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Developmental changes in the lateral diffusion of Leydig cell membranes measured by the FRAP method

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A simple method for isolation and fluorescence labelling of Leydig cells (L-cells) from rat testes was developed. Lateral diffusion coefficients of both lipid and protein membrane fluorescent probes were measured by the method of fluorescence recovery after photobleaching (FRAP). Age-dependent changes in diffusibility of membrane lipids and proteins were discovered.

FRAP; Fluorescent probe; Lateral diffusion; Age dependence; (L-cell)

1. INTRODUCTION

The physical properties of membranes strongly influence the functional activity of membrane-bound enzymes, regulatory proteins and hormonal receptors. Characteristic parameters of the mobility of membrane constituents are the lateral diffusion coefficients D_l. Our previous experiments with the fluorescent probes pyrene and 1,6-diphenyl-1,3,5-hexatriene (DPH) have shown that the fluidity of rat testis membranes decreases during aging (unpublished).

Alterations of the lateral diffusion coefficients D_l of the lipid and protein part of (L-cell) membranes were studied here using the much more sensitive method of fluorescence recovery after photobleaching (FRAP). Fluorescent analogues of lipids, fluorescent labels attached to amino and sulfhydryl groups of proteins, and a fluorescent labelled lectin were used as fluorescent markers.

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2. MATERIALS AND METHODS

2.1. *Fluorescent probes and reagents*

5-(*N*-Octadecanoyl)aminofluorescein (AF18) and 1',1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI18) were purchased from Molecular Probes (USA), fluorescein-succinylated concanavalin A (fConA) from Vector Lab. (USA) and eosin 5-maleimide (EM) from Polyscience (USA). Fluorescein isothiocyanate, isomer I (FITC) and bovine serum albumin (BSA) were obtained from Sigma (USA), dimethylformamide (DMF) and Eagle's medium, Glasgow modification, from BDH (England) and collagenase from *Clostridium histolyticum* from Boehringer (FRG). All other chemicals and reagents were of analytical grade and solutions were prepared with distilled water.

2.2. *Isolation of L-cells*

Male Wistar strain rats were used. They were distributed into four age groups, 1.25, 2.5, 8 and 15 months of age, 3 rats per group. L-cell isolation was carried out after collagenase dispersion of the testis tissue. The cell suspension obtained was

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filtered through 60 μm mesh nylon gauze, centrifuged for 2 min at 200 × g and the cells washed 3 times in fresh Eagle's medium by centrifugation for 4 min at 600 × g. Finally, the pellet containing L-cells, Sertoli cells and a small quantity of spermatozoa was resuspended in Eagle's medium containing 1% BSA and 1% glucose. Aliquots were treated with 0.5% trypan blue for light microscopy.

2.3. *Fluorescence labelling of L-cells: Lipid probes*

0.3 ml cell suspension containing over 10^8 cells were incubated at room temperature for 10 min with AF18 or DiI18, dissolved previously in DMF. The final concentrations of dyes were 0.033 mg/ml and the DMF/cell suspension ratio (v/v) was less than 0.03. The unbound fluorescent probe was removed by washing the cells 3 times with fresh Eagle's medium and by centrifugation for 3 min at 800 × g. Finally, the labelled cells were resuspended in Eagle's medium containing 1% BSA and 1% glucose. In this medium at 20°C the cells remain vital for more than 5 h.

2.4. *Fluorescence labelling of L-cells: Protein probes*

The procedure for fluorescence labelling of cells with FITC, EM and fConA was the same, but the incubation times were 30 min and the final concentrations of the dyes were 0.066 mg/ml.

FITC-labelled BSA in 95% glycerol was used as a model system for measuring D_l by the FRAP method. Fluorescence labelling of BSA with FITC was carried out according to Nairn [1].

2.5. *Measurements of* D_l

These measurements were performed in the Laboratory of P.B.G. (University of Dundee) using the instrument described by Garland [2] and Johnson and Garland [3] employing the 514.5 nm argon laser line, fluorescence microscope with a 40 × water immersion objective and focussed on the cell surface laser spot with radius 1.1 μm. Single fluorescence-labelled L-cells were observed. The coefficients D_l were calculated by non-linear least-squares routine fitting of the experimental data to the theoretical equation described by Axelrod et al. [4] for the case of a Gaussian transverse energy mode of the laser beam.

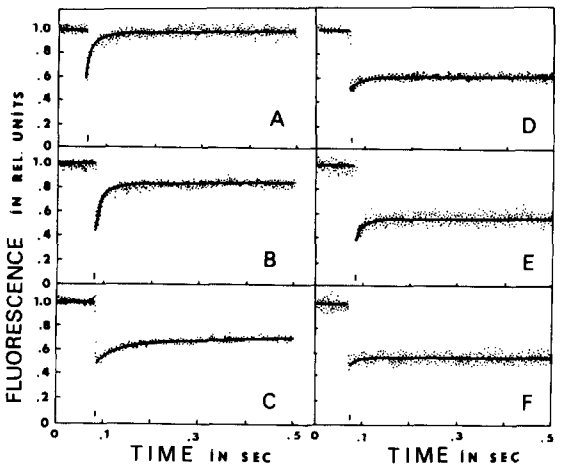

Fig.1. Fluorescence recovery after photobleaching (FRAP) at 20°C in: (A) model system, FITC-labelled BSA in 95% glycerol, and L-cells labelled with different fluorescent probes, (B) AF18, (C) DiI18, (D) FITC, (E) EM, (F) fConA. Fluorescence intensity is shown in arbitrary units, normalized to 1.0 for the mean prebleach level. Time is expressed as channels (or addresses) in the digital storage system, each channel corresponding to 0.125 s.

