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Differential Effect of Triiodothyronine and Thyroxine on Liposomes Containing Cholesterol: Physiological Speculations

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Abstract. The effect of thyroid hormones on the steady-state fluorescence polarization and on the release of the liposomal content was analyzed in liposomes composed of egg phosphatidylcholine and egg phosphatidyl choline:cholesterol in different molar ratios. Depending on liposome cholesterol composition, a dual effect of triiodothyronine was found. The fluorescence polarization of 1,6 diphenyl 1,3,5 hexatriene or 1-(4-trimethylaminophenyl) 6 phenyl-1, 3, 5 hexatriene decreased by the addition of the hormone when cholesterol content was in the range from 0 to 30 moles %, while it increased with cholesterol from 30 to 50 moles %. In the release experiments, the effect of triiodothyronine was also biphasic; the leakage was the highest at 0% and 50% and the lowest at 30 moles % of cholesterol. On the contrary, thyroxine was without effect on liposomes containing cholesterol from 30 to 50 mol %. This fact correlated with a lower incorporation of thyroxine, compared with that of triiodothyronine in liposomes containing up to 30 moles % of cholesterol.

The fact that the above differential incorporation of thyroid hormones was also observed at physiological concentration and that most of the mammalian membrane cells have more than 25 moles % of cholesterol have for physiological implications to the observations reported here.

Key words: Thyroid hormones — Liposomes — Liquid-ordered phase

Introduction

Cholesterol is the major lipid component of most mammalian cell membranes, whereas it is present at considerably lower levels in the membranes of most intracellular organelles (Yeagle, 1988). One of its primary roles is as a modulator of the physical properties of the plasma cell membrane phospholipid bilayer (Yeagle, 1988). Such effects are best studied in model membranes composed of cholesterol and phospholipids (Visit & Davis, 1990, Thewalt & Bloom, 1992, McMullen et al., 1993). At pH 7.4, cholesterol is able to affect the membrane fluidity of PC liposomes, generating a liquid-ordered phase in PC membranes containing more than 25 mol % of cholesterol. The liquid-ordered phase is postulated to be the relevant physical state for many biological membranes that contain substantial amounts of cholesterol. This feature is relatively independent of the precise chemical structure of the PC molecule (Thewalt & Bloom, 1992). In the present paper, the perturbation effects induced by thyroid hormone analogues at pH 7.4 on a membrane model composed of egg-phosphatidylcholine (PC) and cholesterol were studied.

Materials and Methods

PC from egg yolk was obtained as described elsewhere (Luthra & Sheltawary, 1972). Phospholipid preparations were dissolved in chloroform under nitrogen and stored at −20°C. DPH and TMA-DPH, supplied by Molecular Probes (Junction City, OR), were solubilized in dimethylsulfoxide and stored at −20°C. Cholesterol and L-T_3 analogues were purchased from Sigma Chemical Co. All lipids were chromatographically pure as tested by thin layer chromatography on silica gel. (^{125}I) L-T_3 (specific activity, 3,300 μCi/μg) and (^{125}I) L-T_4 (specific activity 5,700 μCi/μg) were obtained from Du Pont-New England Nuclear.

Preparation of Liposomes

Approximately 0.7 mg of egg yolk phosphatidyl choline, together with the appropriate amount of cholesterol, were dissolved in 100 μl of

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chloroform, mixed or not with fluorescent probes, were dried under a nitrogen stream and suspended in 50 mM pH 7.40 sodium phosphate buffer. Small unilamellar vesicles were obtained after sonicating multilamellar vesicles until obtaining a clear solution with a probe-type or bath-type sonicator in a capped glass test tube under nitrogen. Total phospholipid concentrations were determined by phosphate analysis according to Ames (1966).

