buffer solution. Final preparation of SL vesicles (2 mg protein/ml) in 100 mM KCl, 0.4 mM dithiothreitol, 20 mM Hepes (pH 7.4), 0.1 mM EGTA, 0.2 mM $PhMeSO_2F$, and 250 mM sucrose, was used immediately for the steady-state fluorescence and the ATPase activity measurements or was stored for 2–3 weeks at −135°C in the presence of a 0.05% butylated hydroxytoluene (Merck, Darmstadt, F.R.G.) for lipid analysis.

Lipid analysis

Lipids were extracted from SL membrane by the method of Folch et al. [29] or Bligh and Dyer [30], and then separated into neutral lipids and phospholipids by silic acid column chromatography (100 mesh, Mallinckrodt, St. Louis, MO, U.S.A.) employing subsequent elution with chloroform and methanol. A 0.05% butylated hydroxytoluene was present throughout as an anti-oxidant. Phospholipids were separated into individual classes by thin-layer chromatography, described previously by Sarzala and Michalak [31].

Fatty acid methyl esters were obtained either from total lipids by the hydrolysis in methanolic KOH and estrification with diazomethane (Sigma, St. Louis, MO, U.S.A.) according to Eichenberger [32] or from purified phospholipids by transesterification with sodium methoxide (Merck, Darmstadt, F.R.G.) according to Thies [33]. However, it should be mentioned, that 6.4% of saturated acyl chains esterified in skeletal muscle SL phospholipids are linked via diethyl ether bond [34]. They cannot be derivatized and determined by the method used by us. Therefore, the relative proportions of individual fatty acids presented in this work might be slightly different. For gas-liquid chromatography separations a Shimadzu GC-8A chromatograph (Japan) equipped with data storage and retrieval system was used. The fused silica column, 25-m long, 0.25 mm i.d., coated with Carbowax 20 M (chemically bound), was operating at 185–210°C (1 C° min^{-1}) with nitrogen as a carrier gas. Pentadecanoic (15:0) acid methyl ester (Sigma, St.Louis, MO, U.S.A.) was used as an internal standard. Integration of peaks was achieved with a Shimadzu Chromatopac C-R3A integrator (Japan).

Cholesterol was extracted from SL membrane preparations according to Rose and Oklander [35] and its content was determined by GLC on a Pye Unicam (Pye series 104) chromatograph (England) after sililation with *N,O*-bis[trimethylsililo]trifluoroacetamide (Serva, Heidelberg, F.R.G.). Cholestane (Serva, Heidelberg, F.R.G.) was used as an internal standard. A 1.5-m long column, filled with QF-1, was operating at 217°C with argon as a carrier gas. Detector temperature was 250°C.

Steady-state fluorescence measurements

Freshly prepared SL vesicles were diluted 10 times in the medium containing 100 mM KCl, 20 mM Hepes (pH 7.4), 0.4 mM dithiothreitol, 0.1 mM EGTA and 0.2 mM $PhMeSO_2F$, and incubated for 60 min at 22°C with DPH (Sigma, St. Louis, MO, U.S.A.) dissolved in tetrahydrofuran. The probe was used in proportion of one molecule of fluorophore per 800–1000 of phospholipid molecules. Steady-state fluorescence polarization of DPH was measured at the temperature range of 2–49°C (with accuracy of ±0.2 C°) in a cuvette of optical path length of 0.5 cm using Perkin-Elmer LS-5B spectrofluorimeter (U.S.A.) equipped with a thermostated cell holder and Perkin-Elmer 3700 data storage system. Fluorescence intensities were recorded in the basic medium of total 0.7 ml containing 100 mM KCl, 20 mM Hepes (pH 7.4), 0.1 mM EGTA, 0.4 mM dithiothreitol, 0.2 mM $PhMeSO_2F$, 25 mM sucrose, and 0.2 mg/ml of protein, in the presence of fluorophore, at 430 nm (5 nm slit width) with excitation

wavelength set at 355 nm (10 nm slit width). All buffer solutions were saturated with argon prior to use.

Polarization of fluorescence (P) was calculated using Eqn. 1:

$$P = [I_{vv} - I_{vh}(I_{hv}/I_{hh})]/[I_{vv} + I_{vh}(I_{hv}/I_{hh})] \quad (1)$$

where I_{hv}, for example, is the fluorescence intensity measured with the excitation and emission polarizers set horizontally and vertically, respectively.

