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SINGLE AND MULTIPLE DESIPRAMINE EXPOSURES OF CULTURED CELLS

CHANGES IN CELLULAR ANISOTROPY AND IN LIPID COMPOSITION OF WHOLE CELLS AND OF PLASMA MEMBRANES

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Abstract—Effects of the antidepressant drug desipramine (DMI) on fluorescence anisotropy were studied in living cultured human fibroblasts, rat brain astrocytes and rat ROC-1 hybridoma cells (oligodendrocytes × C6). Fluorescence anisotropy, a measure for fluidity, was measured by means of a fluorescence polarization technique using a set of *n*-(9-anthroyloxy) fatty acids as markers. Apparent fluorescence anisotropies were determined in cells following single or multiple dose exposures to 5 μM DMI at 37° and compared to control cells. In all three cell types single doses of DMI led to significant decreases in anisotropies of the deeper layers (12-AS) of the membranes only, suggesting increases in fluidity. Repeated exposures to 5 μM DMI led to cell specific, significant changes in anisotropies of the superficial membrane layers, as determined by 2-AP, 6-, 7- and 9-AS. The resulting anisotropy values of the three different cell types became more alike than prior to DMI exposure. Alterations in anisotropies were accompanied with changes in the phospholipid patterns of whole cells and isolated plasma membrane vesicles. The changes of PC/PE ratios were consistent with changes observed in fluorescence anisotropies. Such alterations may be individual regulatory responses of the cells to the chronic presence of the drug within the membranes.

Desipramine (DMI), a cationic amphiphilic antidepressant drug, produces profound changes in cellular phospholipid metabolism when repeatedly administered to cultured cells in low micromolar concentrations for 1 week [1, 2]. Following chronic exposure to DMI and to physicochemically similar other drugs microscopically visible lamellar bodies are formed in a variety of tissues of man and animals but also in cultured cells. These organelles have been identified as lysosomes containing excess membrane phospholipids [1, 3]. Drug induced phospholipid storage is a result of impaired lysosomal degradation [2, 4]. Although such an effect has been observed with different cationic amphiphilic compounds [5] the accumulation of individual phospholipids seems to be qualitatively and quantitatively different for individual drugs [unpublished results].

In addition, chronic exposure to DMI led to a reduction in β-adrenoceptor binding in different cultured human cells [6] similar to that observed in rat brain [7]. Neurotransmitter receptor binding characteristics may be modulated secondary to changes in membrane fluidity [8]. Membrane fluidity can be determined as fluorescence anisotropy by a fluorescence polarization method using fluorescent markers. In this paper we used a set of *n*-(9-anthroyloxy) fatty acids to investigate the effects of DMI on fluorescence anisotropy. With these fluorescent analogues of fatty acids an anisotropy profile could be measured across the membrane [9–11]. Cultured cells were exposed to a single or to a repetitive dose of 5 μM DMI over several days, each time exchanging the drug containing culture medium. The results of fluorescence anisotropy measurements on intact living cells were related to drug induced changes in the phospholipid composition of whole cells and of purified plasma membrane preparations.

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MATERIALS AND METHODS

Tissue culture. Skin biopsies for fibroblast cultures were obtained after informed consent from healthy persons at the occasion of minor surgery. Fibroblasts were grown from the biopsies and cultured as previously described [12].

Rat brain astrocyte cultures were obtained after decapitation of new-born rats by mechanical suspension of the cleaned whole brain tissue in Dulbecco's minimal essential medium (MEM) supplemented with 10% of fetal calf serum [13]. The medium was buffered at pH 7.4 with bicarbonate and 5% CO_2 in air and contained 50 units of penicillin G per mL (Gist/Brokades, Delft, The Netherlands). The cells were seeded in petri dishes. Subculturing was done by trypsinization with a split of 1:4. Astrocytic cells were used between second and sixth subculture.

