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Monitoring Differential Changes of the Mobility of Cell Membrane Components Following Treatment with a Tumor Promoter by Using Pyrene and a Novel Fatty Acid Fluorophore

TETSURO MOHRI,* TSUNEO TAKADERA, AKIHIRO KAWANAKA and TAKAO OHYASHIKI

Department of Physiological Chemistry, Hokuriku University, School of Pharmacy, Kanazawa 920–11, Japan

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The effects of an active tumor promoter, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), on cell membrane components have been examined using two lipophilic fluorophores, pyrene and 12-[6-(*N*-methylanilino)-2-naphthalenesulfonyloxy]-stearic acid (MANSOS), in Chang liver cells. MANSOS has a high fluorescence quantum yield in water and shows a blue shift (15 nm from the value of 445 nm in phosphate buffer) of the emission maximum on incorporation into the cell membrane. Pretreatment of cells with TPA for 2 h induced differential changes in the fluorescence parameters of pyrene and MANSOS incorporated into the cell membrane; it decreased the lifetime and harmonic mean of the rotational relaxation time and amplified the quenching by KI of pyrene fluorescence, whereas it increased the harmonic mean of the rotational relaxation time and slightly attenuated the quenching of MANSOS fluorescence.

Taken together, these findings suggest different partition characteristics of the two fluorescent probes in the cell membrane components, and differential effects of TPA on the two species of components as judged from the opposite mobility changes of the two probes.

Keywords——phorbol ester; cell membrane; membrane lipid; membrane fluidity; fluorescent probe; liver cell; fatty acid; anilinonaphthalenesulfonate

Introduction

The fluidity of the plasma membrane of cells is implicated in many cellular functions and activities.[1] It has been monitored by various methods[2] including fluorometry and electron spin resonance (ESR) using lipophilic (*e.g.* diphenylhexatriene) or amphipathic (*e.g.* anilinonaphthalenesulfonic acid and fatty acid labeled with *N*-oxydimethyloxazolidine) probes intended to reflect the physical state of either the interior (within the lipids) or the perimembranous regions of the phospholipid bilayers, where these compounds are selectively located.[2b]

It has been reported with a lipophilic probe that the fluidity or the mobility of molecular components of the cell membrane is not necessarily uniform throughout the membrane, whereas that of unimolecular phospholipid vesicles is homogeneous.[3] Capping or clustering of membrane proteins on stimulation of cells by lectins or other mitogens[4] and cell fusogens[5] involves a local separation of the proteins from lipids, which is thought to increase the fluidity of the membrane at the protein-depleted regions. The mobility of constitutive molecules in turn is probably lowered in the protein-rich regions.

Biologically active phorbol esters such as 2-*O*-tetradecanoylphorbol-13-acetate (TPA) have been demonstrated to be localized in the lipid layers or cell membrane, binding at least partly to specific membrane receptors,[6] when added to phospholipid vesicle suspensions[7] or cell suspensions.[8] They significantly perturb the lipids and have a mitogenic action on the cells, greatly increasing the fluidity of the bulk lipid of the membranes.[9]

We have exploited in the present experiments two kinds of fluorescent probe in an attempt to elucidate the changes of the mobility or flexibility of different molecules constituting the cell membrane of Chang liver cells on treatment with TPA. One of the probes is pyrene, which can be deeply immersed in the domain of hydrocarbon chains of the membrane lipids because of its high lipophilicity, and the other is a novel stearate derivative which possesses a fluorescent core linked to the hydrocarbon chain of the fatty acid. Experiments with these probes have revealed that the treatment of the cells with TPA produces opposite effects on the membranous microcompartments for which these probes have reasonably specific binding affinities. On the basis of these results, the reorganization of membrane structure upon stimulation of the cells by phorbol ester is discussed.

Materials and Methods

Materials——Commercially available reagents were obtained from the following sources: sodium 6-hydroxy-2-naphthalenesulfonate, Tokyo Kasei Ind. Co.; 12-hydroxystearic acid and pyrene, Wako Pure Chemical Co.; TPA and 4α-phorbol 12,13-didecanoate (4α-PDD), Sigma Chemical Co.

