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EFFECTS OF DIFFERENTIATION INDUCERS ON DIPHENYLHEXATRIENE FLUORESCENCE POLARIZATION IN INTRACYTOPLASMIC AND PLASMA MEMBRANES FROM FRIEND ERYTHROLEUKEMIA CELLS

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Treatment of Friend leukemia cells with dimethylsulfoxide or hexamethylenbisacetamide, which induced erythroid differentiation, resulted in enhancement of fluorescence polarization of diphenylhexatriene in not only plasma membranes, but also in intracellular membranes. In a cell variant resistant to induction, the polarization values of intracellular membranes were not affected by the inducing agents, whereas plasma membranes had the same enhancement of polarization values as in sensitive cells. Therefore, Friend cell differentiation can be associated with the effect of the inducers on intracellular membranes, but not with the effect on plasma membranes.

Friend leukemia (FL) cells are virus-transformed erythroid precursors thought to be arrested at the proerythroblast stage of maturation (1). Treatment of FL cells with dimethylsulfoxide (DMSO), hexamethylenbisacetamide (HMBA) or a variety of other compounds results in hemoglobin synthesis and in the production of a terminally differentiated erythroid cell (1,2). The plasma membrane was suggested to be the primary site of action of chemical inducers (2-5).

In recent studies on membrane dynamics, we showed that the degree of fluorescence polarization of diphenylhexatriene (DPH)-labeled FL cells is altered during DMSO or HMBA-induced differentiation (7-9). This change was attributed to modifications in membrane protein-lipid interactions (8) resulting from the action of the inducers rather than from the differentiated state of the cell (9). Furthermore, this change was not seen in a cell variant resistant to DMSO induction (RFL cells) (7).

In the present study, we have compared the degree of DPH fluorescence polarization in plasma and intracellular membranes obtained from FL and RFL cells. Changes were observed upon DMSO and HMBA-treatments, and their relation to the induction of hemoglobin synthesis was analyzed.

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MATERIALS AND METHODS

Cell culture. The FL cell line derived from the clone 745 A of Friend virus-transformed mouse spleen cells (1), and its cell variant RFL resistant to DMSO induction, isolated in our laboratory (7), were cultivated as previously described (8). Cultures were treated with 280 mM DMSO or with 4 mM HMBA for 4 days. Hemoglobin synthesis was determined by benzidine staining, as already described (7) and expressed as percent of benzidine positive (B^+) cells.

Isolation of plasma and intracytoplasmic membranes. Membranes were prepared by subcellular fractionation, according to a method used previously (10). All steps were carried out at 4°C. Cells were harvested, washed 3 times in 0.15 M NaCl, and lysed in hypotonic buffer (1 mM $NaHCO_3$, 2 mM $CaCl_2$, pH 8) for 10 min. At this time, most cells were broken, the nuclei being still intact. After homogenization in a Dounce homogeniser (6-8 strokes), isotonicity was restored in the cell lysat by adding 0.1·vol. of 1.5 M NaCl, 20 mM $CaCl_2$. After pelleting the nuclei by a 300 g centrifugation for 10 min, the supernatant was centrifuged at 27000 g for 20 min. The "whole membrane" pellet was slowly resuspended with 8% sucrose in 5 mM Tris buffer (w/w), pH 8. Sucrose concentration was brought to 45% by dropwise addition of 62% sucrose under gentle vortexing. Six ml of this suspension were put in a nitrocellulose tube. Four layers of 41% (12 ml), 37% (8 ml), 33% (6 ml) and 29% (6 ml) sucrose solutions were successively laid on. This discontinuous gradient was centrifuged for 3 h at 131000 g in a Beckman SW-27 rotor. Four interfacial bands (A at 29%/33%, B at 33%/37%, C at 37%/41% and D at 41%/45%) were collected, diluted with distillated water and centrifuged at 27000 g for 40 min. The different membrane fractions were assayed for protein content (11), and specific activity of four membrane marker enzymes : 5'-nucleotidase (EC 3.1.3.5) for plasma membrane, glucose-6-phosphatase (EC 3.1.3.9) for endoplasmic reticulum, acid phosphatase (EC 3.1.3.2) for lysosomes and cytochrome c oxidase for mitochondria, according to established procedures (12-15). Acid phosphatase activity was detectable only in fraction D, which showed also a 15-fold enrichment in cytochrome c oxidase over cell lysat. Fraction C represented mainly endoplasmic reticulum membranes (5 to 7-fold enrichment in glucose-6-phosphatase) with a contamination by mitochondrial membranes. Fraction A was enriched 9 to 12-fold in 5'-nucleotidase, and had very low glucose-6-phosphatase and cytochrome c oxidase activities. Fraction A, which seemed to correspond to fraction C found by Rawyler et al. (16), was therefore considered to be representative of plasma membrane material. Finally, fraction B exhibited the same enrichment in 5'-nucleotidase, but was contaminated by endoplasmic reticulum material.

Fluorescence polarization. This technique, based on the well-established theory of Shinitzky et al. (see review in ref.6), was used with the probe 1,6-diphenyl-1,3,5-hexatriene (DPH) embedded in hydrophobic regions of cell membranes. Aliquots (containing 100 µg of protein) of each membrane fraction, and of unfractioned "whole membranes" were labeled with 2×10^{-6} M DPH in phosphate buffer saline (PBS) at 37°C for 30 min, and steady-state polarization (P) measurements were performed with a MV-1 Elscint instrument at 37°C, according to the previously described methods (6,10,17). DPH labeling and P measurements were also carried out as described above with intact cells (10×10^6) after being washed three times in PBS.

