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TRANSVERSE MOTION OF SPIN-LABELED 3,3',5-TRIIODO-L-THYRONINE IN PHOSPHOLIPID BILAYERS

Ching-San Lai,* W. Korytowski,* Chien-Hau Niu,† and Sheue-Yann Cheng§

*National Biomedical ESR Center, Medical College of Wisconsin, Milwaukee, WI 53226

†Laboratory of Biochemical Pharmacology, National Institute of Arthritis, Diabetes, Digestive and Kidney Disease and §Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20205

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SUMMARY Using electron spin resonance stop-flow technique, the transverse motion (flip-flop) of 3-{[α-carboxy-4-(4-hydroxy-3-iodophenoxy)-3,5-diiodophenethyl]carbamoyl}-2,2,5,5-tetramethyl-3-pyrrolin (T_3-SL)in dipalmitoyl L-α-phosphatidylcholine (DPPC) membranes was evaluated. At 22°C, the electron spin resonance spectra of T_3-SL in DPPC vesicles were compared before and after the addition of sodium ascorbate, a membrane impermeable reducing agent. The addition of ascorbate reduces the signal amplitude by 67% in 3 min but yields no further reduction for at least 60 min. These results indicate that T_3-SL does not flip-flop at any appreciable rate in the membranes. This finding suggests that once partitioned into the membrane, T_3 remains in the outer half of the lipid bilayer, thus reducing the possibility that T_3 enters the cell by passive diffusion. © 1985 Academic Press, Inc.

The thyroid hormone, 3,3',5-triiodo-L-thyronine (T_3), plays an important role in regulating tissue differentiation and development and influences many metabolic processes. Recently, there has been growing interest in understanding the mode of T_3 entry into cells. Evidence has been presented to indicate that the uptake of T_3 by cells might be receptor mediated (1-6). Furthermore, saturable and stereospecific T_3 binding sites on plasma membranes have been identified in rat liver (7,8), erythrocyte membranes (9,10), and several culture cell lines (6,11,12). These binding sites have been postulated to mediate the uptake of T_3 (6,11-13).

Abbreviations:

DPPC, dipalmitoyl L-α-phosphatidylcholine; DPPC-SL, spin-labeled DPPC; ESR, electron spin resonance; T_3, 3,3',5-triiodo-Lthyronine; T_3-SL, 3-{[α-carboxy-4-(4-hydroxy-3-iodophenoxy carbamoyl}-2,2,5,5-tetramethyl-3-pyrrolin.

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In the above studies, however, a rather high non-saturable T_3 binding component (30-50%) was observed. The non-saturable binding component could be due to the binding to nonspecific proteins and/or simply due to the partition of T_3 in the lipid components of the membranes. If it is the latter, it is important to see whether T_3 could flip-flop across the membrane as this could relate to the mode of entry of T_3 into cells. To study this problem, we have synthesized a spin-labeled derivative of T_3 (14). This analog was shown to bind to thyroid hormone nuclear receptor with an 18% potency of that of T_3. Using electron spin resonance (ESR) techniques, we first characterized the rotational and lateral diffusion of T_3-SL in vesicles derived from DPPC. T_3-SL was found to diffuse freely in phospholipid bilayers (14). In this report, we have determined the flip-flop rate of T_3-SL in phospholipid bilayers and found that T_3-SL does not flip-flop at any appreciable rate in the membranes.

MATERIALS AND METHODS

Dipalmitoyl L-α-phosphatidylcholine (DPPC) and L-α-phosphatidylcholine from egg yolk were obtained from Sigma (St. Louis, MO). Spin-labeled DPPC (DPPC-SL, see Fig. 1 for chemical structure) was prepared essentially as described by Kornberg and McConnell (15). Spin-labeled T_3 was synthesized as described previously (14).

DPPC multilamellar vesicles, containing either DPPC-SL or T_3-SL were prepared as described previously (14). Small, unilamellar vesicles were prepared by sonicating the multilamellar vesicles with a Branson Sonifier Cell Disruptor model 185 at 22°C for 1 min with 1 min cooling; this process was repeated until the solution turned transparent. This procedure yielded at least 95% of small, unilamellar vesicles, which is in agreement with observations made by other investigators (16). The method for preparing unilamellar egg lecithin vesicles was the same as described above for DPPC membranes, except that the egg lecithin membrane was kept at 4°C during sonication.