Synthesis of MANSOS——12-[6-(*N*-Methylanilino)-2-naphthalenesulfonyloxy]-stearic acid (MANSOS) was prepared by the reaction of 12-hydroxystearic acid with 6-(*N*-methylanilino)-2-naphthalenesulfonyl chloride (2,6-mansyl chloride; mp 131—133 °C), which was synthesized from sodium 6-hydroxy-2-naphthalenesulfonate according to the method described by Cory *et al.*[10]

2,6-Mansyl chloride (1 g) was added in small portions with stirring to a solution of 12-hydroxystearic acid (960 mg) in dry pyridine (6 ml) in an ice bath. The reaction mixture was stirred for an additional 3 h at room temperature and then pyridine was removed *in vacuo* below 30 °C. Water (5 ml) was added to the residue and the mixture was extracted with three 20 ml portions of CH_2Cl_2. The combined extract was dried over $MgSO_4$ and evaporated. The resulting residue was chromatographed on silica gel using $CHCl_3$–AcOEt (9 : 1) as an eluent to give MANSOS, which was further purified by rechromatography.

MANSOS: Colorless paste. IR ν_{max}^{Nujol} cm^{-1}: 1180 and 1360 (SO_2–O–), 1710 (C=O). ^{1}H-NMR ($CDCl_3$): 0.8—2.4 (34H, m, aliphatic H), 3.41 (3H, s, N–CH_3), 7.0—8.3 (11H, m, aromatic H). UV $\lambda_{max}^{H_2O}$ nm: 226, 258, 330. *Anal.* Calcd for $C_{35}H_{49}O_5S$: C, 70.55; H, 8.29; N, 2.35. Found: C, 70.31; H, 8.53; N, 2.08. The above data are consistent with the proposed MANSOS structure shown in Chart 1. MANSOS was stored in a freezer or in ethanol as a 1 mM solution at 4 °C.

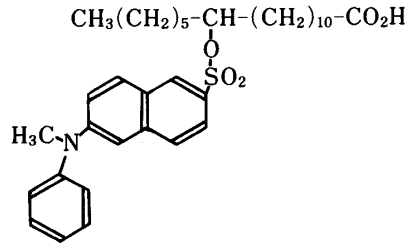

Chart 1

Culture of Cells——Chang liver cells were grown in suspension with medium composed of 90% (v/v) Eagle's minimum essential medium (MEM, NISSUI, No. 4) and 10% (v/v) bovine serum at 37 °C up to a population of around 10^6 cells/ml at harvest.

Pretreatment of Cells with TPA or 4α-PDD and Labeling with Pyrene or MANSOS——The cells were pelleted by centrifugation (1500 rpm, 5 min) and rinsed with choline–Hepes medium (CHM, 133 mM choline chloride, 5 mM KCl, 1 mM $MgSO_4$, 1% glucose and 10 mM Hepes–Tris, pH 7.4). The cells were divided into two portions, suspended in MEM containing 10 mM Hepes–Tris (each portion in 20 ml), and incubated at 37 °C for 2 h, one portion in the presence of 10^{-7} M TPA or 4α-PDD and the other with the vehicle only. Cell concentrations in the suspension were 110—120 μg/ml as cellular protein. TPA and 4α-PDD were stored as 10^{-4} M ethanol solutions. At the end of the incubation the cells in the two tubes were separately centrifuged and resuspended in CHM. A 0.2 ml aliquot of each suspension (450—488 μg protein/ml) was mixed with 3.0 ml of CHM containing MANSOS or pyrene to make final concentrations of 5 and 4 μM, respectively. After incubation for 10—30 min, the cells of each sample were pelleted and suspended in 5 ml of CHM. Pyrene was dissolved in ethanol at 2 mM before use.

Analyses by Spectrofluorometry of Pyrene, 6-(*N*-Methylanilino)-2-naphthalene Sulfonate (2,6-Mansate) and MANSOS——The fluorescence intensities and polarizations of pyrene, 2,6-mansate and MANSOS were measured

with a Hitachi MPF-4 spectrofluorometer equipped with a rhodamine B quantum counter at 25 °C, except that the polarization was measured at temperatures ranging from 20 to 43 °C. Excitation and emission wavelengths used for pyrene and MANSOS incorporated into the cell membrane were 340 and 330 nm and 392 and 430 nm, respectively, unless otherwise indicated.

