Biochimica et Biophysica Acta, 465 (1977) 661—666

BBA Report

BBA 71290

INTRACELLULAR DISTRIBUTION OF LIPOPHILIC FLUORESCENT PROBES IN MAMMALIAN CELLS

RICHARD E. PAGANO[a], KEIKO OZATO[b] and JEAN-MARIE RUYSSCHAERT[c]

Carnegie Institution of Washington, Department of Embryology, 115 West University Parkway, Baltimore, Md. 21210 (U.S.A.)

(Received December 13th, 1976)

Summary

The intracellular distribution of several hydrophobic fluorescent probes (1,6-diphenyl-1,3,5-hexatriene (DPH), perylene, and 2-*p*-toluidinyl-6-naphthalene sulfonate (TNS)) in mouse lymphocytes and a fibroblast cell line was examined using radiolabeled fluorescent probes and the technique of high resolution EM autoradiography. Following a short term incubation, DPH and perylene were found largely internalized in cells, while TNS was localized predominantly at the cell surface. These findings suggest that fluorescence polarization studies using such probes with intact cells do not necessarily monitor only the cell surface membrane and must be interpreted with caution.

The use of small lipophilic fluorescent molecules for probing the physical state of lipid membranes has been a subject of recent intensive investigation by membranologists. Such probes partition to varying degrees into the hydrophobic phase of the lipid bilayer and can be used to detect, by fluorescence, subtle changes in the molecular dynamics and physical state of the

[a] To whom correspondence should be addressed.

[b] Present Address: Good Samaritan Hospital, O'Neill Memorial Laboratory, Baltimore, Md. 21239 (U.S.A.).

[c] Permanent Address: Laboratoire de Chimie Physique des Macromolécules aux Interfaces, Campus Plaine, Université Libre de Bruxelles, Bruxelles, 1000 (Belgium).

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; TNS, 2-*p*-toluidinyl-6-naphthalene sulfonate.

lipid molecules in the vicinity of the probe. This approach has been particularly fruitful using model lipid dispersions or liposome systems [1—8]. Recently these probes have also been used with intact cells, including erythrocytes, cultured fibroblasts, lymphocytes, and plant cells [7, 9—16]. It is frequently assumed in such studies that the fluorescent molecules do not penetrate the intact cell, but are localized at the cell surface, and thus report on the "fluidity" or "microviscosity" of the plasma membrane lipid bilayer. Since a partitioning of such probes into internal compartments of the cell would greatly complicate the interpretation of these fluorescence measurements, it is essential that this assumption be rigorously tested. In the present study we examine the location of several lipophilic fluorescent probes in whole cells using EM autoradiographic methods.

1,6-Diphenyl-1,3,5-hexatriene (DPH), perylene, and 2-*p*-toluidinyl-6-naphthalene sulfonate (TNS) were obtained from commercial sources, and radiolabeled by exposure to pure tritium gas in an ionizing electric field [17]. The tritiated samples were purified by crystallization (4 times) from the appropriate organic solvents and had specific activities of approximately 50 μCi/mg (DPH), 600 μCi/mg (perylene) and 100 μCi/mg (TNS). Fluorescence spectra of the radiolabeled and unlabeled probe species were indistinguishable. Mouse thymocytes were prepared using 4—8-week-old CBA male mice (Jackson Laboratory, Bar Harbor, Maine) as described [18]; about 1% of the cells in the isolated thymocyte population were erythrocytes. The latter were also analyzed and served as an internal control (see below). Chinese hamster V79 fibroblasts were maintained in culture [19,20] and dissociated into single cell suspensions using a Ca^{2+} and Mg^{2+} free saline containing 1 mM EDTA. One volume of cell suspension ($0.5 \cdot 10^7$–$2 \cdot 10^7$ cells/ml balanced salt solution) was added to one volume of a $2 \cdot 10^{-6}$ M solution of ^{3}H-labeled fluorescent probe in 0.15 M KCl, and the mixture incubated at 25° C for 30 min. These incubation conditions did not impair the viability of the treated cells as assessed by trypan blue staining. The labeled cells were then washed twice in a balanced salt solution, fixed in glutaraldehyde, postfixed in OsO_4, and processed for EM autoradiography using Ilford L-4 emulsion as previously described [20,21]. In separate grids containing no sectioned material, background grains were random and less than 5 grains per grid square (200 mesh).