3. RESULTS

The cell suspension containing interstitial cells, obtained as described in section 2, consists of 3 major populations, strongly different morphologically: L-cells, being largest, with a diameter of about 10 μm, Sertoli cells of diameter 2–5 μm and spermatozoa. In the light microscopy mode it is easy to select only L-cells for measurements. Moreover, the living cells are too different in their fluorescence characteristics from the dead ones and we can investigate the vital cells only. All the probes used, incorporated into the cell membrane, gave a strong fluorescence signal. The background fluorescence was 1–2% of the signal of the cells, excluding the case of DiI18 where the background was about 10%. Comparing the fluorescence from the membrane and the cell interior, it was found that the probes were situated in the cell membrane, excluding DiI18 which slightly penetrates through the membrane and gave a cytoplasmic signal less than 20% from the intensity of the membrane signal. None of the other probes gave a significant cytoplasmic signal.

Fig.1 shows typical FRAP curves at 20°C for L-

Table 1

Diffusion characteristics of different fluorescent probes in L-cell membranes

Fluorescent probe	Labelled membrane component	R (%)	D_l ($\times 10^{-10}$ cm^2/s)	n
AF18	lipids	54.96 ± 6.64	16.09 ± 5.90	5
DiI18	lipids	64.88 ± 20.50	3.41 ± 5.90	4
FITC	proteins	38.70 ± 18.90	6.27 ± 2.30	4
EM	proteins	36.78 ± 11.50	6.29 ± 1.94	4
fConA	lectin receptors	23.84 ± 4.30	3.20 ± 1.80	4
FITC	model system BSA in 95% glycerol	96.37 ± 2.80	50.66 ± 3.00	5

R, recovery of fluorescence after photobleaching; D_l, lateral diffusion coefficient; n, number of cells observed. The cells were isolated from 2.5-month old rats. Data are means ± SE

cells isolated from 2.5-month-old rats. The FRAP curve for the model system FITC-labelled BSA in 95% glycerol is presented for comparison. Data for D_l and recovery are given in table 1. The highest is D_l for the model system and for the lipid probe AF18 and lowest for the surface lectin receptors. In table 2 are shown the data for all markers and ages under investigation. Using the Student's test demonstrates a decrease in D_l with age for the lipid label AF18, but at a level of significance of $p < 0.2$ only. No significant changes in D_l were observed for the lipid label DiI18. More significant

Table 2

Diffusion characteristics of different fluorescent probes in L-cell membranes, isolated from rats of different ages

Age (months)	Fluorescent probe	Labelled membrane component	R (%)	D_l ($\times 10^{-10}$ cm^2/s)	n
1.25	AF18	lipids	60.98 ± 6.1	53.00 ± 27.92	4
2.5	AF18	lipids	54.96 ± 6.6	16.09 ± 27.91	5
8	AF18	lipids	77.33 ± 9.0	30.18 ± 11.00	9
15	AF18	lipids	77.60 ± 0.6	23.19 ± 4.20	4
2.5	DiI18	lipids	64.88 ± 20.05	3.49 ± 2.69	4
15	DiI18	lipids	43.10 ± 2.60	4.45 ± 0.98	4
1.25	FITC	proteins	35.47 ± 3.4	11.42 ± 4.3	4
2.5	FITC	proteins	38.70 ± 18.9	6.27 ± 2.3	4
8	EM	proteins	29.10 ± 9.1	31.43 ± 9.7	4
15	fConA	lectin receptors	20.00 ± 3.0	62.50 ± 9.0	5

R, recovery of fluorescence after photobleaching; D_l, lateral diffusion coefficient; n, number of cells observed. Data are means ± SE

were the age-induced alterations in mobility of the membrane proteins. D_l increases in this case significantly at $p < 0.1$. Comparison of protein D_l values for 1.25–15 and 2.5–15 months shows significant increase with age at $p < 0.05$.

4. DISCUSSION

The method used for isolation of L-cells was useful, rapid and easy and gave us the opportunity of simultaneously investigating L-cells, Sertoli cells and spermatozoa.

L-cells are a target for gonadotropin hormones and contain membrane-bound gonadotropin receptors and a hormone-sensitive adenylate cyclase system [5,6]. These cells synthesize important steroid hormones, such as testosterone. Our investigations were focussed on these cells because alterations in the physical properties of L-cell membranes during ageing are quite important for translation of the hormonal signals to and from these cells.

Experiments with pyrene and DPH as fluorescent probes have shown an enhanced membranous 'rigidity' with ageing (unpublished). A slight fall was observed in the present work as well for the lipid mobility in L-cell membranes.

The developmental changes in membrane composition [7] lead to changes with age in the membrane proteins as well, but surprisingly their mobility was enhanced during the course of ageing. One possible explanation of this phenomenon might be the change in fractal properties of the molecular trajectories in the fluid membrane [8,9]. Protein molecules are much larger than lipid molecules and their lateral movement through the fractal microruggedness on the membrane surface should be quite different from the Brownian movement. In terms of the fractal theory our results can indicate an increased microruggedness of the L-cell membrane with ageing.

An additional factor for explanation of this phenomenon might be the altered interactions between membrane proteins and the cytoskeleton during development and ageing. A decrease in the forces connecting the membrane to the cytoskeleton will lead to an increase in number of mobile proteins and subsequently to enhancement of their lateral mobility [10,11].

Reported studies in this area are quite few and it is difficult to generalize on the factors influencing the diffusion of membrane components. The developmental changes that we find in D_l of lipids and proteins in L-cell membranes are undoubtedly related to the previously observed developmental changes in functional properties of membrane-bound enzymes [12] and gonadotropin receptors [13] as well as to the composition [7] of rat testis membranes. Investigations of these effects are important for understanding the phenomena observed during development and ageing in cellular membranes.

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