The degree of fluorescence polarization, P, was calculated as the value of $(I_V - I_H)/(I_V + I_H)$, where I_V and I_H are the intensities of vertically and horizontally polarized emission with vertically polarized exciting light, respectively. The harmonic mean of the rotational relaxation times, ρ_h, was estimated from the slope of a $1/P$ *versus* T/η plot using the following equation,[11]

$$\rho_h = \left(\frac{1}{P_0} - \frac{1}{3}\right) \frac{3\tau}{\text{the slope}} \cdot \frac{\eta}{T}$$

where P_0 is the limiting polarization at $T/\eta = 0$, η the viscosity of the solvent, τ the fluorescence lifetime and T the absolute temperature.

The quantum yields of 2,6-mansate and MANSOS in H_2O and several organic solvents were determined by the usual method using quinine sulfate as a standard. The concentrations used for 2,6-mansate were 16.7 μM in H_2O, 0.667 μM in ethanol and 1.0 μM in ethylene glycol. Those for MANSOS were 3.33 μM in H_2O and ethanol, 0.662 μM in $CHCl_3$ and 10 μM in ethylene glycol. Excitation wavelengths used for 2,6-mansate and MANSOS were 315 and 330 nm, respectively.

Determination of the Quenching of Fluorescence by KI——Cells were incubated with or without TPA (10^{-7} M) for 2 h and then labeled with pyrene or MANSOS as described above. After being washed with CHM they were allocated into 3 ml samples in CHM in a range of cellular protein concentration from 400 to 550 μg/ml. The fluorescence intensities at emission wavelengths of 392 and 430 nm, using excitation wavelengths of 340 and 330 nm for pyrene and MANSOS, respectively, were recorded before and after successive additions of 5 μl aliquots of 1 M KI solution to each sample to make 0—16.4 mM KI as indicated in the results. The quenching constant (k_q) was calculated by using the Stern–Volmer equation,[12]

$$I_0/I = 1 + k_q\tau[\text{KI}]$$

and from the slope of an I_0/I *versus* [KI] plot, where I is the fluorescence intensity at [KI], I_0 is I at [KI] = 0 and τ the lifetime, ns, of fluorescence.

Determination of the Fluorescence Lifetimes of Pyrene and MANSOS Incorporated into the Cell Membrane——Fluorescence lifetime measurements were performed by the single photon technique with an Ortec PRA-3000 nanosecond fluorometer with a 7100 multichannel analyzer (Photochemical Research Associates Inc., Canada). Analysis of data was carried out using a Digital MINC-11 computer system (Digital Equipment Co., U.S.A.). Cells were labeled with the dyes in the same way and wavelengths used were the same as in the measurement of the fluorescence intensities.

Results

Fluorometry of MANSOS in Solvents

Figure 1 shows the spectral profiles of maximal fluorescence of MANSOS (10^{-7} M) in various solvents including water. Excitation at 330 nm was the most effective in all of the solvents tested. Shift of the fluorescence peak to progressively shorter ranges of wavelength and simultaneous increase of the intensity were observed with increase of lipophilicity of the alcohols. In water, the fluorescence peak is in a far shorter range of wavelength than in any alcohol tested and was greatly intensified. The fluorescence maximum of MANSOS is at 455 nm in chloroform, comparable to that in H_2O. The values of the quantum yield of the fluorescence in several solvents are shown in Table I, compared with those of 2,6-mansate. The characteristics elevation of the quantum yield of MANSOS in water suggests restriction of the mobility of the molecules in this solvent, possibly by micelle formation. Very low quantum yields of MANSOS in ethanol and ethylene glycol may indicate a specific interaction of the alcoholic hydroxyl group with the dye molecule, decreasing the energy of the fluorescence.

Interaction of MANSOS with the Cell Membrane

The fluorescence maximum of MANSOS (10^{-7} M) in phosphate-buffered saline (PBS), pH 7.4, at 445 nm shifted to 430 nm when cultured cells were added to this solution at a final