Fig. 1a shows a typical autoradiogram obtained for mouse thymocytes treated with [^{3}H] DPH. A large fraction of the silver grains appear to be localized in both cytoplasmic and nuclear regions of the cell. A similar grain distribution was seen using [^{3}H] perylene. In contrast, thymocytes treated with [^{3}H] TNS (Fig. 1b) showed heavy labeling of the cell surface with relatively little internalized probe. These observations were quantitated as follows. Micrographs of thymocytes treated with the radioactive fluorescent probes were taken at 14 000 ×, and the outline of individual cells and location of grains traced on paper, as indicated in Fig. 1c. Each traced cell was then divided into two compartments and the number of silver grains in each compartment determined. A cell surface compartment was defined by a zone 0.3 μm wide and centered over the cell surface membrane contour. The remaining portion of the cell defined the internal compartment. Grain

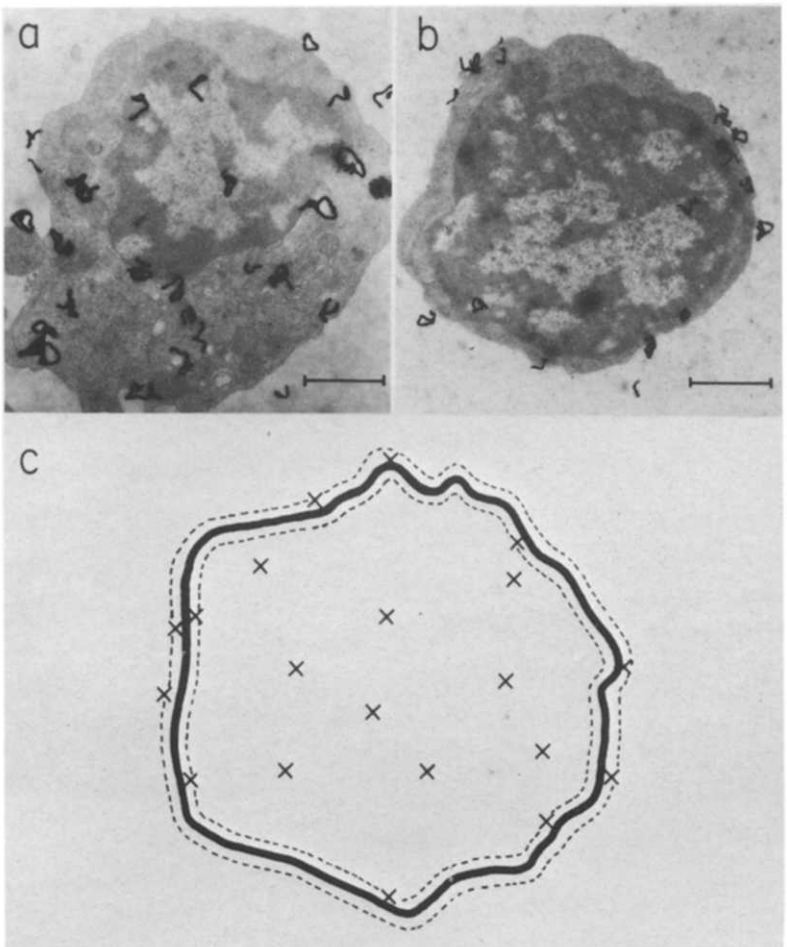

Fig. 1. Electron microscope autoradiograms of mouse thymocytes treated with (a) [^{3}H] DPH and (b) [^{3}H] TNS. Bar is 1 μm. Quantitative analysis of the autoradiograms was made by tracing the outline of each cell and dividing it into a cell surface and interior compartment as shown in (c). The position of silver grains (x's) in each compartment was then scored and the grain densities calculated (see text).

densities were obtained by dividing the number of grains in each compartment by the area of that compartment. The ratio of surface grain density to interior grain density was then calculated, and the average ± S.E.M. calculated. The results of such measurements are summarized in Table I. It is readily seen, both from the percentage of silver grains found in the surface compartment, as well as the ratio of surface to internal compartment grain densities, that DPH and perylene are largely internalized in the treated cells, while TNS is confined mostly to the cell surface.

Although our quantitative analysis of the intracellular distribution of fluorescent probes is limited to mouse thymocytes, qualitatively similar distributions were seen using Chinese hamster V79 fibroblasts. A typical EM autoradiogram of a V79 cell treated with [^{3}H] DPH is shown in Fig. 2.