Determination of the activation energy for the ATPase-catalysed reation

CaM-stimulated (Ca^{2+}-Mg^{2+})-ATPase activity was measured spectrophotometrically at the temperature indicated in the abscissa presented in Fig. 4 using a coupled enzyme assay system [9], in the presence or absence of 0.2 μM CaM. Incubation mixture contained 100 mM KCl, 20 mM Hepes (pH 7.4), 0.1 mM ATP, 1 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM EDTA, 0.2 mM NADH, 0.5 mM phospho(*enol*)pyruvate, 1 unit/ml of pyruvate kinase, 1 unit/ml of lactate dehydrogenase and 0.02 mg/ml of membrane protein. $CaCl_2$ was added to the assay mixture to obtain 1 μM free Ca^{2+} concentration. Both enzymes used for assay of the ATPase activity, as well as ATP, NADH and phospho(*enol*)pyruvate, were purchased from Behringer, Mannheim, F.R.G. All other chemicals were of highest grade commercially available.

The activation energy (E_a) for the reaction catalysed by (Ca^{2+}-Mg^{2+})-ATPase was calculated from the Arrhenius plots of the enzyme activity ($\log V$) versus reciprocal absolute temperature ($1/T$) according to Eqn. 2:

$$E_a = 2.3 \cdot R \cdot (\log V_1 - \log V_2)/(1/T_2 - 1/T_1) \quad (2)$$

where R is the gas constant (1.9869 cal/K per mol), and V_1 and V_2 are values of the enzyme activity in the respective temperatures (T_1 and T_2).

Other determinations

Phospholipid phosphorus content and protein concentration were measured as described previously [36] CaM from bovine brain was purified according to the method of Brzeska et al. [37]. Statistical significance of the obtained differences between control and hyperthyroid animals was estimated by the Student's t-test. A probability level of 0.05 or less was considered a significant difference. The concentration of free Ca^{2+} was calculated using a computer program employing the dissociation constants published by Fabiato and Fabiato [38].

Results

Effect of hyperthyroidism on sarcolemma lipid composition

Results presented in Table I and Figs. 1 and 2 clearly indicate that the elevation of thyroid hormones level in blood serum resulted in changes in the lipid composition and their fatty acid content of SL membrane preparations. The amount of total phospholipids per mg of membrane proteins significantly increased (Table I), whereas the amount of cholesterol was only slightly changed. It resulted in a drastic decrease in the cholesterol to phospholipid ratio. It has to be stressed that the decrease in this ratio may reflect increased membrane fluidity. In addition, HR increased significantly the amount of phosphatidylethanolamine, while the increase in the amount of phosphatidylcholine was less pronounced.

Another important factor which may influence membrane fluidity is the fatty acid composition [16,23,25,26]. The unsaturated fatty acid fraction of SL phospholipids contains mainly oleic (18:1, n–9), linoleic (18:2, n–6), and arachidonic (20:4, n–6) acids. Thyroxine pretreatment resulted in a significant increase in the amount of arachidonic acid and a decrease of oleic one (Fig. 1). The relative amounts of other unsaturated fatty acids, i.e. linoleic (see Fig. 1), docosatetraenoic (22:4, n–6), and docosahexaenoic (22:6, n–3) did not differ significantly in preparations obtained from both groups of animals. The same was true for palmitate (16:0) and stearate (18:0), which represent the main fraction of saturated fatty acids (Fig. 1). The calculated fatty acids unsaturation index (Σmol% of each fatty acid × number of double bonds of the same fatty acid) was higher for the membranes isolated from HR animals (131.3 ± 2.0) than for control preparations (112.8 ± 1.8) ($P < 0.01$ vs. euthyroid). The arachidonic/palmitic acid ratio was 1.2 ± 0.1 and 0.8 ± 0.1 ($P < 0.005$ vs. euthyroid) for preparations of

TABLE I

Effect of hyperthyroidism on sarcolemma membrane lipid content and composition

Lipids	Thyreometabolic status	
	EU [a]	HR [a]
Total cholesterol [b]	0.68 ± 0.03	0.58 ± 0.02
Total phospholipids [b]	1.22 ± 0.05	2.08 ± 0.05 [e]
Cholesterol/phospholipid [c]	0.56 ± 0.04	0.28 ± 0.03 [e]
Phosphatidylcholine [d]	49.90 ± 0.84	53.52 ± 0.91
Phosphatidylethanolamine [d]	22.31 ± 0.41	27.21 ± 0.50 [f]
Phosphatidylserine [d]	10.13 ± 0.50	5.60 ± 0.30 [e]
Sphingomyelin [d]	15.72 ± 0.32	11.32 ± 0.21 [f]
Others [d]	1.41 ± 0.23	1.91 ± 0.32

[a] Five separate SL preparations of each thyreometabolic status were analysed and three determinations per SL preparation were performed; mean values ± S.E. are expressed as: [b] μmol/mg protein, [c] mol/mol and [d] mol% of total phospholipids; [e] $P < 0.001$ vs. euthyroid; [f] $P < 0.005$ vs. euthyroid.