Fluorescence Polarization Measurement

The DPH and TMA-DPH can be used as indicators of the relative internal motions of phospholipid acyl chains of the liposomal membrane. These probes are frequently referred to as membrane fluidity indicators. However, they are largely sensitive to only the angular reorientation of lipid acyl chains. The fluorescence anisotropy of these probes is primarily determined by the restricted angular range of the motion rather than its rate (Haugland, 1992). In the present paper, physical changes in the membrane caused by thyroid hormones are expressed as a difference of polarization (Δ polarization) between the polarization values obtained in the absence and in the presence of the hormones. The fluorescent probes were introduced in the lipid bilayer at a molar ratio of 0.2% by adding DPH or TMA-DPH solution to the lipid suspension before drying, according to Shinitzky and Barenholz (1987). The steady-state fluorescence polarization (P) was measured in 1.5 ml of 50 mM pH 7.40 sodium phosphate buffer. Both phospholipid and thyroid hormone concentration were 50 μM. The degree of polarization: $P = (I_{II} - I_I)/(I_{II} + I_I)$ was determined by using a SLM Instrument model 4800 fluorimeter (SLM Aminco, Urbana, IL) equipped with a thermostatic cuvette holder. The excitation wavelength was selected at 360 nm, and all the fluorescence was collected through a cutoff filter (Corning 3–73). The hormone effect was recorded 1–2 min after the addition of aliquots of hormone solution. Before hormone addition, the liposomes were equilibrated at room temperature (22–26° C) for 10 min. L-T_3 and their analogues were solubilized in 1 mM NaOH. No changes in pH and turbidity were noted after hormone addition.

Measurement of Liposomal Leakage

The kinetics of the release of the liposomal content was measured according to Ellens, Bentz & Szoha (1984), using the ANTS/DPX dequenching assay. The residual fluorescence of the liposomes containing 50 mM DPX and 50 mM ANTS was taken as 0% release. The maximal fluorescence, obtained after disruption of the liposomes with Triton X-100 (0.5%) was taken as 100% release. The transfer of fluorescence energy between Tb and dipicolinic acid (Bentz et al., 1983) method was also used. The leakage experiments were started by injection of a small volume of the hormones (10–50 μl) with a Hamilton syringe into a cuvette containing 1.5 ml of 50 mM sodium phosphate buffer. The highest release of liposomal content occurred within the first 15 sec after hormone addition. In our experimental system, it was not possible to follow faster events. After the initial burst, the fluorescence level remained constant. One likely explanation for these results is that the effect was instantaneous, and on the other hand that L-T_3 and L-T_4 were unable to redistribute after incorporation to the membrane since this would gradually cause lysis of the entire liposome population.

Incorporation Assay of (^{125}I)L-T_3 and (^{125}I)L-T_4 to Liposomes

50 μM of liposomes were incubated with 50 μM or 0.050 nM of hormones in 1.2 ml of 50 mM pH 7.40 sodium phosphate buffer. After incubation, bound and free hormones were immediately separated by filtration through an anionic Dowex exchange resin (Dowex 1 × 8 Cl 200–400 mesh), as reported by Farias et al. (1995).

Reproducibility of the Results

Fluorescence polarization and leakage values were obtained at least in three independent experiments. In the leakage experiment, when two different curves were analyzed, the individual values obtained showed a maximum variability of 5%.

Abbreviations

DPH, 1,6 diphenyl-1,3,5 hexatriene; TMA-DPH, 1-(4-trimethylamoniophenyl)-6 phenyl-1,3,5-hexatriene; PC, egg phosphatidylcholine; L-T_3, 3,5,3′ triiodo L-thyronine; D-T_3, 3,5,3′ triiodo D-thyronine; L-T_4, L-thyroxine, D-T_4, D-thyronine; DIT2, 3,5 diiodothyronine; ANTS, 8-aminonaphthalene-1,3,6-triisulfonic acid; DDX p-xylylene-bis (pyridium bromide)

Results

Dose-Response Studies

Figure 1 shows the Δ polarization and leakage values of PC-liposomes as a function of L-T_3 concentration. A similar pattern was observed with L-T_4, but the Δ polarization and leakage values are lower than for L-T_3. The effect of L-T_3 and L-T_4 on 50 μM of PC liposomes increased almost linearly with the hormone concentration. Under our experimental conditions, higher than 50 μM L-T_3 concentration could not be used, since the scatter of the sample interferes with the fluorescence polarization determinations. DIT_2 didn't show any effect. The latter indicates that the presence of the iodine atom in the thyronine β ring is necessary for membrane perturbation. The alanine chain group may not participate in the phenomenon since a similar response could be observed with the thyroid hormone isomers D-T_3 and D-T_4 (*not shown*).

DPH is known to be located within the hydrophobic core of the liposomal membrane and provides information on this region. Cationic TMA-DPH is anchored in proximity to the bilayer surface, so it only provides information on the bilayer surface (New, 1990).