RESULTS AND DISCUSSION

The ESR spectra of DPPC-SL in small DPPC vesicles at 22°C, before and after addition of sodium ascorbate, are shown in Fig. 1a and 1b, respectively. The presence of 0.25 M sodium ascorbate diminished the signal amplitude by 68% in 1 min and produced no further change during 60 min. Sodium ascorbate, known to reduce the paramagnetic nitroxide radical to the nonparamagnetic hydroxylamine derivative, is impermeable at 22°C to the DPPC vesicle, which has a main phase transition temperature of 41°C (16). The ESR spectrum in Fig. 1b arises from

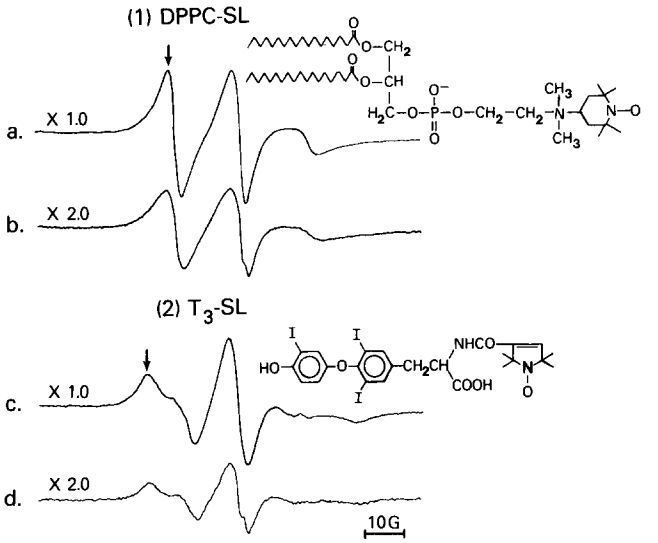

Fig. 1. Effects of sodium ascorbate on the ESR spectra of 1) DPPC-SL and 2) T_3-SL. The concentration of spin-labeled derivative of DPPC or T_3 was about 1×10^{-4} M and the molar ratio of spin-labeled molecule to lipid was about 1:100. The membrane vesicles containing different spin-labeled molecules prepared in buffer A (10 mM Tris, 100 mM NaCl and 2 mM EDTA, pH 7.0) were mixed with an equal volume of buffer A (a and c, control) or with an equal volume of 0.5 M sodium ascorbate in water (b and d). All samples were incubated at 22°C for 1 hr prior to ESR measurement at the same temperature. Spectra were recorded with a Varian Century-line spectrometer, operating at 9.4 GHz. The modulation amplitude was 2.0 G, the field sweep 100 G, and the microwave power 1 mW. The arrows indicate the peak positions used for monitoring the signal decay due to ascorbate reduction during stop-flow experiment (see Fig. 2).

the fraction of DPPC-SL in the internal monolayer of the vesicle which is shielded from the reducing agent. By comparing the line shapes of Fig. 1a and Fig. 1b, DPPC-SL in the inner half of the vesicle (Fig. 1b) appears to be more immobilized than the probe in the outer half (Fig. 1a). This probably is due to closer packing of phospholipids in the internal monolayer. A plot of the signal amplitude of DPPC-SL versus time at 22°C, after treatment with ascorbate, is shown in Fig. 2B. A rapid destruction of the spin probe is followed by a flat plateau (about 30%). We conclude that the rate of flip-flop of phospholipid spin label in the membrane is very slow. The decay curve of DPPC-SL (Fig. 2B) is essentially similar to that reported by Birrell et al. (17) in which the translipid motion of DPPC-SL and spin-labeled chlorophyll derivatives in egg yolk phosphatidylcholine was compared. Using the same ascorbate reduction method, they found that the flip-flop rate of the spin-labeled chlorophyll derivatives was $T_{1/2} \sim 4$ min at 4°C, whereas that of DPPC-SL was nondetectable.

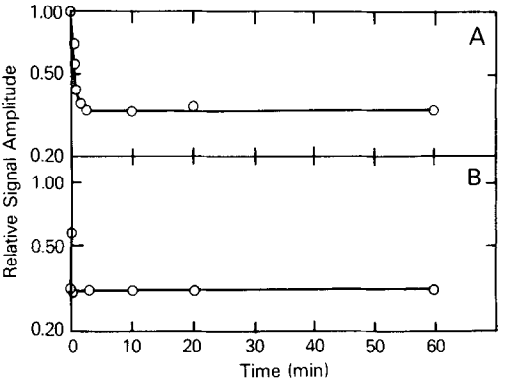

Fig. 2. Decay curves for relative signal amplitudes of the ESR spectra of (A) T_3-SL and (B) DPPC-SL in DPPC unilamellar vesicles at 22°C after addition of sodium ascorbate. Rapid mixing of membrane vesicles containing different spin-labeled molecules with sodium ascorbate was carried out using a Harvard apparatus (infusion/withdrawal pump) with a mixing time of about 100 msec. For these kinetic measurements, the dial of the magnetic field was turned off and the recorder pen was set at the top of the peak of the low-field component (see arrows in Fig. 1). Other instrumental settings were the same as described in Fig. 1.

