DRUG INDUCED MEMBRANE EFFECTS IN METABOLICALLY IMPAIRED AND NONIMPAIRED HUMAN T (JURKAT) LYMPHOBLASTOID CELLS

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Abstract. Steady-state fluorescence polarization measurements showed that N,N-dimethylaminoethyl hydrochloride esters of phthaloyl-glycine (Pht-Gly) and phthaloyl-leucine (Pht-Leu) induce increase in the lipid order parameter and membrane rigidization, while quaternary ammonium salts of Pht-Gly and Pht-Leu cause diminution of lipid packing density in the polar head-group region and produce fluidization of the human leukemic T cells plasma membrane inner surface. Progressive metabolic impairment of cells leads to changes in plasma membrane properties, modulation, and finally loss of membrane effects of these antibacterial agents.

Key words: N-phthaloyl-aminoacid derivatives, serum starvation, plasma membrane fluidity, lipid order parameter, steady-state fluorescence anisotropy, TMA-DPH.

INTRODUCTION

Plasma membranes of cells are constituted from lipid and protein molecules exhibiting a certain degree of static order and a variety of motional dynamics. Both order and dynamics of membrane components are measurable by spectroscopic techniques [1–4, 6]. Using appropriate lipophilic probes and monitoring changes in their steady state fluorescence anisotropy changes occurring in lipid dynamics and order in various regions of the membrane bilayer, can easily be detected. The technique allows to follow up membrane effects of biologically active chemicals, as well as the influence of cells metabolic state leading to modulation of these effects.

Newly synthesized N,N-dimethylaminoethyl esters of some phthaloyl-aminoacids and their quaternary ammonium salts were described as potent antimicrobial agents having negligible effects on cholesterol containing artificial lipid membranes permeability [5].

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Using the human leukemic T cell line, Jurkat, and the lipid probe TMA-DPH, selected to monitor fluidity/lipid packing density in the polar head-group regions of the plasma membrane lipid bilayer, the present paper reports data revealing modulation by the cells actual metabolic state of the membrane effects induced by these bacteria killing drugs.

MATERIALS AND METHODS

MATERIALS

The fluorescent lipid probe 1[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) was from Molecular Probes, Hepes buffer substance from Sigma Chemical Co., while all other chemicals were the best research grade available. Phtaloyl derivatives of aminoacids: Pht-Leu-OH (1), Pht-Leu-$OCH_2CH_2Me_2$.HCl (2), Pht-Leu-$OCH_2CH_2N^+Me_3I^-$ (3), Pht-Gly-$OCH_2CH_2NMe_2$.HCl (4), Pht-Gly-$OCH_2CH_2N^+Me_3I^-$ (5) were synthetized in the Laboratory of Organic Chemistry of Bucharest University [5].

Chemicals were controlled as not to interfere with the spectroscopic properties of the fluorescence probe used.

CELLS

Human T lymphoblasts, Jurkat, were cultured in RPMI 1640 medium supplemented with 10% FCS and antibiotics in a 5% CO_2 incubator at 37 °C and 80% humidity. For fluorescence anisotropy measurements cells were centrifuged at 100g for 5 min and resuspended in Hepes buffer HPMI (100 mM NaCl, 5.4 mM KCl, 0.4 mM $MgCl_2$, 0.04 $CaCl_2$, 10 mM Hepes, 20 mM glucose, 24 mM $NaHCO_3$ and 5 mM Na_2HPO_4) at pH 7.4. Cell suspensions with viability levels assessed by Trypan blue exclusion, higher than 95% were only used.

METABOLIC IMPAIRMENT

Metabolic impairment of cells was obtained by serum starvation at temperatures below 37˚C. Jurkat cells were kept various time periods (1– 48 hours) at room temperature (~ 25°C) or at 4°C, without any nutritional supplement (serum, aminoacids or vitamins) in the glucose-containing Hepes buffer HPMI. We consider metabolically non-impaired, samples constituted of suspensions of cells freshly transferred in the serum-free buffer.

SAMPLES

Samples were constituted by continuously stirred, thermostatted human T lymphoblastoid cell (Jurkat) suspensions of cell densities of $2 \cdot 10^6$ cells/ml, prepared in HPMI buffer.

LABELING OF CELLS WITH FLUORESCENT LIPID PROBES

$5 \cdot 10^{-4}$ M stock solution of TMA-DPH prepared in dimethylformamide, was kept at –20 °C until use within a month. Human T cells (Jurkat) were labeled with TMA-DPH by incubating them in the measuring cuvette with 1.25 μM dye at 37 °C for 3 min at a cell density of $2 \cdot 10^6$ cells/ml.