ROC-1 cells are hybridoma clones between C6 rat glioblastoma cells and rat oligodendrocytes kindly provided by Dr F. A. McMorris (Wistar Institute,

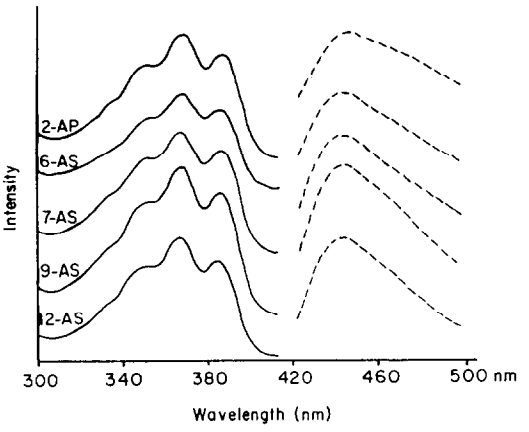

Fig. 1. Excitation (——) and emission (– – – –) spectra of 0.5 μM 2-AP and 6-, 7-, 9- and 12-AS in a suspension of trypsinized Roc-1 cells at an optical density of 0.220 (absorption at 385 nm) in Hank's solution, pH 7.4, after 20 min of preincubation.

Philadelphia, U.S.A.) and were used as a model for oligodendrocytic cells. Growing and subculturing was done as described for rat brain astrocytes.

Fluorescence polarization measurements. Cells were prepared for polarization measurements by washing the cultures with Eagle's MEM and twice with Hank's balanced salt solution, pH 7.4, followed by trypsinization (5 min at 37°) and centrifugation (5 min, 500 *g*). Cells were resuspended in Hank's balanced salt solution, pH 7.4, and the suspension was diluted to a final optical density of 0.220, as measured in a Beckmann DB-GD spectrophotometer at 385 nm [14].

Depth dependent fluorescence anisotropies were determined with a Shimadzu RF-540 spectrofluorophotometer (L-format), equipped with a constant temperature four cell holder and two automatically rotating polarizers, which avoided the necessity to open the sample compartment. Fluorescence intensities at four positions of the polarizers were determined and anisotropy $r(G)$ was calculated as follows:

$$r(G) = (Ivv - G \times Ivh)/(Ivv + 2 \times G \times Ivh)$$

$$G = Ihv/Ihh$$

where $r(G)$: fluorescence anisotropy; G: correction factor for the optical system; $Ivv(vh)$: vertical excitation, vertical (horizontal) emission polarizer; $Ihh(hv)$: horizontal excitation, horizontal (vertical) emission polarizer; excitation: 385 nm; emission: 450 nm.

Excitation and emission spectra of 2-anthroyloxy palmitic (AP), 6-, 7-, 9- and 12-anthroyloxy stearic acid (AS) are shown in Fig. 1.

Plasma membrane isolation by vesiculation. Cell cultures were incubated with 25 mM formaldehyde and 2 mM dithiothreitol in Ca^{2+} and Mg^{2+} containing phosphate buffered saline at 37° for 90 min [15]. The formed plasma membrane vesicles (PMV) were washed away from the cells with isotonic Tris–HCl buffer (10 mM) containing 0.1% BSA. Detached cells and cell debris were separated from the vesicles by precentrifugation (500 *g*). The PMV were pelleted at 48,000 *g*, collected and centrifuged in hypotonic Tris-buffer to remove soluble proteins from the lysed PMV. This final pellet was stored at −20° prior to analyses [16].

Determination of total and individual phospholipids, of cholesterol and of protein in whole cells and plasma membrane vesicles. Trypsinized cells or plasma membrane vesicles were washed in saline solution and stored frozen (−20°). For analyses pellets were resuspended in saline solution and sonicated for 5 sec at 50 W. Total phospholipids were determined fluorometrically according to the method of Jouanel *et al.* [17], using a lecithin standard from bovine brain. Determination of total cholesterol was done enzymatically with a test kit (Boehringer GmbH, Mannheim, F.R.G.). Proteins were measured with the BCA protein assay reagent (Pierce Chemical Company, Rockford, IL). Total cellular lipids were extracted according to Folch *et al.* [18]. Aliquots of the lipid extracts were applied to HPTLC silica gel plates (No. 5641, Merck, Darmstadt, F.R.G.). The plates were developed with chloroform, methylacetate, propanol-2, methanol and KCl 0.9% in water (25/25/25/10/9, v/v) for 30 min [19], dried (5 min at 180°) and subsequently cooled for 5 min. Spots corresponding to individual