TABLE I

DISTRIBUTION OF AUTORADIOGRAPHIC GRAINS OVER MOUSE THYMOCYTES TREATED WITH ^{3}H-LABELED FLUORESCENT PROBES

Probe	Total Grains Counted	Grains in Surface Compartment (%)	(Surface Grain Density)/ (Interior Grain Density) ± S.E.M.
[^{3}H] TNS	302	69	11.4 ± 1.3
[^{3}H] DPH	990	16	2.0 ± 0.3
[^{3}H] Perylene	918	27	2.4 ± 0.1

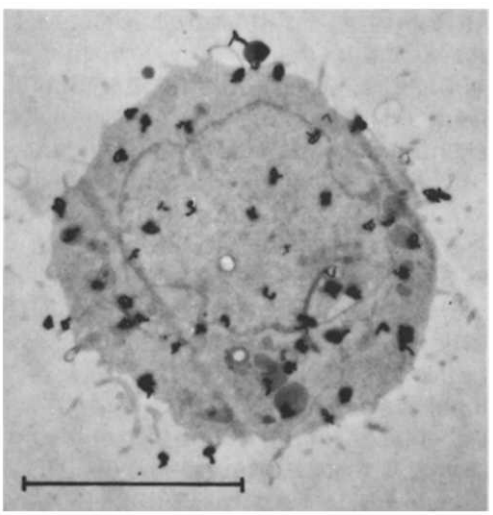

Fig. 2. Electron microscope autoradiogram of a Chinese hamster V79 fibroblast incubated with [^{3}H] DPH. Bar is 5 μm.

As for thymocytes, most of the radiolabeled probe was found internalized in the cell.

Analysis of autoradiograms obtained for mouse erythrocytes treated with tritium labeled DPH, perylene, or TNS demonstrated that the radioactive probes were symmetrically distributed about the red cell membrane, with approximately 50% of the silver grains found within about ± 0.15 μm of the membrane surface (Fig. 3). This distribution demonstrates that, within the resolution of the EM autoradiographic technique (≈0.15 μm; ref. 22), the radiolabeled fluorescent probes are localized in the erythrocyte surface membrane. Furthermore, these data argue against a significant nonrandom redistribution of the probes during fixation and embedding for EM, since this would likely result in a skewed distribution of silver grains, as well as a high background outside the cell.

Our autoradiographic findings show that the more hydrophobic probes, DPH and perylene, when incubated with intact lymphocytes or fibroblasts, partition not only into the cell surface membrane but also into cytoplasmic and nuclear regions of the cell. Presumably this partitioning

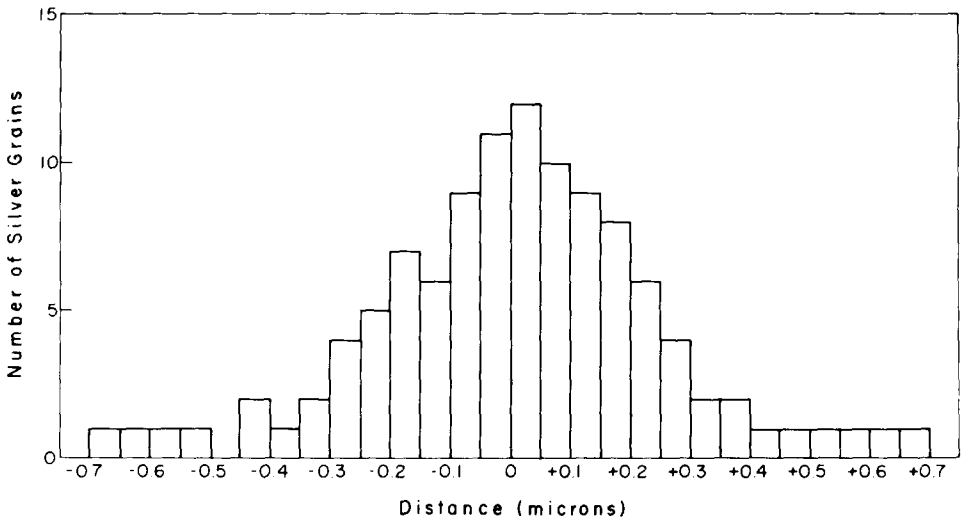

Fig. 3. Distribution of silver grains in autoradiograms of mouse erythrocytes treated with [^{3}H] DPH. Number of silver grains is plotted vs. their distance from the plasma membrane (+, outside; —, inside). Quantitatively similar distributions were obtained using [^{3}H] TNS and [^{3}H] perylene.