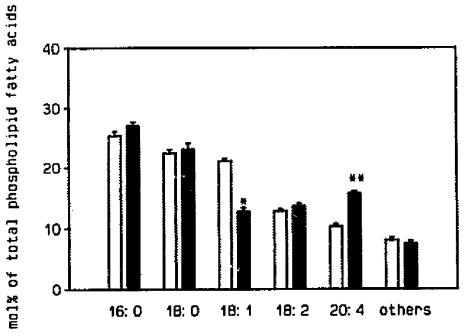

Fig. 1. Effect of hyperthyroidism on fatty acid composition of the total phospholipid fraction of sarcolemma membrane. Four independent SL preparations of each thyreometabolic status (EU, empty bars; HR, filled bars) were analysed in triplicates; mean values ± S.E. are shown and fatty acid content is expressed in mol% of total phospholipid fatty acids; * $P < 0.001$ vs. euthyroid; ** $P < 0.005$ vs. euthyroid.

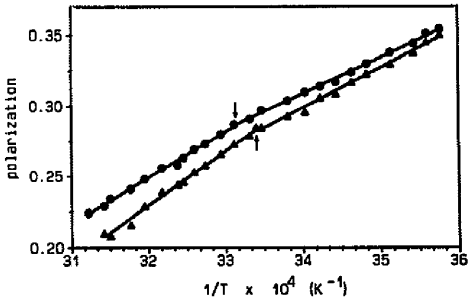

Fig. 3. Temperature dependence of DPH fluorescence polarization in sarcolemma membrane. SL preparations isolated from euthyroid (●) and hyperthyroid (▲) animals were incubated with DPH and steady-state fluorescence polarization was measured as described under Materials and Methods. Each point represents an average value of three independent SL preparations analysed in triplicates, which varied within standard error of 3–5%. Arrows indicate break-point temperatures (25.9 ± 0.3 °C and 28.9 ± 0.2 °C for SL membrane isolated from hyperthyroid and euthyroid rabbits, respectively).

hyperthyroid and control animals, respectively. There was no effect of thyroid status on SL membrane free fatty acid content (results not shown).

Fig. 2 shows the fatty acid composition of two main SL phospholipid classes (PE and PC). Thyroxine caused the elevation in arachidonic and linoleic acid amounts together with a decrease in oleic acid content. Hormone effect was more pronounced in PE than PC. An overall increase in the proportion of palmitic acid in the phospholipid fraction is caused by changes in PC, whereas changes in PE are responsible for the overall elevation of stearic acid content. The unsaturation index for fatty acid of both phospholipid classes increased from 137.4 ± 1.9 to 151.1 ± 2.5 ($P < 0.01$ vs. euthyroid) for PE and from 123.0 ± 2.2 to 131.8 ± 2.0 ($P < 0.01$ vs. euthyroid) for PC. Thyroid hormone administration did not change the proportions between arachidonic and linoleic acids (1.1 ± 0.1 and 1.5 ± 0.1 in PC and PE, respectively).

The effect of hyperthyroidism on the fluidity of SL membrane

In view of observed changes in the membrane lipid composition it was mandatory to compare the fluidity of SL preparations isolated from control and hyperthyroid animals. Variations of DPH steady-state fluorescence polarization with temperature have been used to measure changes in molecular ordering of membrane lipids. Arrhenius plots (Fig. 3) shows that fluorescence polarization was higher for the probe inserted into the control SL preparation. It indicates lower fluidity of the membrane. The discontinuity of the plot (break-point) was found at lower temperature (25.9°C) for membrane preparations obtained from hyperthyroid animals as compared to the control preparations (28.9 °C).