Table 1. Anisotropy values $r(G)$ of cultured cells in the absence (controls) and in the presence of 5 μM DMI

Fluorescent ligand	Exposure	Human fibroblasts	Rat brain cells	Rat Roc-1 cells
6-AS	Control	0.121 ± 0.005	0.115 ± 0.006	0.116 ± 0.004
	5 μM DMI	0.127 ± 0.005	0.118 ± 0.005	0.118 ± 0.003
	t-test	NS	NS	NS
12-AS	Control	0.084 ± 0.005	0.084 ± 0.004	0.087 ± 0.003
	5 μM DMI	0.073 ± 0.006	0.074 ± 0.004	0.081 ± 0.004
	t-test	$P < 0.01$	$P < 0.001$	$P < 0.01$

Fluorescence anisotropy $r(G)$ of trypsinized cultured cells was determined in absence (controls) or presence of 5 μM DMI at 37° in Hank's balanced salt solution, pH 7.4, using the fluorescent dyes 6- or 12-AS (0.5 μM).

Data represent the mean ± SD of at least four independent experiments. Statistical analysis was done by Student's *t*-test.

NS, not significant.

Table 2. Anisotropy values $r(G)$ of different membrane domains of cultured control cells and of cells after chronic exposure to 5 μM DMI

Fluorescent ligand	Exposure	Human fibroblasts	Rat brain cells	Rat Roc-1 cells
2-AP	Control	0.125 ± 0.002	0.132 ± 0.005	0.114 ± 0.005
	5 μM DMI	0.120 ± 0.005	0.121 ± 0.005	0.121 ± 0.004
	t-test	$P < 0.05$	$P < 0.001$	$P < 0.001$
6-AS	Control	0.123 ± 0.005	0.125 ± 0.005	0.109 ± 0.008
	5 μM DMI	0.116 ± 0.004	0.117 ± 0.005	0.118 ± 0.006
	t-test	$P < 0.001$	$P < 0.001$	$P < 0.05$
7-AS	Control	0.117 ± 0.005	0.120 ± 0.003	0.101 ± 0.003
	5 μM DMI	0.111 ± 0.005	0.110 ± 0.005	0.108 ± 0.005
	t-test	$P < 0.001$	$P < 0.001$	$P < 0.05$
9-AS	Control	0.101 ± 0.005	0.108 ± 0.006	0.096 ± 0.007
	5 μM DMI	0.097 ± 0.005	0.097 ± 0.005	0.101 ± 0.008
	t-test	$P < 0.01$	$P < 0.001$	NS
12-AS	Control	0.077 ± 0.002	0.081 ± 0.003	0.073 ± 0.003
	5 μM DMI	0.074 ± 0.004	0.075 ± 0.002	0.073 ± 0.002
	t-test	$P < 0.05$	$P < 0.001$	NS

Anisotropy values of cells exposed to seven doses of 5 μM DMI were compared to controls (fed with seven changes of medium alone). Determinations were performed using the fluorescent dyes 2-AP; 6-, 7-, 9- and 12-AS.

Data represent the mean ± SD of at least four independent experiments. Statistical analysis was done by Student's *t*-test.

NS, not significant.

phospholipids were visualized by immersing the plates into Cu-(II)-acetate (0.5 g/100 mL ethanol) for 10 sec and into H_3PO_4 (20.7 mL of 85% H_3PO_4 in 220 mL methanol), for another 10 sec. After drying (6 min at 180°) the phospholipids were quantified by reflection densitometry (Camag TLC scanner I, Muttenz, Switzerland) according to Kolarovic and Traitler [20].

Single dose exposure to DMI. At time $t = 0$ the fluorescent dye as well as DMI were added to 3 mL of a suspension of trypsinized cells in Hank's balanced salt solution at pH 7.4 and 37°. Anisotropy measurements were performed at $t = 15$, 20 and 25 min, respectively.