FLUORESCENCE ANISOTROPY MEASUREMENTS

Steady state fluorescence and anisotropy measurements were carried out in a Jobin-Yvon SpectroFluo JY3 spectrofluorimeter, equipped with thermostatted cell holders, stirring devices, Polaroid HN polarizers and connected to IBM PC computers using a dedicated data acquisition software: EASYEST. The excitation and emission wavelengths were selected at 340 nm and 425 nm, respectively. Steady state fluorescence anisotropy values (r) were obtained by quasi-simultaneous (within 12 s) measurements of the intensity components I_{VV} and I_{VH}, where VV and VH stand for vertical/vertical (parallel) and vertical/horizontal (perpendicular) positions of the excitation and emission polarizers respectively. A correction factor $G = \frac{I_{HV}}{I_{HH}}$ for unequal transmission by the optical elements of the vertically and horizontally polarized intensity components also was determined and thus the steady-state fluorescence anisotropy values were calculated every 20– 25 s according to the formula:

$$r = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2 \cdot G \cdot I_{VH}} \tag{1}$$

The small autofluorescence of cells and the unavoidable scattered light contribution, determined by measuring unlabeled controls under the same experimental conditions as samples, were substracted from each intensity component.

The lipid order parameter in the polar head-group region of the plasma membrane bilayer, S, was computed as function of limiting initial r_0 and long-time r_∞ values of the fluorescence anisotropy:

$$S = \sqrt{\frac{r_\infty}{r_0}} \tag{2}$$

where $r_0 = 0.362$, while the limiting long-time fluorescence anisotropy values r_∞ were approximated using the empirical curve obtained by van Blitterswijk *et al*. [6] as:

$$r_\infty = 1.270 \cdot r - 0.076 \quad \text{for} \quad 0.13 < r < 0.28 \tag{3}$$

$$r_\infty = 1.100 \cdot \mathrm{r} - 0.032 \quad \text{for} \quad 0.28 < r < 0.34 \tag{4}$$

where r represents the TMA-DPH fluorescence anisotropies, calculated from measured fluorescence intensities according to the formula (1).

Membrane fluidity, f, was expressed as a reciprocal of the lipid order parameter.

The energetic parameter, characteristic of the degree of order in the lipid head-group region of plasma membrane bilayer, was calculated according to a formula analogous with that given by Shinitzky [3]:

$$E_\mathrm{a} = \frac{R \cdot T_1 \cdot T_2}{T_2 - T_1} \cdot \ln \frac{S_{T_1}}{S_{T_2}} \tag{5}$$

TREATMENT OF SAMPLES WITH BIOLOGICALLY ACTIVE COMPOUNDS

Exposure of cells to the action of the studied chemicals was realized by mixing dense cell suspensions with comparable volumes of the compounds concentrated solutions. Final concentration of the active substances in cell suspensions of $1–2 \cdot 10^6$ cells/ml was 1%.

STATISTICAL ANALYSIS

Anisotropy, lipid order parameter and membrane fluidity values and changes occurred in these parameters under the influence of laser irradiation are presented as $\mathrm{mean} \pm \mathrm{S.D.}$ calculated from at least three independent measurements. Unpaired analysis of data series obtained by measurements made on drug treated and control cells, was performed by Student's *t*-test. Significance was accepted at $p < 0.05$.

RESULTS AND DISCUSSION

At 37 °C the positively charged lipid probe, TMA-DPH, reported in the head-group region of the plasma membrane lipid bilayer of Jurkat cells an average fluorescence anisotropy of 0.25 ± 0.01. The corresponding values of the second rank order parameter and fluidity of membrane lipids were 0.81±0.02 and 1.24±0.03

respectively. The values of the same parameters at 25°C were 0.28±0.01, 0.87±0.01 and 1.15±0.02, respectively.

Under the influence of the different newly synthetized drugs the steady-state fluorescence anisotropy of TMA-DPH incorporated in human T lymphoblasts (Jurkat) plasma membrane appeared altered in different manner and to a different extent (Fig. 1 and Table 1).