RESULTS

Erythroid differentiation of FL cells was induced by addition of DMSO or HMBA to the culture medium. After 4 days of treatment, the percentage of hemoglobin-synthesizing (B^+) cells was 70-75%, while remaining at 0-2% in

The numbers themselves together with simple arithmetic give a surprising amount of information on the molecule. We see that the A chain (m/z 2338) remains intact being devoid of tryptic cleavage sites. The B chain, on the other hand, splits as predicted into fragments at m/z 859, 930, 2486 and 3326, with some intact B chain remaining at m/z 3397. The mass difference between the heaviest fragment (3326) and the intact molecule (3397) of 71 u. shows that the C-terminus of the B chain is ALANINE. In this case the complete structure is mapped in the one experiment.

In more complex analyses further subdigests or derivative formations and FAB-MAPS are recommended particularly if the problem demands location of a point mutation, blocked N-terminus, or the C-terminus of a protein. Such problems may be solved by continuing the FAB-MAPPING strategy with, for example, carboxypeptidase B digestion and/or n cycles of Edman degradation.

Examples of this idea are shown in Figure 3 (b) and (c). Figure 3(b) shows the partial FAB-MAP around m/z 2300 after treating the tryptic digest of the reduced Insulin with carboxypeptidase B. The signal at m/z 2338 remains unchanged indicating the absence of a C-terminal LYS or ARG (unless preceded by PRO) in the peptide; since this was derived by tryptic digestion, this is a special case of a C-terminal peptide assignment i/e. the signal remains unchanged on carboxypeptidase B digestion. In contrast m/z 2486 in

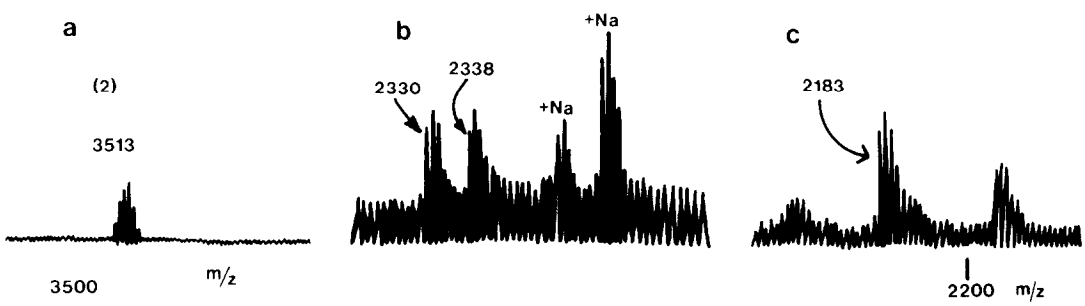

Figure 3. (a) Partial FAB MAP of reduced and carboxymethylated Insulin showing the addition of 2 carboxymethyl units to the B chain.
(b) Partial FAB MAP of the carboxypeptidase B-treated tryptic digest of reduced Insulin. The sodium cationised signals derive from the commercial enzyme preparation.
(c) Partial FAB MAP of the Edman degradation product of the carboxypeptidase B-treated tryptic digest of reduced Insulin.

Table 2 EFFECT OF DMSO AND HMBA ON FLUORESCENCE POLARIZATION OF DPH IN INTACT CELLS, WHOLE AND FRACTIONED MEMBRANES FROM NON INDUCIBLE RFL CELLS.

	Untreated	DMSO		HMBA	
Intact cells	0.211	0.212	(0.001)	0.206	(-0.005)
Whole membranes	0.210	0.209	(-0.001)	0.213	(0.003)
Fraction A	0.223	0.257	(0.034)	0.259	(0.036)
Fraction B	0.225	0.256	(0.031)	0.254	(0.029)
Fraction C	0.210	0.215	(0.005)	0.212	(0.002)
Fraction D	0.192	0.189	(-0.003)	0.193	(0.001)

All experimental procedures are described in Materials and Methods. Results are expressed as in Table 1.

indicating that inducers had no effect on intracytoplasmic membranes P values of RFL cells. In contrast, fractions A and B had the same Pvalue enhancement (0.030-0.040) as in differentiated FL cells. This result shows that DMSO and HMBA induced a similar effect on plasma membrane P values in either sensitive or resistant Friend cells.

DISCUSSION

DPH fluorescence polarization technique is widely used to study the physical state of lipids (6). When DPH is embedded in cellular membranes, the degree of P can be considered only as the average relative freedom of the probe, reflecting dynamics of membrane components, including several mechanisms (19). Our recent work on human leukemia cell membranes supports the view that protein modifications are involved in the polarization values (20). In this respect, changes in protein-lipid interactions were associated with alterations of whole cell-P values previously found in differentiating FL cells (7,9). Such membrane dynamic alterations could be attributed to the direct action of chemical inducers (8), suggesting that they might be involved in the processes of differentiation (7-9).

We show here that the effect of DMSO and HMBA is not specific for plasma membrane, since also exerted on microsomal, lysosomal and mitochondrial membranes. Moreover, the effect on plasma membrane occurs also in non inducible RFL cells. Although we cannot exclude the possibility that in those cells the "primary signal" of differentiation remains without effect because of a modification at the level of a subsequent step, it appears that the

observed plasma membrane change is not related to induction of hemoglobin synthesis, but is likely due to pleiotropic action of the chemical agents.

In contrast, the effect of DMSO and HMBA on intracytoplasmic membrane dynamics, being not seen in RFL cells, can be associated with erythroid differentiation. It has been reported that DMSO alters the physical state of phospholipids (3) as well as the conformation and activity of proteins (18,21). Thus, the inducers could affect, either directly or through lipid-protein interactions, some regulating molecules bound to endoplasmic reticulum (e.g. enzymes or receptors). Such a mechanism of action of Friend cell differentiation inducers remains to be established. Nevertheless, data presented here do not favor the hypothesis that they act primary at the plasma membrane.

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