Chronic exposure to DMI. Cells were incubated in Eagle's MEM (fibroblasts) or in Dulbecco's modified MEM (rat brain astrocytes and ROC-1 cells), both containing 10% of fetal calf serum and 5 μM DMI. Between days 1 and 4 the DMI containing media were changed seven times. On day 5 cell suspensions were prepared as described. Apparent anisotropies of 2-AP and 6-, 7-, 9- and 12-AS were determined in aliquots of suspended cells. Results of DMI treated cells were compared to those of appropriate control cells (exposed to repeated drug free media changes only).

Chemicals. MEM dry powdered media with Earle's salt were purchased from Seromed (Munich,

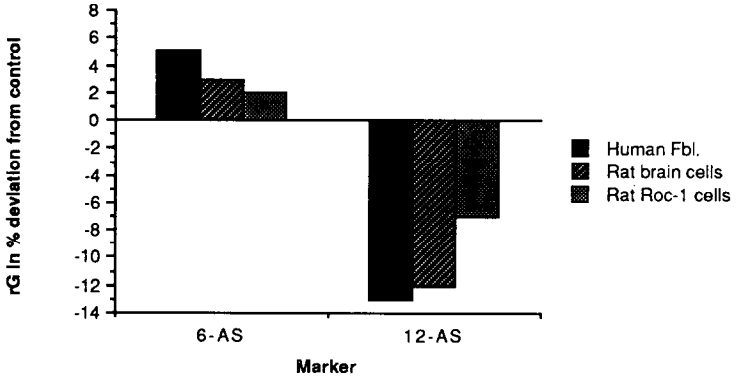

Fig. 2. Trypsinized cultured cells were exposed to a single dose of 5 μM DMI at 37°. Anisotropy measurements were performed at times 15, 20 and 25 min, respectively, and expressed as the mean thereof. Fluorescence anisotropies of 6-AS (superficial) and 12-AS (deeper layers) are presented in per cent deviation from control values (controls = 0%).

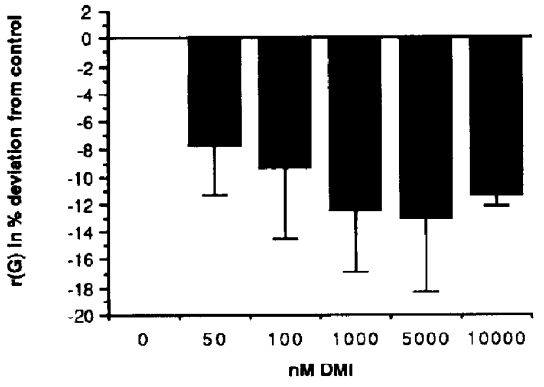

Fig. 3. Trypsinized cultured cells were exposed to increasing doses of DMI between 0.05 and 10 μM at 37°. Anisotropy values of the deeper layers of the membrane, represented by 12-AS, are shown in per cent (±SD) deviation of control values (controls = 0%).

F.R.G.). Fetal calf serum was from Boehringer. Crystalline bovine serum albumin was purchased from the Sigma Chemical Co. (St Louis, MO). All phospholipid standards were purchased from Supelco (Gland, Switzerland). The fluorescent markers were obtained from Molecular Probes, Inc. (Eugene, OR) and kept as 1 mM ethanolic stock solutions in the dark at 4°. DMI was kindly provided by Ciba Geigy (Basel, Switzerland). All other chemicals, solutions and solvents were of analytical purity grade and were purchased from Merck.

RESULTS

Anisotropy

Control values. Trypsinized human fibroblasts, rat brain astrocytes and ROC-1 glioma cells showed only minor differences in fluorescence anisotropies (Table 1). Anisotropy values decreased with increasing depth of the membrane in all cell types as determined with 6-AS (superficial layers) and 12-AS (deeper layers), respectively. Values obtained with 6-AS were slightly higher in human fibroblasts than in the two rat brain derived cell strains.