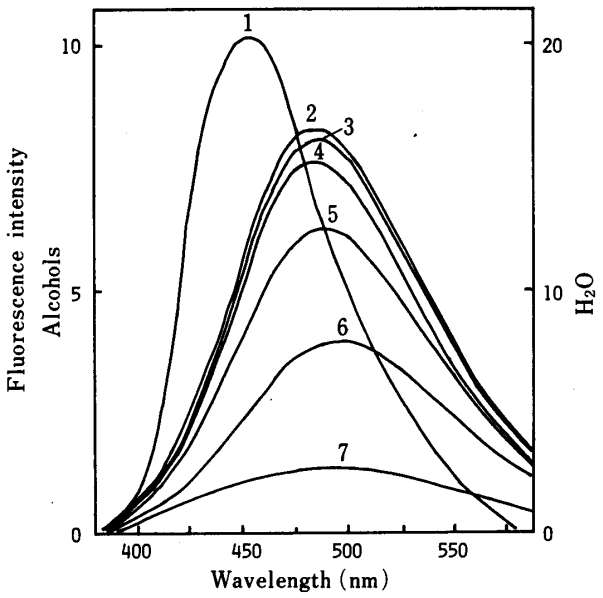

Fig. 1. Fluorescence Emission Spectra of MANSOS in Solvents

MANSOS stock solution in ethanol was added to each solvent to make 10^{-7} M dye. Excitation at 330 nm. The ordinate is expressed in arbitrary units. Refer to Materials and Methods for more details of the fluorometry. Solvents: 1, H_2O; 2, isobutyl alcohol; 3, 2-propanol; 4, *n*-butanol; 5, *n*-propanol; 6, ethanol; 7, methanol.

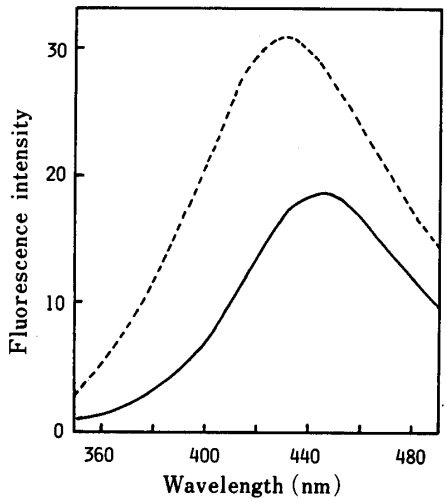

Fig. 2. Change of the Maximum MANSOS Fluorescence upon Incubation with Cells

MANSOS was added at 10^{-7} M to PBS (——) or the buffer containing Chang liver cells (103 μg protein/ml) in suspension (----).

TABLE I. Quantum Yields of MANSOS and 2,6-Mansate in Solvents

Solvent	Quantum yield			
	MANSOS*	S.D.	2,6-Mansate	S.D.
H_2O	0.2419	0.0273	0.0037	0.0001
$CHCl_3$	0.3043	0.0211	ND	
Ethanol	0.0574	0.0016	0.3697	0.0091
Ethylene glycol	0.0261	0.0001	0.2411	0.0067

The concentrations of the fluorophores used are given in Materials and Methods. S.D., standard deviation. ND, not determined.

concentration of 103 μg protein/ml (Fig. 2). The fluorescence intensity increased 1.6-fold on addition of the cells. These findings indicate a hydrophobic interaction of the fluorophore with the lipid bilayers of the plasma membrane.

Fluorescence Parameters of MANSOS in the Cell Membrane and the Effects of TPA

Each cell suspension (3.2 ml) was incubated with 5 μM MANSOS in Hepes-buffered choline medium (CHM), pH 7.4, for 30 min at a range of cellular protein concentration from 450—488 μg/ml. Cell pellets were obtained by centrifugation at 1500 rpm for 5 min, rinsed twice, and suspended in 5 ml CHM. The cells had previously been incubated with or without TPA (10^{-7} M) at 37 °C for 2 h as described in Materials and Methods. The cell suspensions were subjected to determination of the fluorescence polarization and lifetime. Figure 3 shows the Perrin plots of the relation of fluorescence polarization with temperature in TPA-pretreated and control cells. A detectable slope is observed in the plot for the control, but not

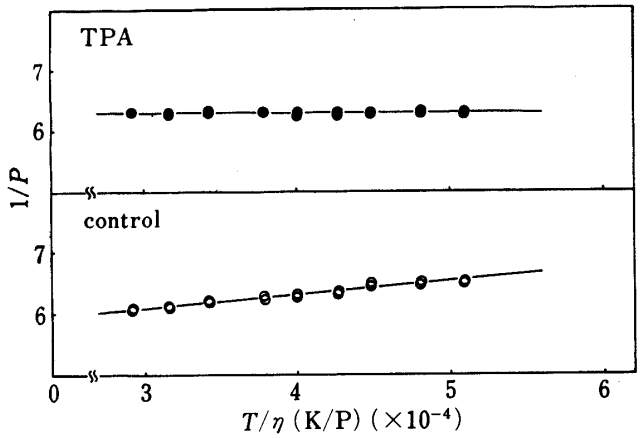

Fig. 3. Temperature-Dependence Profiles of MANSOS Fluorescence Polarization after Treatment of Cells with or without TPA