The ESR spectra of T_3-SL in the DPPC vesicle at 22°C, before and after treatment with sodium ascorbate, are depicted in Figs. 1c and 1d, respectively. The addition of ascorbate also reduced the signal amplitude by 67% in 3 min and yielded no further reduction during 60 min. Fig. 2A also shows a rapid reduction of the signal amplitude of T_3 derivative, followed by a flat plateau of about 33% signal amplitude. The data indicate that T_3 derivative does not flip-flop at any appreciable rate in the membrane under these conditions. Since the experiments were carried out at 22°C, which is below the main phase transition of DPPC membranes, the question is raised whether T_3-SL would flip-flop in a lipid system at a temperature above the phase transitions. To test this possibility, we have determined the flip-flop rate of T_3-SL at 4°C in unilamellar egg lecithin vesicles, which have a main phase transition temperature of about -10°C. Again, no flip-flop of T_3-SL could be detected (data not shown).

The use of ESR technique to study the transverse motion of T_3 across the membranes necessitates the incorporation of a paramagnetic probe into a T_3 molecule. The introduction of a nitroxide moiety at the amino group renders T_3-SL more hydrophobic. However, T_3-SL still retains 18% of T_3 nuclear binding activity (14). Since the underivatized T_3 is Zwitterionic at physiological

pH, possessing both a negatively charged carboxyl group and a positively charged amino group, it would be reasonable to expect the underivatized T_3 to be anchored at the membrane surface even more firmly than T_3-SL. The translocation of T_3 across the lipid bilayer of responsive cells is probably mediated by mechanisms other than passive diffusion. Recently, many laboratories have demonstrated the presence of specific T_3 binding sites on plasma membranes of many tissues and cultured cells (1-13). One of the functions of these binding sites has been postulated to mediate the entry of T_3. Our data presented in this report are consistent with a requirement for receptor-mediated uptake of T_3. Furthermore, the present study also suggests that the T_3 recognition site on the receptor molecule is likely to be located near the extracellular side of the plasma membrane of responsive cells.

REFERENCES

1. Holm, A.-C., and Jacquemin, C. (1979) Biochem. Biophys. Res. Commun. 89, 1006-1017.
2. Parl, F., Koreck, L., Siegel, J.S., and Tabachnick, M. (1977) FEBS Lett. 83, 145-147.
3. Krenning, E.P., Docter, R., Bernard, H.F., Visser, T.J., and Hennemann, G. (1978) FEBS Lett. 91, 113-116.
4. Rao, G.S., Eckel, J., Rao, M.L., and Breuer, H. (1976) Biochem. Biophys. Res. Commun. 73, 98-104.
5. Eckel, J., Rao, G.S., Rao, M.L., and Breuer, H. (1979) Biochem. J. 182, 473-491.
6. Cheng, S.-Y., Maxfield, F.R., Robbins, J., Willingham, M.C., and Pastan, I. (1980) Proc. Natl. Acad. Sci. USA 77, 3425-3429.
7. Pliam, N.B., and Goldfine, I.D. (1977) Biochem. Biophys. Res. Commun. 79, 166-172.
8. Charbi-Chihi, J., and Torresani, J. (1981) J. Endocrinol. Invest. 4, 177-181.
9. Holm, A.C., and Jacquemin, C. (1979) Biochem. Biophys. Res. Commun. 89, 1006-1017.
10. Butta, J.A., de Mendoza, D., Monero, R.D., and Zaviar, R.N. (1983) J. Biol. Chem. 258, 6690-6693.
11. Cheng, S.-Y. (1983) Endocrinology 112, 1754-1762.
12. Cheng, S.-Y. (1983) Endocrinology 113, 1155-1158.
13. Maxfield, F.R., Willingham, M.C., Pastan, I., Dragsten, P., and Cheng, S.-Y. (1981) Science 211, 63-65.
14. Lai, C.-S., and Cheng, S.-Y. (1984) Arch. Biochem. Biophys. 232, 477-481.
15. Kornberg, R.D., and McConnell, H.M. (1971) Biochemistry 10, 1111-1120.
16. Paphadjopoulos, D., and Miller, W. (1967) Biochim. Biophys. Acta 135, 624-638.
17. Birrell, G.B., Boyd, S.A., Keana, J.F.W., and Griffith, H. (1980) Biochim. Biophys. Acta 603, 213-219.