Effects of repetitive feeding on control cultures. Anisotropy values, determined by 6- and 12-AS, of cells fed with seven media changes in 4 days differed slightly from those fed only twice in seven days. Anisotropy measured in the superficial layers (6-AS) remained unchanged in fibroblasts, but increased in rat brain astrocytes ($P < 0.001$) and decreased in rat ROC-1 cells ($P < 0.01$). Anisotropy values measured in the deeper layers of the membrane (12-AS) were significantly lowered by repetitive feeding in all three cell types (Tables 1 and 2).

Effects of DMI on membrane anisotropy. Single dose exposure to DMI: In the superficial layers, determined by 6-AS, anisotropy values in presence of 5 μM DMI were only insignificantly increased over control. Anisotropy values of the deeper, hydrophobic layers of the membrane (12-AS), however, were significantly decreased in all cell types ($P < 0.01$) (Table 1), indicating a fluidizing effect of DMI on the core of the membrane. This effect was more expressed in human fibroblasts (12%) and rat brain astrocytes (13%) than in rat ROC-1 glioma cells (7%) (Fig. 2).

Figure 3 shows the dose dependence of the decrease in anisotropy measured with 12-AS in trypsinized DMI exposed human fibroblasts. Because of an interaction between the drug and the fluorescent dye the drug effects on anisotropy were counteracted by fluorescence quenching phenomena at DMI concentrations above 10 μM. Measurements of fluorescence lifetime of 12-AS indeed showed a marked decrease (data not shown).

Multiple dose exposure to DMI: Chronic exposures of the cells to 5 μM DMI led to alterations in fluorescence anisotropies in various depths of the membrane, measured with a set of *n*-(9-anthroyloxy) fatty acids (Table 2). These data are presented as per cent deviation from control anisotropies (values in control cells = 0%) measured in different membrane layers (Fig. 4).

In human fibroblasts DMI induced a decrease in anisotropy values across all levels of the membrane. The effect was similar in rat brain astrocytes. In rat ROC-1 cells, however, the changes were inverse to those observed in the other cell strains.

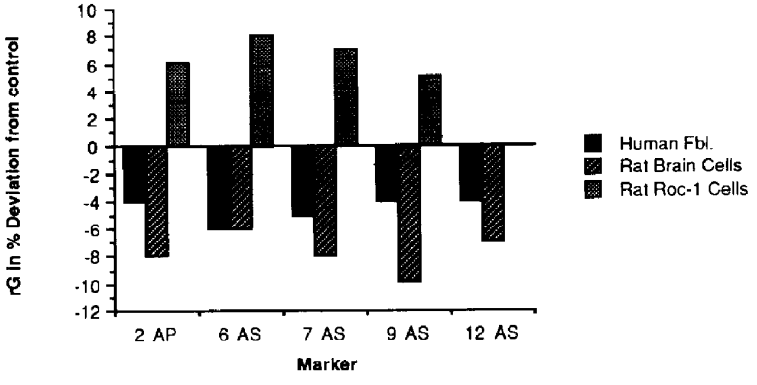

Fig. 4. Effects of seven doses of 5 μM DMI in 4 days on anisotropy profiles were measured in three cell types. 2-AP, 6-AS, 7-AS, 9-AS and 12-AS were used as markers. Results are given in per cent deviation from control values (controls = 0%).

Phospholipid, cholesterol and protein contents in whole cells and plasma membranes

Comparable data from cellular and from plasma membrane lipids are restricted to fibroblasts and to ROC-1 cells. The number of available rat brain cell cultures was too small for the preparation of plasma membrane vesicles from these cells.