Cells were preincubated in MEM containing Hepes–Tris, pH 7.4, with or without 10^{-7} M TPA for 2 h at 37 °C. Cell suspensions in CHM were subjected to measurement of fluorescence polarization with increasing temperature after labeling of the cells with MANSOS by the procedures described in Materials and Methods. The correlation coefficients of the least-squares lines are 0.979 and −0.281 for the control and TPA, respectively.

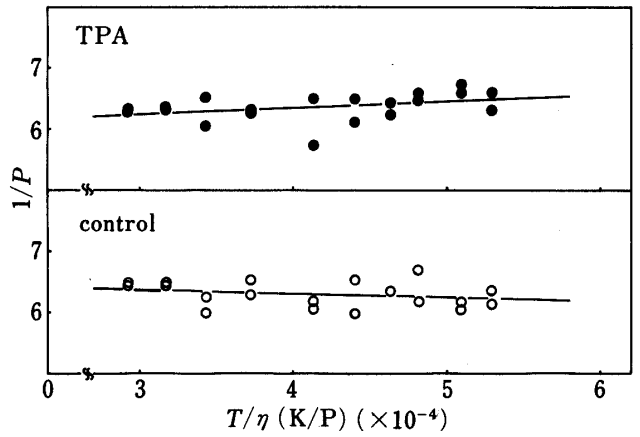

Fig. 4. Temperature-Dependence Profiles of Pyrene Fluorescence Polarization after Treatment of Cells with or without TPA

The experimental procedures were similar to those in the legend to Fig. 3, except that pyrene was used in place of MANSOS. The correlation coefficients of the least squares lines are −0.275 and 0.334 for the control and TPA, respectively.

TABLE II. Effect of Pretreatment of Cells with TPA on the Fluorescence Lifetimes (τ) and Harmonic Means of the Rotational Relaxation Time (ρ_h) of MANSOS and Pyrene Incorporated into the Cell Membrane

Fluorophore	Treatment	τ (ns)	Slope × 10^{-4} [a]	ρ_h (μs)
MANSOS	Control	12.5	0.211	0.271
	TPA	12.5	≒0	[b]
Pyrene	Control	139	≒0	[b]
	TPA	133	0.092	7.03
	Control	169	≒0	[b]
	4α-PDD	165	≒0	[b]

Cells were preincubated in MEM containing Hepes–Tris, pH 7.4, with or without 10^{-7} M TPA for 2 h at 37 °C. Cell suspensions were subjected to measurement of the fluorescence lifetimes and polarizations as described in Materials and Methods. *a*) The slope was obtained from the plots shown in Figs. 3, 4 and 5. *b*) Infinitely large on the polarization time scale.

in that for the treated cells. The lifetime of MANSOS fluorescence in the membrane showed a single component in both control and TPA-pretreated cells (Table II). The harmonic mean of the rotational relaxation time (ρ_h) of MANSOS calculated from the data thus obtained was 0.271 μs for the control cells, but it could not be determined for TPA-treated cells, since the slope in the Perrin plot was lost in these cells. The value is assumed to be infinitely large on the polarization time scale. These results suggest a decrease of the average mobility of MANSOS molecules embedded in the membrane upon treatment of cells with TPA.

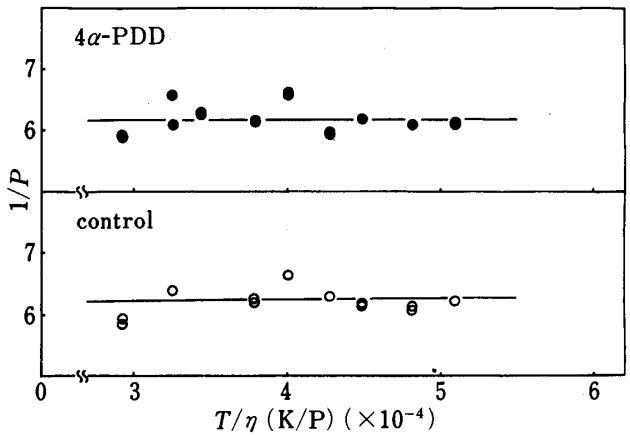

Fig. 5. Temperature-Dependence Profiles of Pyrene Fluorescence Polarization after Treatment of Cells with or without 4α-PDD

The experimental procedures were similar to those in the legend to Fig. 4, except that pretreatment of cells with TPA for 2 h was replaced by that with 4α-PDD. The correlation coefficients of the least squares lines are 0.0847 and −0.0706 for the control and 4α-PDD, respectively.