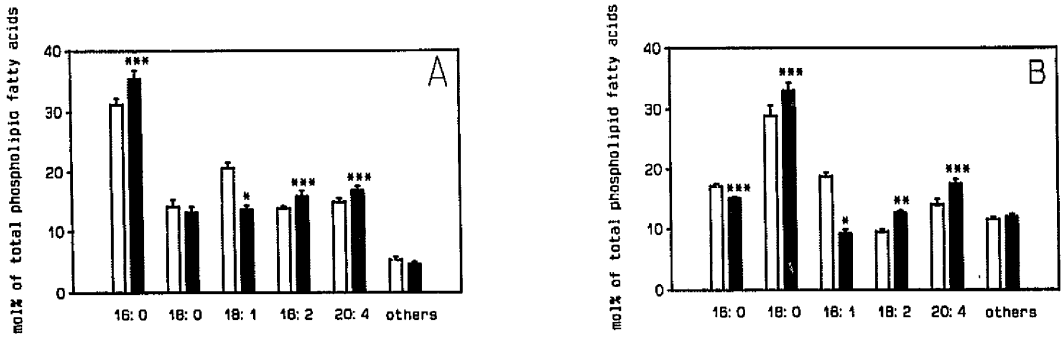

Fig. 2. Effect of hyperthyroidism on fatty acid composition of phosphatidylcholine (A) and phosphatidylethanolamine (B) of sarcolemma membrane. Four separate SL preparations of each thyreometabolic status were analysed (EU, empty bars; HR, filled bars) and for each membrane preparation three to five determinations were performed; mean values ± S.E. are presented; * $P < 0.001$ vs. euthyroid, ** $P < 0.005$ vs. euthyroid; *** $P < 0.01$ vs. euthyroid.

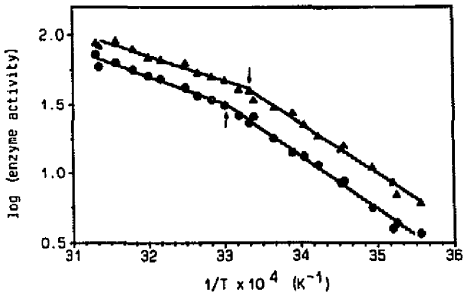

Fig. 4. Arrhenius plots for CaM-stimulated (Ca^{2+}-Mg^{2+})-ATPase activity of sarcolemma membrane. The ATPase activity (expressed in nmol P_i/mg protein per min) of SL preparations isolated from control (●) and hyperthyroid (▲) animals was assayed as described under Materials and Methods, in the range of temperature given on the abscissa. The temperature accuracy has an error of ±0.5 C°. Each point represents an average value for the enzyme activity of five separate SL preparations of each thyreometabolic status (standard error of 4–6%). Three independent measurements per each preparation were performed. Slope differences are significant at a level of $P < 0.01$ vs. euthyroid. Arrows indicate transition temperatures 26.9 ± 0.3°C and 30.0 ± 0.4°C for SL membranes prepared from hyperthyroid and euthyroid animals, respectively).

Effect of hyperthyroidism on the activation energy for the reaction catalysed by (Ca^{2+}-Mg^{2+})-ATPase

Since we have stated that HR changed the membrane fluidity, it was neccessary to measure the activity of CaM-stimulated (Ca^{2+}-Mg^{2+})-ATPase in SL membranes. The temperature dependence of the enzyme activity was analysed for membrane preparations obtained from animals of both thyreometabolic statuses. Results were expressed as the Arrhenius plots (Fig. 4). The activity of CaM-stimulated (Ca^{2+}-Mg^{2+})-ATPase was higher in SL preparation of T_4-treated animals within assayed temperature limits, what is consistent with our previous findings for the ATPase activity measured at 37°C [9,10]. HR lowered the temperature of the Arrhenius plot break-point (26.9 ° C), while this value was clearly higher for membrane preparation obtained from control animals (30 ° C). This means that hyperthyroidism rendered SL membrane more fluid. Hormonal treatment resulted also in the decrease in the activation energy for the reaction catalysed by CaM-stimulated (Ca^{2+}-Mg^{2+})-ATPase from 9.8 ± 0.1 kcal/mol to 8.2 ± 0.2 kcal/mol ($P < 0.01$ vs. euthyroid) above break-point and from 17.1 ± 0.2 kcal/mol to 16.5 ± 0.1 kcal/mol below it.

Discussion

Although it has been demonstrated that the thyroid status influences cell organelle lipids from many tissues [16–26], no studies on the thyroxine overload effect on skeletal muscle SL lipid phase were done so far. The present work shows that hyperthyroidism caused a prominent increase in the total phospholipid amount and a fair decrease in the cholesterol content of these membranes. Enrichement in the phospholipid content (70%) prevails heavily the diminution of cholesterol moiety (15%). It resulted in the significant reduction in cholesterol/phospholipid molar ratio. Similar changes were observed for heart SL preparations, however, occurring to a lesser extent [11]. The uneven effect of HR on the phospholipid content has been shown for other membrane preparations of different origin. In case of liver mitochondria it caused a minor increase in total phospholipid content [18,23], while liver microsomes responded either with the decrease [18,19,23] or no changes [22]. HR changed the proportions between individual phospholipid classes of skeletal muscle SL membrane, as was also demonstrated for liver microsomes and mitochondria [18,19,23].