As expected, in the absence of L-T_3, the increase of the molar fraction of cholesterol resulted in an increase in polarization values of both DPH and TMA-DPH probes (polarization of 0.1513 for DPH and 0.2756 for TMA-DPH in PC liposomes and 0.2713 for DPH and 0.3314 for TMA-DPH in PC:cholesterol liposomes 50:50 moles). The effects of L-T_3 on DPH and TMA-DPH fluorescence polarization as a function of the molar fraction of cholesterol are shown in Fig. 2*A*. In the presence of 50 μM L-T_3, the Δ polarization values of both fluorescence probes increased in PC liposomes and de-

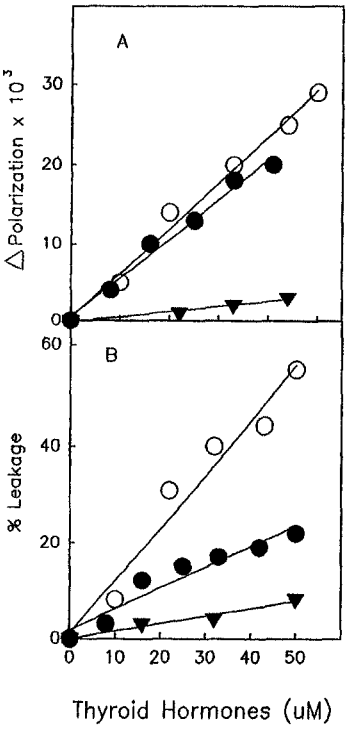

Fig. 1. Dose-dependence response of: Δ polarization of DPH (*A*) and leakage of liposomal content (*B*) induced by L-T_3 (○) L-T_3 (●) and DIT_2 (▼). Fluorescence polarization and release were determined as described in Materials and Methods.

creased in PC liposomes containing 50 mol % of cholesterol, with Δ polarization being zero at 30 mol % of cholesterol, for both DPH and TMA-DPH. This behavior did not change with the assay temperature, since similar results were obtained at 5, 22 or 45° C (*not shown*). The leakage induced by the addition of L-T_3 was biphasic, since it decreased in the range from 0 to 30% and increased from 30 to 50% of cholesterol (Fig. 2*C*). The release was null with liposomes composed of PC and cholesterol in 7:3 molar ratio. L-T_4 did not show any effect on PC liposomes containing cholesterol more than 30 moles % of cholesterol (Fig. 2 *B* and *D*).

DIT_2 did not induce any change on Δ polarization and release parameters in PC liposomes containing from 10 to 50 moles % of cholesterol (*not shown*).

Incorporation of L-T_3 and L-T_4 into Liposomes

The incorporation of L-T_3 and L-T_4 was expressed as a function of phospholipid present in the liposomes.

Cholesterol is able to induce a decrease in hormone incorporation, but the effect of cholesterol on the incorporation of L-T_4 or L-T_3 was different (Fig. 2*E* and *F*). The incorporation of L-T_4 to liposomes containing 40 and 50 moles % of cholesterol was negligible.

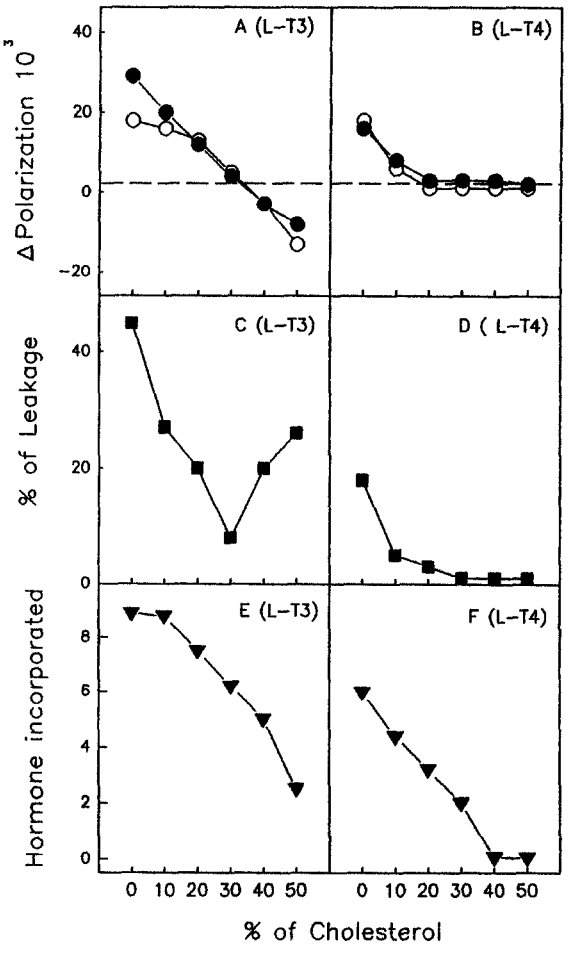

Fig. 2. Cholesterol dependence of: Δ polarization of DPH (●) and TMA-DPH (○) (*A* and *B*), release of liposomal content (■) (*C* and *D*) and hormone incorporation (*E* and *F*). The incorporation results are expressed as nmoles of hormone incorporated by 100 nmoles of phospholipids. L-T_3 (*A, C* and *E*); L-T_4 (*B, D* and *F*).