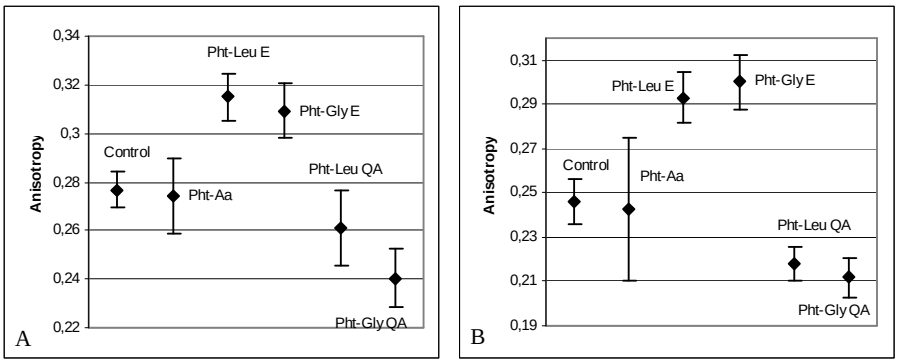

Fig.1. TMA-DPH fluorescence anisotropy (r) data, demonstrating significant drug membrane effects in human T cells, Jurkat, at both room temperature, 25 °C (A) and 37 °C (B).

The inactive source substance – the phtaloyl aminoaminoacid (Pht-Aa) – had no measurable membrane effect at any of these temperatures.

Table 1

TMA-DPH fluorescence anisotropy (*r*) data reporting drug effects at different temperatures on membrane lipid order (*S*) and fluidity (*f*) in the lipid head-group region of plasma membrane of metabolically intact human T cells

		Control	Pht-Aa	Pht-Leu E	Pht-Gly E	Pht-Leu QA	Pht-Gly QA
25 °C	*r*	0.277±0.007	0.274±0.016	0.315±0.010	0.309±0.011	0.261±0.016	0.240±0.012
	S	0.870±0.011	0.864±0.028	0.932±0.016	0.923±0.018	0.840±0.033	0.795±0.026
	f	1.150±0.015	1.158±0.038	1.073±0.018	1.084±0.021	1.193±0.049	1.258±0.040
37 °C	*r*	0.246±0.010	0.243±0.032	0.293±0.012	0.300±0.012	0.218±0.008	0.212±0.009
	S	0.808±0.022	0.798±0.076	0.898±0.018	0.908±0.021	0.745±0.018	0.730±0.022
	f	1.238±0.033	1.262±0.124	1.114±0.023	1.102±0.025	1.342±0.033	1.371±0.042

The statistical significance of induced changes was higher than that corresponding to $p < 0.05$ in cases of all bactericid phtaloyl-aminoacid derivatives at both temperatures. At 37 °C the statistical significance of the observed differences was higher than that corresponding to $p < 0.0005$ in case of all studied

drugs: dimethylaminoethyl hydrochloride esters or quaternary ammonium salts of phtaloyl aminoacids.

The energetic characteristic of lipid order in the head-group region of plasma membrane bilayer of Jurkat cells of around 5 kJ/mol was not modified by phtaloyl aminoacids, but was slightly increased by their hydrochloride esters and drastically reduced by quaternary ammonium salts of these compounds.

Metabolic impairment of Jurkat cells caused gradual decrease of lipid order parameter and increase of membrane fluidity in the head-group region of bilayer. In case of mild metabolic impairment caused by 4h serum starvation (membrane depolarization less than 25%, viability checked by Trypan blue exclusion, higher than 92%, data not shown) the differences observed in membrane fluidity parameters were already significant ($p < 0.05$). 24h hour starvation caused further decrease in the lipid order parameter in the head-group region of the plasma membrane bilayer, the statistical significance of differences became higher than that corresponding to $p < 0.0005$, though the changes were yet not very dramatical.

In contrast membrane effects of the studied bactericide drugs were dramatically changed (Fig. 2, Table 2).

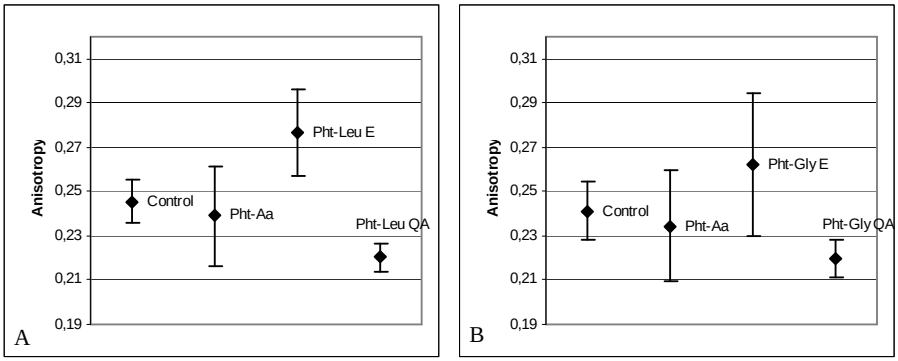

Fig. 2. TMA-DPH fluorescence anisotropy (r) data, demonstrating decrease and loss of significance of drug membrane effects in human T cells (Jurkat) exposed to various periods (4h – A; 24h – B) of previous serum starvation, as compared to metabolically intact cells.