Cholesterol content did not change significantly in liver microsomes [19], whereas in mitochondria a 25% decrease was observed [23]. It seems that the above mentioned HR-induced changes in the lipid composition of cellular membranes do not form a general pattern and they may be tissue-specific.

The common model for thyroid hormone action on membrane function and structure is the experimentaly evoked hypothyroidism. Reversal of this status by administering T_3 to hypothyroid animals serves as a positive control. Therefore, it is interesting to compare the effect of both thyreometabolical statuses on the lipid composition of cellular membranes. The most surprising finding is that hypothyroidism also resulted in a decrease in cholesterol/phospholipid molar ratio of rat colonic apical plasma membrane [26]. It was mainly caused by a decrease in cholesterol content of these membranes. In case of SR isolated from fast-skeletal muscles no significant changes in cholesterol content were observed [25], whereas liver microsomes exhibited an increased cholesterol content in hypothyroid status [20]. In last two cases administration of T_3 led to the decrease in cholesterol content. In view of these findings and our observations it can be concluded that elevation of T_3 blood levels causes a decrease in the cholesterol content of cellular membranes. It has to be stressed that this phenomenon apparently takes place despite of different thyreometabolic status of animals used for experiments.

It is widely accepted that cholesterol/phospholipid ratio is mirrored by changes in membrane fluidity, since cholesterol is believed to regulate the mobility of phospholipid acyl chains [39,40]. The decrease in the molar ratio of these membrane constituents, observed either in plasma membranes from hypothyroid [26] or hyperthyroid (this report) animals, led to more fluid state of the membrane. However, it has to be emphasized that this effect is due to possibly different regulatory mechanisms of cholesterol synthesis [41], transport

[42], as well as degradation [43] and absorption [44] pertaining to thyreometabolic statuses. Our results show that HR causes a concomitant decrease in percentage contribution of sphingomyelin in total lipid content of SL preparation. Sphingomyelin, similarly to cholesterol, also renders lipid matrix less fluid [40].

Another important determinant of membrane fluidity, the composition of phospholipid fatty acids, is most frequently reported to be thyroid-dependent [16–26]. In the present study an increase in the proportions of polyunsaturated acids (mainly linoleic and arachidonic acid) and a concomitant decrease of monounsaturated (especially oleic acid) ones, were found. As a net result, the increase in the unsaturation index of phospholipid fatty acids was observed inferring higher fluidity of skeletal muscle SL membrane from hyperthyroid animals. It has to be emphasized, that alterations in fatty acids composition occurred within the two major pools of membrane phospholipids, i.e. PC and PE. Similar observations, concerning changes in the phospholipid fatty acids composition, were made for SL preparations obtained from rabbit hearts [11], mitochondria and to some extent microsomes isolated from rat liver [23]. Contrary to this hypothyroidism evoked the opposite effect. This was true for liver microsomes [22] and colonic apical cell membranes [26].

Altered percentage contribution of arachidonic, oleic, and linoleic acids esterified in total skeletal muscle SL phospholipids could suggest stimulation of the desaturases activity [19,22]. However, the arachidonic/linoleic ratio was practically unchanged in PE and PC subfractions of skeletal muscle SL. The plausible explanation for changes in fatty acid pattern could be an alteration in the acylation-deacylation cycle caused by thyroid hormones [11,45–47].

Observed changes in SL lipid matrix indicated that HR causes an increase of SL membrane fluidity, which was confirmed by fluorescence polarization measurements (this study). Changes in the values of DPH fluorescence polarization reflect the transition temperature of membrane lipid phase. In case of skeletal muscle SL isolated from hyperthyroid animals it has a lower value (25.9°C) than in control one (28.9°C).

The Arrhenius representation of CaM-stimulated (Ca^{2+}-Mg^{2+})-ATPase activity in the function of temperature demonstrated that the changes in the SL membrane lipids and hence membrane fluidity caused the enzyme activation [48,49]. The Arrhenius plot break-point for CaM-stimulated (Ca^{2+}-Mg^{2+})-ATPase activity occurred at lower temperature, when measured in SL preparations isolated from hyperthyroid animals (26.9°C versus 30.0°C in control). Also the activation energy of the ATPase reaction was lower (above as well as below the transition temperature) in HR in comparison to euthyroid state. Thus, the decreased activation energy may contribute, apart from the enhancement in enzymic protein content caused by thyroxine [9,10], to the overall activation of the ATPase.

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