Hormonal Incorporation at Physiological Concentration Range

The hormone concentration employed in the experiments showed in Fig. 2 was 50 μM. While this hormone concentration was dictated by the sensitivity of the membrane perturbation methods employed, it was higher than the physiological concentrations of the hormones. In plasma, the total L-T_3 and L-T_4 concentrations are about 1 nM and 100 nM, respectively whereas the free hormone concentrations are in the range of 0.025–0.050 nM for L-T_3 and 0.017–0.034 nM for L-T_4 (Najad et al., 1975). Using 0.050 nM of both hormones, a differential incorporation of L-T_3 and L-T_4 to liposomes containing cholesterol was also found. The incorporation ratio into liposomes containing 50% and 0% moles of cholesterol was 0.24 ± 0.05 for L-T_3 and 0.012 ± 0.001 for L-T_4 (data of three independent experiments).

RED BLOOD CELL LIPID-LIPOSOMES

When similar experiments, as seen in Fig. 2 *E* and *F*, were carried out using liposomes made with lipids extracted from a natural membrane, such as that of human red blood cells which contains about 40–45 moles % of cholesterol (Yeagle, 1988), a differential incorporation of L-T_4 and L-T_3 was also found. Using physiological concentrations of L-T_3 and L-T_4 (0.050 nM) the incorporation's for L-T_3 and L-T_4 in three independent experiments were of 5.18 ± 0.12 and 0.022 ± 0.001 fmoles/100 nmoles of total phospholipids, respectively.

Discussion

The effect of thyroid hormones on the degree of order or mobility of dimyristoyl, dipalmitoyl or egg yolk phosphatidyl choline liposomes was reported by Farias et al (1995). The freedom of molecular motion above the phase transition temperature was decreased, while below the transition, the mobility was actually increased by the incorporation of L-T_3 to liposomes. While L-T_4 decreases the mobility in the liquid crystalline state, it cannot increase it in the gel state. These facts were correlated with the differential incorporation of the hormones to liposomes above and below the phase transition temperature of dimyristoyl and dipalmitoyl phospholipid choline. In gel state, a low incorporation of L-T_4 compared with L-T_3 was reported (Farias et al., 1995).

In the present paper, we show that when the liposomes are in a liquid-ordered phase, containing up to 25 moles % of cholesterol, a differential membrane perturbation induced by L-T_3 and L-T_4 was observed. It is possible that the larger size of L-T_4 compared to L-T_3 blocks their incorporation into the cholesterol-phospholipid liquid-ordered phase, similar to that which occurred in the gel phase of the phospholipids (Farias et al., 1995).

The interaction of L-T_3 and L-T_4 with lipid vesicles could be considered to be a nonphysiological issue, and details concerning incorporation and membrane perturbation could add very little to our understanding of how thyroid hormones elicit their metabolic responses. However, the thyroid hormones must cross the plasma membrane of the target cell to reach the deiodinase enzymes, the cytosolic binding proteins and the nuclear receptors before they can express their biological activities (Botta et al., 1983, Oppenheimer & Schwartz, 1985, Angel, Botta & Farias, 1989, Fanjul & Farias, 1991, Fanjul & Farias, 1993*a,b*). The differential incorporation of physiological concentrations of L-T_3 and L-T_4 in the red blood cell lipid membrane model shown in the present paper could have physiological implications and great significance on the interpretation of the hormone transport experiments since differences in membrane transport and in the intracellular concentrations of L-T_3 and L-T_4 in human red blood cells were reported (Yoshida & Davis, 1980, Osty et al., 1988, Osty et al., 1990). The relationship between the liposomal membrane perturbation reported herein and the well-known in vivo effect of the thyroid hormones on the membrane composition (Clejan, Collipp & Middaiah, 1980; Pasquini et al., 1980; Faas & Carter, 1981; Hoch, F.L., 1988; Pilarska et al., 1991) is also an attractive field for future studies.

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