Whole cells. Results from the effects of chronic drug exposure of cultured cells on cellular cholesterol and on phospholipid (PL) contents are summarized in Table 3. Data from human fibroblasts and from rat ROC-1 cells that were exposed to seven doses of 5 μM DMI were compared with results in control cells that were fed with the same schedule but without drug. PL contents per mg of protein increased in chronically DMI exposed cells. This increase was less pronounced and statistically not significant in ROC-1 cells. Cholesterol and PL contents per mg of cell protein were similarly increased. The cholesterol to PL ratios therefore remained unchanged. Chronic DMI exposure led to changes in the PL patterns of both cell types. The drug induced changes were highly significant in fibroblasts and less striking in ROC-1 cells. The most impressive change was the increase in phosphatidylinositol (PI) in fibroblasts. The ratio of phosphatidylcholine (PC) to phosphatidylethanolamine (PE) increased in fibroblasts while it decreased in ROC-1 cells. This difference was significant in ROC-1-cells (Table 3).

Plasma membrane vesicles. Purified plasma membrane preparations were obtained by vesiculation induced by treatment of monolayer cultures with formaldehyde and dithiothreitol. This preparation proved to be virtually free of contaminating mitochondria and lysosomes according to determinations of marker enzyme activities. The plasma membrane marker enzyme 5′-nucleotidase was considerably enriched. The PL to protein ratio was 3–4-fold increased and a 7–8-fold higher cholesterol to protein ratio was found compared to the results in whole cells.

PL and cholesterol contents per protein were decreased in membranes of chronically drug exposed cells (Table 3). This was due to an increase in the amount of protein, since the cholesterol to phospholipid ratio was not different from that in control membrane preparations. Although effects of DMI exposure on individual PL concentrations were only minor the PC to PE ratios were significant in both cell types ($P < 0.05$) but in opposite directions. The changes were similar to those obtained in whole cells (Table 3).

Table 3. Lipid contents of whole cells and of plasma membranes in control and in chronically DMI exposed cultured cells

Cell-type	Preparation	Exposure	PL/Prot (mg/g)	Chol/Prot (mg/g)	Chol/PL (mg/g)	Sph (%)	PC (%)	PS (%)	PI (%)	PA (%)	PE (%)	PC/PE (%)
Human fibroblasts	Whole Cell	Control (N = 6)	355	34.4	97.8	8.0	42.6	5.6	4.4	2.3	37.0	1.15
		5 μM DMI	472.9	45.4	98.1	6.4	45.0	5.1	6.8	1.9	36.6	1.23
		t-test	$P < 0.01$	$P < 0.05$	NS							$P < 0.05$
	Vesicles	Control (N = 6)	1213.5	221	184.1	12.8	46.3	6.2	3.3	3.3	28.1	1.65
		5 μM DMI	1091.5	208.2	192.4	13.9	47.1	6.0	3.5	3.7	25.8	1.83
		t-test	NS	NS	NS							$P < 0.05$
Rat Roc-1 cells	Whole Cell	Control (N = 6)	336.8	28	83.1	5.9	46.8	3.4	5.2	1.6	37.1	1.26
		5 μM DMI	370.9	30.2	81.3	5.6	42.8	3.0	5.4	1.5	41.8	1.02
		t-test	NS	NS	NS							$P < 0.01$
	Vesicles	Control (N = 6)	1260.5	245.4	194.7	9.8	51.4	4.7	3.3	0.5	30.3	1.7
		5 μM DMI	1104.3	216.8	196.5	9.8	48.1	4.6	3.8	0.7	33.0	1.46
		t-test	$P < 0.05$	$P < 0.05$	NS							$P < 0.05$

Cells were treated as described in the legend to Table 2. The following abbreviations are used: PL, phospholipids; Chol, cholesterol; Sph, sphingomyelin; PC, phosphatidyl-choline; PS, phosphatidyl-serine; PI, phosphatidyl-inositol; PA, phosphatidic acid; PE, phosphatidyl-ethanolamine. Individual phospholipids are expressed as per cent of total PL.

DISCUSSION

Human skin fibroblasts have been used because they can be grown from patients with genetic disorders and may be used in individual drug studies. Dissociated rat brain cells and ROC-1 cells on the other hand are closer to the target organ of psychotropic drugs since human brain cells are not usually available for investigation in cultures.

