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Increase of DPH fluorescence polarization during development of *Dictyostelium discoideum* cells

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Whole cells of the eukaryotic microorganism *Dictyostelium discoideum* have been assayed with membrane fluorescence polarization probes. With diphenylhexatriene (DPH) the steady-state fluorescence polarization remained stable during growth, but significant rise was found as a function of time of development. This variation, discussed as being specific to the plasma membrane, is the first report of a biophysical change occurring at this level during cellular aggregation of *D. discoideum*. In contrast, with trimethylammonium (TMA)-DPH no change was observed during development. The differences obtained with the two probes are discussed in the light of complementary fluorescence anisotropy decay measurements.

Fluorescence polarization; Diphenylhexatriene; Trimethylammonium diphenylhexatriene; Membrane fluidity; (*D. discoideum*)

1. INTRODUCTION

The eukaryotic cellular slime mold *Dictyostelium discoideum* has been widely studied [1,2]. Upon starvation, isolated growing cells enter into a developmental process, beginning with the aggregation of 10^4–10^5 cells and leading to an organized multicellular structure containing only two major differentiated cell species.

In other species the plasma membrane fluidity has been related to various biological processes, such as growth [3], differentiation [4], membrane receptor function [5] and enzyme activities [6]. For *D. discoideum*, the fluidity of the plasma membrane has already been studied, mainly by ESR [7–12]. One study was also performed by fluorescence polarization using DPH [10], the probe proposed by Shinitzky and Barenholz [13] to measure the microviscosity of the plasma membrane. The common feature of these studies was the failure to detect any major change in the membrane fluidity, either after incorporation of polyunsaturated fatty acids during growth, or as a function of time or temperature during development. This led to the general conclusion that, for this organism, the many known biochemical changes occurring at the plasma membrane level cannot be sensed by the bulk lipid fluidity measurements. However, experiments with plasma membranes, prepared during development in agitated suspensions, seemed to contradict the claimed invariability of the lipid fluidity during development (Tatischeff, I. et al., unpublished). Therefore, it was thought worthwhile to reconsider the question of an eventual variation of the membrane fluidity of *D. discoideum*, during both growth and differentiation steps.

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Abbreviations: DPH, diphenylhexatriene; TMA-DPH, trimethylammonium diphenylhexatriene

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Moreover, a new fluorescent probe TMA-DPH [14], a good candidate for monitoring the plasma membrane of whole cells [15,16], was compared to its parent molecule DPH. Fluorescence anisotropy decay measurements were used in addition to steady state fluorescence polarization measurements. Hence, more detailed information, regarding both fluidity and structural changes within the microenvironment of the probes accompanying growth and development events, could be ascertained. A decrease in membrane fluidity during development, unobserved when using ESR, was evidenced by DPH fluorescence polarization.

2. MATERIALS AND METHODS

2.1. *Cell growth and development conditions*

Cloned *D. discoideum* cells, strain AX2, were grown in HL5 medium [17] in rotatory agitated suspensions (175 rpm) at 22°C. To initiate the development, cells were harvested during the exponential phase of growth, centrifuged at 700 × *g* for 2 min, washed twice in 17 mM potassium phosphate buffer (pH 6.8) and resuspended, in the same buffer, at a final density of 10^8 cells/ml. The fluorescence polarization measurements were either performed immediately with these t_0 cells (0 h of development) or the cellular suspension was kept at 22°C and shaken at 175 rpm until the fluorescence measurements at time t_x (after x h of starvation-induced development).

2.2. *Fluorescence probe labelling of the cells*

The probes used were DPH (Fluka) and TMA-DPH (Molecular Probes). Stock solutions were kept at −20°C (2 mM DPH in tetrahydrofuran, 2 mM TMA-DPH in dimethyl formamide). For each probe, a fresh solution in potassium phosphate buffer (pH 6.8) was prepared daily, by mixing appropriate volumes of the stock solutions and phosphate buffer, followed by vigorous vortexing during 30 s. For incorporation of the probes, 5×10^6 cells/ml were incubated for 15 min at 22°C with 5×10^{-6} M (final concentration) of either DPH or TMA-DPH in a 10 × 10 mm quartz cell. This seemed optimal for a good signal to noise ratio in our experimental conditions. Some experiments were performed with 10^{-6} M probe and 10^6 cells/ml.