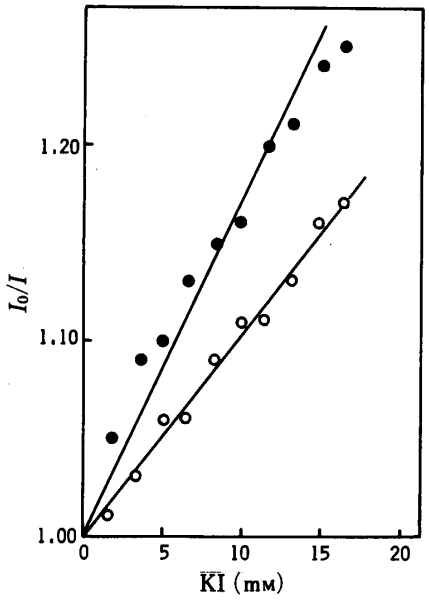

Fig. 6. Quenching Profiles of Pyrene Fluorescence with KI after Treatment of Cells with or without TPA

Cells were preincubated in MEM containing Hepes–Tris, pH 7.4, with or without 10^{-7} M TPA for 2 h at 37 °C. Cell suspensions in CHM were subjected to measurement of the fluorescence intensity with various concentrations of KI as indicated after labeling the cells with pyrene by the procedures described in Materials and Methods. ○, control; ●, TPA.

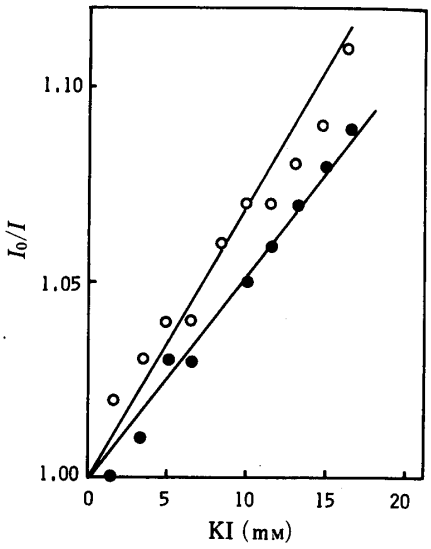

Fig. 7. Quenching Profiles of MANSOS Fluorescence with KI after Treatment of Cells with or without TPA

The experimental procedures were similar to those in the legend to Fig. 6, except that MANSOS was used in placed of pyrene. ○, control; ●, TPA.

Fluorescence Parameters of Pyrene in the Cell Membrane and the Effects of TPA

Cells were incubated with or without TPA for 2 h, then incubated with 4 μM pyrene for 10 min for labeling, under conditions similar to those used in the case of MANSOS. The Perrin plots of the change of polarization with increasing temperature showed different slopes, very small in control and 0.0917×10^{-4} P/K in TPA-pretreated cells (Fig. 4, Table II). The lifetime of pyrene was analyzed and dissociated into two components, shorter (1.4 ns) and larger (139—169 ns) in control cells. The larger component was shortened after TPA treatment as shown in Table II. The rotational relaxation time at 25 °C calculated from the data was 7.03 μs for the treated cells. It was not defined for the control.

These results suggest a substantial increase of the fluidity of the microenvironment in the cell membrane where pyrene molecules are located after the TPA treatment, and a resultant

increase of the mobility (rotation) of the dye molecules.

Effect of 4α-PDD on the Fluorescence Parameters of Pyrene in the Cell Membrane

As analogue of TPA, 4α-PDD, has been confirmed to be inactive in skin tumor promotion and not to be inhibitory on the binding of biologically active phorbol esters to their cell membrane receptor(s).[13] It was therefore tested for its effects on the fluorescence parameters of pyrene incorporated into the membrane.

Cells were incubated with or without 4α-PDD (10^{-7} M) for 2 h, then labelled with pyrene as described above. The 4α-PDD pretreatment of cells did not affect the Perrin plot of the polarization of pyrene (Fig. 5). The lifetime of pyrene was very slightly shortened by the treatment (Table II).