Both extent and statistical significance of drug induced membrane effects were substantially decreased due to metabolic impairment of cells. After 4h serum starvation the effects of bactericide derivatives of phtaloyl-leucine were reduced to half, as compared to those observed in metabolically non-impaired cells (from ~19% increase of lipid order parameter to only ~9% in case of dimethylaminoethyl ester, Pht-Leu-E, and from ~11% to only ~7% decrease in lipid order in the head-group region of plasma membrane bilayer in case of quaternary ammonium salt of phtaloyl leucine, Pht-Leu-QA), while the statistical significance of effects observed

was one order of magnitude lower than that observed in metabolically non-impaired cells ($p < 0.005$ instead of $p < 0.0005$).

Table 2

TMA-DPH fluorescence anisotropy (r) data reporting drug effects on membrane lipid order (S) and fluidity (f) in the lipid head-group region of plasma membrane of metabolically impaired and non-impaired human T cells. (non-imp. – metabolically non-imapired; 4h, 24h – metabolically impaired due to serum starvation of 4h and 24h, respectively; n.d. – not determined)

		Control	Pht-Aa	Pht-Leu E	Pht-Gly E	Pht-Leu QA	Pht-Gly QA
Non-imp.	r	0.246±0.009	0.245±0.030	0.294±0.011	0.301±0.012	0.218±0.008	0.211±0.008
	S	0.808±0.020	0.803±0.068	0.898±0.018	0.909±0.018	0.746±0.019	0.727±0.018
	f	1.239±0.031	1.252±0.112	1.114±0.022	1.101±0.022	1.342±0.034	1.375±0.035
4 h	r	0.239±0.003	0.236±0.016	0.260±0.011	n.d.	0.223±0.002	n.d.
	S	0.794±0.007	0.786±0.038	0.838±0.019	n.d.	0.757±0.008	n.d.
	f	1.260±0.011	1.274±0.064	1.193±0.027	n.d.	1.322±0.013	n.d.
24 h	r	0.225±0.011	0.224±0.005	n.d.	0.240±0.022	n.d.	0.221±0.010
	S	0.760±0.025	0.758±0.012	n.d.	0.794±0.048	n.d.	0.753±0.023
	f	1.317±0.043	1.319±0.021	n.d.	1.264±0.078	n.d.	1.330±0.041

24h of serum starvation caused loss of the significant membrane effects observed in metabolically intact cells (Fig. 2 and Table 2). Though viability of cells was still high (> 85% of cells were yet Trypan excluding), the slight modifications of membrane lipid order parameter and fluidity in the head-group region of human T cells plasma membrane bilayer, induced by both dimethylaminoethyl hydrochloride ester and quaternary ammonium salt of phtaloyl glycine (less than 5% increase in the lipid order parameter, induced by Pht-Gly-E, and less than 1% decrease induced by Pht-Gly-QA), were not statistically significant ($p > 0.1$).

CONCLUSION

Steady-state fluorescence polarization measurements and data analysis show that, inactive phtaloyl-aminoacids (PHT-Aa) have no measurable membrane effect in either metabolically impaired or non-impaired human T cells. Dimethylaminoethyl hydrochloride esters of phthaloyl-aminoacids (Pht-Gly-E and Pht-Leu-E) induce increase in the lipid order parameter and membrane rigidization, while quaternary ammonium salts of these phtaloyl-aminoacids (Pht-Gly-QA and Pht-Leu-QA) cause diminution of the lipid packing density and order in the head-group region of the bilayer and produce fluidization of the metabolically intact human T cells (Jurkat) plasma membrane. Mild metabolic impairment of cells leads to diminution of phtaloyl-aminoacid derivatives membrane effects, while overnight serum starvation causes loss of statistically significant sensitivity to phtaloyl-aminoacid derived bactericide drugs of human T cells (Jurkat) plasma

membrane. In consequence, we suggest that, care has to be taken in drawing the conclusion of insensitivity to certain drugs of human cells plasma membrane on basis of experimental tests made on artificial membranes of similar composition.

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