represents a rapid equilibration of the probes between the cell surface and hydrophobic elements within the cell, e.g. intracellular membranes. Such an equilibration is readily observed with DPH when two populations of artificial membranes are mixed with one another [6]. In contrast, TNS was found to be localized predominantly at the cell surface. This finding is consistent with the studies of model lipid dispersions using TNS, which suggest that this probe resides at the interface between the lipid polar head groups and the aqueous phase [23]. Our findings raise serious questions about the validity of using probes such as DPH with intact cells to estimate the "microviscosity" or "fluidity" of the cell surface membrane. Since large quantities of the fluorescent probe are internalized by treated cells, it seems quite likely that fluorescence polarization measurements represent some complicated average of fluorescence signals from various regions of the cell rather than from the cell surface alone. Thus, a rigorous interpretation of such data must await a determination of the relative amounts (and degrees of quenching) of a given probe in each of these regions.

We gratefully acknowledge the expert technical assistance of Mr. W. Duncan and thank Drs. A. Sandra and B.J. Litman for critically reading this manuscript. This work was supported by National Institutes of Health Research Grant GM 22942 to R.E.P., and by NATO Research Grant No. 771.

References

1 Shinitzky, M., Dianoux, A.C., Gitler, C. and Weber, G. (1971) Biochemistry 10, 2106—2113
2 Shinitzky, M. and Barenholz, Y. (1974) J. Biol. Chem. 249, 2652—2657
3 Papahadjopoulos, D., Jacobson, K., Poste, G. and Shepherd, G. (1975) Biochim. Biophys. Acta 394, 504—519
4 Andrich, M.P. and Vanderkooi, J.M. (1976) Biochemistry 15, 1257—1261

5 Lentz, B.R., Barenholz, Y. and Thompson, T.E. (1976) Biochemistry 15, 4521—4528
6 Lentz, B.R., Barenholz, Y. and Thompson, T.E. (1976) Biochemistry 15, 4529—4537
7 Stubbs, D.W., Litman, B.J. and Barenholz, Y. (1976) Biochemistry 15, 2766—2772
8 Suurkuusk, J., Lentz, B.R., Barenholz, Y., Biltonen, R.L. and Thompson, T.E. (1976) Biochemistry 15, 1393—1401
9 Inbar, M., Shinitzky, M. and Sachs, L. (1974) FEBS Lett. 38, 268—270
10 Kishiye, T., Toyoshima, S. and Osawa, T. (1974) Biochem. Biophys. Res. Commun. 60, 681—686
11 Fuchs, P., Parola, A., Robbins, P.W. and Blout, E.R. (1975) Proc. Natl. Acad. Sci. U.S. 72, 3351—3354
12 Inbar, M. and Shinitzky, M. (1975) Eur. J. Immunol. 5, 166—170
13 Wiley, J. and Cooper, R.A. (1975) Biochim. Biophys. Acta 413, 425—431
14 Borochov, A., Halevy, A.H. and Shinitzky, M. (1976) Nature 263, 158—159
15 Shattil, S.J. and Cooper, R.A. (1976) Biochemistry 15, 4832—4837
16 Shinitzky, M. and Inbar, M. (1976) Biochim. Biophys. Acta 433, 133—149
17 Dorfmann, L.M. and Wilzbach, K.E. (1959) J. Phys. Chem. 63, 799—801
18 Ozato, K., Ebert, J.D. and Adler, W.H. (1975) J. Immunol. 115, 339—344
19 Stambrook, P.J. and Sisken, J.E. (1972) J. Cell Biol. 52, 514—525
20 Huang, L. and Pagano, R.E. (1975) J. Cell Biol. 67, 38—48
21 Caro, L.G., van Tubergen, R.P. and Kolb, J.O. (1962) J. Cell Biol. 15, 173—188
22 Salpeter, M.M. and Bachman, L. (1972) in Principles and Techniques of Electron Microscopy (Hayat, M.A., ed.), Vol. 2, Ch. 6, Van Nostrand Reinhold Co., New York
23 Huang, C. and Charlton, J.P. (1972) Biochemistry 11, 735—740