Quenching of the Fluorescence by KI and the Effect of TPA

The pyrene and MANSOS fluorescences of labeled cells were quenched to various extents by KI in accordance with the Stern–Volmer equation (Figs. 6 and 7). The quenching constants for pyrene and MANSOS were calculated to be 6.9×10^7 and $4.7 \times 10^7\ s^{-1}.M^{-1}$, respectively, in control cells. After the treatment of cells with TPA (10^{-7} M), the quenching of the fluorescence of pyrene was promoted (Fig. 6) and a value $11.1 \times 10^7\ s^{-1}\,M^{-1}$ was obtained for the quenching constant. The quenching of the fluorescence of MANSOS was very slightly retarded by the pretreatment with TPA (Fig. 7), and the quenching constant was $3.6 \times 10^7\ s^{-1}\,M^{-1}$.

Discussion

We have suggested that the differential changes of the fluorescence parameters of pyrene and MANSOS in the cell membrane after pretreatment of Chang liver cells with TPA are attributable to differences in the partition of these fluorophores in the membrane components and also to differential effects of TPA on the various components. Increase and decrease of the harmonic mean of the rotational relaxation time of MANSOS and pyrene after the treatment of the cells with TPA indicate depression and promotion of the rotational mobility of these fluorescent molecules in the membrane, respectively. The enhancement of KI quenching of pyrene fluorescence implies an increase of solvent access to pyrene molecules.

Pyrene is highly lipophilic, so it is reasonable to assume that it is embedded in the internal hydrocarbon domain of the lipid bilayers of the cell membrane. On the other hand, MANSOS possesses a carboxyl group at one end of the molecule and is therefore expected to be able to bind to cationic groups of membrane components such as proteins. Sulfonic dyes, 2,6-mansate[14] and 1-anilinonaphthalene-8-sulfonate[15] have been used to study lipid-protein interactions in various membrane systems and are capable of accepting energy from tryptophan of the proteins in erythrocyte ghosts and model membrane complexes, indicating that the binding sites of the dyes in these systems are very close to proteins.

Packard *et al.*[16] demonstrated by a photobleaching method with a fluorescent phospholipid as a probe that treatment of kidney cells with TPA or another active tumor promoter brought about an increase of the lateral diffusivity of the lipid phase of the cell membrane, but a decrease in the recovery of a fraction of fluorescence bound to locally clustered membrane proteins. The experimental results implied that reorganization of membrane components, especially of macromolecular scaffolding, is induced by TPA and phorbol 12,13-didecanoate.

In contrast, no difference between active (TPA and PDD) and inactive (4α-PDD) phorbol esters was found in terms of their effects on the main transition temperature, and none of these phorbol esters, whether active or inactive, had any effect on the fluorescence anisotropy, cationic permeability, electrophoretic mobility or conductivity of planar bilayers, in model membranes constructed of dipalmitoylphosphatidylcholine.[7]

Our experiments confirmed the concept of differential effects by using two different fluorophores with very contrasting nature. That is, the fluidity of the bulk membrane lipids (monitored by pyrene) is increased, or the microviscosity is decreased, whereas the residual parts of the membrane, presumably consisting of clustered proteins and boundary lipids adjacent to these proteins, to which MANSOS is preferentially bound, are decreased in fluidity by exposure of the cells to the tumor promoter.

Biologically inactive 4α-PDD did not induce changes in the fluorescence parameters of pyrene in the membrane, indicating an intimate correlation between the tumor-promoting activity and the membrane-perturbing effect of phorbol esters.

It is now believed that the receptor(s) for tumor-promoting phorbol esters in the cell membrane are related to protein kinase C.[17] It is possible that the effects of TPA on the cell membrane described above are secondary to its receptor-mediated metabolic effects, because the treatment with TPA was long enough to cause phosphorylation of cellular proteins mediated by activation of protein kinase C. Several other membrane enzymes and functional proteins such as Na^+, K^+-adenosine triphosphatase [18] and Na^+/H^+ antiporter[19] have also been found to be stimulated by treatment of cells with phorbol diesters. Further research should be concentrated on the problem of relating the TPA-induced topological and kinetic alterations in the membrane enzymes to the genetic alteration of cells leading to ultimate malignacy.

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