2.3. *Steady-state fluorescence polarization*

A home-built spectrofluorimeter [18] was coupled to a Hewlett-Packard microcomputer 9835A and transformed for the measurement of the steady-state fluorescence polarization. The excitation beam was set at 360 nm, depolarized by a quartz beam depolarizer from Lyot (Fichou, Fresnes, France) followed by a quartz beam-splitter. One beam was focused onto a quantum counter (3 g/l rhodamine B in methanol), the second one onto the sample through a vertically oriented sheet polarizer (HNP'B Polaroid). The fluorescence emission was analyzed at 90° through two polarizers (HNP'B Polaroid) parallel and perpendicular to the direction of polarization of the excitation beam, then through a monochromator set at 430 nm. A stepping motor alternatively positioned each polarizer and a photocounter (Ortec 9315) measured the parallel (I_v) and perpendicular (I_h) components of the fluorescence emission. The background contribution of the cells was deduced by measuring each component (B_v, B_h) with an unlabelled cell suspension, taken at the same time of development t_x. The polarization of the probe in the membrane was given by:

$$p = (C(I_v - B_v) - (I_h - B_h))/(C(I_v - B_v) + (I_h - B_h)), \quad (1)$$

and its anisotropy by:

$$r = (C(I_v - B_v) - (I_h - B_h))/(C(I_v - B_v) + 2(I_h - B_h)), \quad (2)$$

where C is an instrumental correction factor (I_h/I_v) measured with a solution of 10^{-6} M DPH in hexane. The experiments were performed at 22°C, controlled with a thermostat (Haake).

2.4. *Time-resolved fluorescence measurements*

Time-resolved measurements were performed with the apparatus and methods described in [19]. Fluorescence lifetimes were best described by a double exponential decay according to:

$$I(t) = A_1 \cdot \exp(-t/T_1) + A_2 \cdot \exp(-t/T_2), \quad (3)$$

where $I(t)$ is the decay of the total fluorescence as a function of time; T_1, T_2, the fluorescence lifetimes under double exponential approximation

and A_1, A_2 their respective decay amplitudes. The mean excited state lifetime was:

$$T = (A_1 \cdot T_1^2 + A_2 \cdot T_2^2)/(A_1 \cdot T_1 + A_2 \cdot T_2). \quad (4)$$

The anisotropy decay was deconvoluted either with a monoexponential or with a biexponential function. This allows determination of the rotational correlation time ϕ, and of the residual anisotropy r_∞. The steady-state anisotropy, r_{ss}, can be deduced from these measurements by integration of the $r(t)$ function. The difference $r_{ss} - r_\infty$ represents the dynamic contribution, r_f, from the fast decaying component [20]. The orientational constraint may be characterized by the order parameter [21]:

$$S = (r_\infty/r_0)^{1/2}, \quad (5)$$

the limiting anisotropy r_0 being taken to be 0.390 [14].

In the Kinosita hard cone model [22], the DPH probe is assumed to wobble in a cone of semi-angle θ which can be deduced from the relation:

$$S = 1/2 \cos\theta(1 + \cos\theta). \quad (6)$$

The cone angle characterizes the range of motion of the probe. The rotational correlation time ϕ, characterizes the rate of motion of the probe. This rate depends on the viscosity of the medium in which the probe is assumed to wobble.

3. RESULTS AND DISCUSSION

3.1. *Steady-state fluorescence polarization measurements during growth*

No clear influence of the phase of growth (exponential or stationary) on the polarization values was observed, either with DPH or with TMA-DPH, but the fluorescence polarization was lower with DPH (fig.1). This can be explained by a higher hindered motion in the membrane for the TMA-DPH probe, due to its cationic part interacting electrostatically with the polar head groups of the phospholipids, in the outer leaflet of the plasma membrane [14]. Thus, as confirmed by time-resolved fluorescence anisotropy, TMA-DPH reports a more rigid environment than DPH in the plasma membrane of whole cells.

Our results with both probes show that the membrane fluidity of *D. discoideum* is not dependent upon the phase of growth in axenic conditions.