Membrane fluidity is quantitatively expressed by the mobility of lipid molecules and the rate of rotational motions and is related to local microviscosity. Fluorescence anisotropy predominantly

measures mobility of the apolar fatty acid parts of phospholipids and of the cholesterol. It is not a measure for lateral mobility, e.g. of protein molecules. As fluorescence anisotropy decreases with increasing depth of the fluorescent probes in the membrane, fluidity increases [21].

In our study, fluorescent markers allowing determination of depth dependent membrane anisotropies $r(G)$ were used. The fluorescent analogues of anthroyloxy fatty acids have been shown to partition readily in plasma membranes of various cells [22]. The anthracene ring is assumed to be locked at a defined depth within the bilayer structure by virtue of an interaction of the carboxyl group at the water lipid interface [23]. Thus *n*-(9-anthroyloxy) fatty acids may be used to localize drug action on fluidity in defined domains of the membrane.

Fluorescence anisotropy measurements on intact cultured cells indicated that a single dose of DMI being present during the assay fluidized the deeper, hydrophobic layers of the membranes. These results are consistent with findings by Cater *et al.* [24], describing drug induced lipid phase transitions by differential scanning calorimetry. Fluidizing effects have been explained by drug interference with the hydrophobic forces between lipids in the deeper layers of the membranes [25]. The drug induced decreases in anisotropy of the deeper layers of the membranes were dose dependent. The presence of the drug within the lipid bilayer decreased the fluorescence lifetimes of anthroyloxy fatty acids. Such interference would result in an increase in the apparent anisotropy values, but the observed changes were in the opposite direction. This may explain why at higher DMI concentrations ($>10\ \mu M$) the effects on anisotropy become blurred.

Anisotropy values were changed by repetitive feeding of cell cultures with medium alone. These changes may be related to shifts in the phases of the cell cycle and to alterations in cellular differentiation [26].

Anisotropy determinations in chronic drug experiments were performed after trypsinizing and careful washing of the suspended cells. Chronically drug exposed cells accumulated about 2.5 times the amount of DMI taken up during single exposure. About half of the intracellular DMI was released into the washing solutions as previously shown [27]. Thus the cellular DMI contents during anisotropy measurements were comparable to those in cells exposed to a single DMI dose. The changes in anisotropy values following chronic drug exposures are the result of the effect of DMI still present in the cells and of a cellular response to the chronic presence of the drug.

Chronic drug effects on membrane anisotropies were cell specific and the changes of anisotropies even occurred in different directions. The ROC-1 cells, that were originally more fluid, became more rigid, while the initially more rigid cells like the fibroblasts and the brain cells were fluidized by the chronic DMI exposure, such that the resulting anisotropies became more alike. Those changes were particularly evident in the superficial layers of the membranes.

Highly purified plasma membrane preparations can be obtained by vesiculation of cells *in situ* using formaldehyde and dithiothreitol [15]. The anisotropy measurements of these vesicles were very artefactual because of a strong influence of the vesiculation procedure itself (Zuehlke and Honegger, unpublished results). These plasma membrane vesicles, however, could be used for lipid analyses. Their lipid contents and composition showed DMI induced changes. These were cell specific and qualitatively but not quantitatively similar to those in whole cells. A relative increase in the PC to PE ratios was shown to result in a fluidization while a decrease had a rigidifying effect on membranes [28]. In our experiments the basically more rigid fibroblasts had a lower relative proportion of PC resulting in a lower PC to PE ratio when compared to the respective values in ROC-1 cells which were initially more fluid. This again applied to both whole cells and to purified plasma membranes. After chronic DMI exposure the changes in PC to PE ratios were as to be expected to explain the changes in the anisotropy values observed.

In conclusion, single low micromolar concentrations of DMI themselves appeared to effect the deeper layers of the membranes physico-chemically. Chronic exposures of cultured cells to 5 μM DMI led to cell specific changes in membrane fluidity, which were accompanied by appropriate changes in the PC to PE ratios and were mostly related to the superficial layers of the membranes. Both the changes of phospholipid composition and the changes of anisotropies were to be considered as individual regulatory responses of cells to chronic presence of DMI. This was also suggested by the fact that different cells could modify themselves in different directions.

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