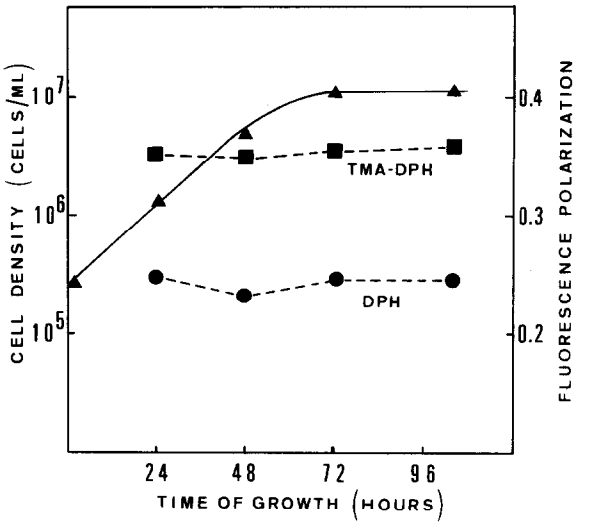

Fig.1. Influence of growth on the fluorescence polarization. The fluorescence polarization of DPH (●) or TMA-DPH (■) was compared with the growth curve (▲). Cellular density, 5×10^6 cells/ml; probe concentration, 5×10^{-6} M; excitation wavelength, 360 nm; emission wavelength, 430 nm; temperature, 22°C. The average accuracy of the polarization measurements was estimated as 5%.

3.2. *Steady-state fluorescence polarization measurements during development*

When the influence of the time of starvation during development was investigated, the results were different depending on the probe used. With DPH labelling, an increase of the fluorescence polarization was observed upon the time of starvation, whereas the TMA-DPH polarization remained constant. However, the extracellular medium itself, when incubated with DPH showed a significant level of highly polarized fluorescence. The influence of the extracellular medium was less noticeable with TMA-DPH, as the fluorescence intensity was very low compared to the one associated with the cells (not shown).

Checking also for a possible depolarization artefact [23–25], control experiments showed that there was no significant contribution to fluorescence depolarization due to light scattering.

In order to monitor only the fluorescence polarization associated with the cells, the experiments were performed after eliminating the extracellular medium, by a mild centrifugation just

before the incubation with the probes. With this procedure, the cells labelled with DPH showed three different levels of fluorescence polarization as function of time of development at 22°C: p = 0.250 for the first 8 h of starvation, p = 0.270 between t_9 and t_{15}, and p = 0.300 for longer time of starvation (fig.2). With TMA-DPH it was not possible to detect the changes observed with DPH.

There are, at least, three possibilities to explain these results. They deal with the different specificities of the probes, their different localizations in the plasma membrane, and their different structural and dynamic properties.

First, the changes recorded with DPH could arise from other cellular components than the plasma membrane [26,27]. However, purified plasma membrane of *D. discoideum* also revealed an increase of DPH fluorescence polarization after 8 h of starvation (Tatischeff, I. et al., unpublished), in agreement with the present data. Thus, the effect being specific to the plasma membrane the different localizations of the two probes inside this membrane [14] could explain the variations observed with DPH as occurring in the inner leaflet not probed by TMA-DPH. In fact, the changes evidenced with DPH are explained by the comparison of the time-resolved fluorescence measurements obtained with both probes.

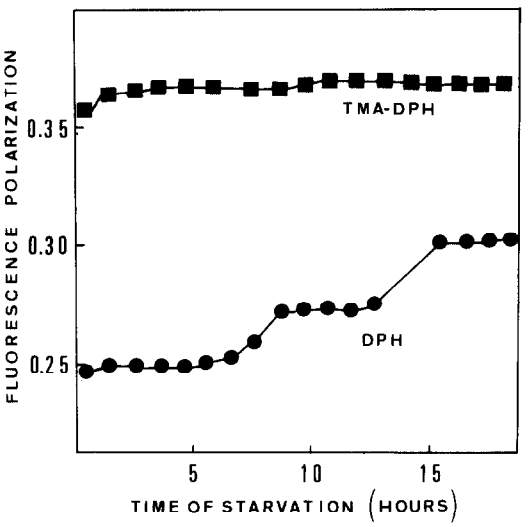

Fig.2. Influence of development on the fluorescence polarization. The fluorescence polarization of DPH (●) or TMA-DPH (■) was measured as a function of time of starvation in agitated suspension (experimental conditions as in fig.1).

3.3. *Time-resolved fluorescence anisotropy*

As DPH probes the structural order of the lipids more than their real fluidity [28–30], it is important to check the origin of the observed increase in steady-state fluorescence polarization by complementary time-resolved fluorescence polarization measurements.

In table 1, the values obtained from time-resolved measurements at early and late stages of starvation-induced development are reported for cells labelled with either DPH or TMA-DPH. It is worthy of note that the absolute values of fluorescence polarization were always higher (≃ 10%) as deduced from time-resolved measurements than with the steady-state measurements. This is attributed to apparatus limitations, leading to different deviations from the theoretical value p = 1 for a diffusing solution (1 mg/ml glycogen) with emission and excitation wavelengths set at 430 nm. The measured values were p = 0.99 for the decay apparatus and p = 0.96 for the steady-state apparatus. Nevertheless, rather than the absolute values, it is more interesting to compare the relative contributions of r_∞ versus r_{ss} for the two probes, together with their changes during starvation.

The average fluorescence lifetimes were about 8 ns for DPH, and 6.5 ns for TMA-DPH, in the range of previously published values [14,31]. For both probes, a residual value of the anisotropy decay (r_∞) was found. In the case of TMA-DPH, r_∞ was very important compared to the steady-state polarization (r_{ss}).

For the DPH labelled cells, the steady-state anisotropy change between the early and late stages of starvation is mainly accompanied by an increase of the rotational correlation time (ϕ), the residual anisotropy being relatively constant. So, the observed increase of fluorescence polarization is really correlated with a decrease in plasma membrane fluidity and not with a structural change. In contrast, for cells labelled with TMA-DPH, the fast decaying component was negligible compared to the residual anisotropy, therefore the apparent rotational correlation time could not be obtained with accuracy. The values obtained for the order parameter (S) and the semi-cone angle (θ), showed

Table 1

Measured values of fluorescence and anisotropy decay parameters for DPH and TMA-DPH, at early and late stages of development

Probe	t_x (h)	p_{ss}	r_{ss}	r_∞	ϕ (ns)	T (ns)	S	θ (°)
DPH	6.5	0.269	0.197	0.171	2.96	8.34	0.66	41.00
	17	0.303	0.225	0.174	3.75	8.04	0.67	40.60
TMA-DPH	5	0.392	0.301	0.293	–	6.40	0.87	24.72
	17	0.393	0.302	0.294	–	6.60	0.87	24.57

t_x, time of starvation-induced development; p_{ss}, steady-state fluorescence polarization; r_{ss}, steady-state fluorescence anisotropy; r_∞, limiting anisotropy; ϕ, rotational correlation time; T, mean-average fluorescence lifetime (eqn 4); S, order parameter (eqn 5); θ, semi-cone angle (eqn 6). Experiments were performed by using 10^6 cells/ml and a probe concentration of 10^{-6} M, at 22°C

that TMA-DPH displays strong structural constraints and that its range of motion in the membrane is very limited compared to its parent molecule DPH (about half as much).

In conclusion, it can be stressed that, despite the important biochemical changes known to occur at the plasma membrane level during acquisition of aggregation-competence, the biophysical parameter of membrane fluidity remains remarkably constant over 8 h, in agreement with previous works using ESR [7,8]. However, a subsequent decrease in membrane fluidity was clearly evidenced with DPH. Perhaps, this could be correlated with a decreased lateral mobility of Con-A receptors in the plasma membrane of *D. discoideum* during development [8]. The ESR probes seem in appropriate to detect such a dynamic change in membrane fluidity. Alternatively, this change could be specific for post-aggregative development in suspension and, therefore, is not observed for development on a solid substratum [7,8]. Finally, our work shows that the inability of the TMA-DPH probe to detect this change is linked to its low sensitivity to variations in the dynamic component of the fluorescence polarization. Thus, DPH, despite its known limitations, has some advantage over TMA-DPH for monitoring plasma membrane fluidity variations during biological processes.

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