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The Effect of a Phorbol Ester on the Lipid Microviscosity of Two Endoplasmic Reticulum Membrane Fractions Isolated From Krebs II Ascites Cells

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Abstract This paper deals with microviscosity parameters and thermoinduced structural transitions in the lipids of smooth and heavy rough endoplasmic reticulum membranes isolated from Krebs II ascites cells incubated with the phorbol ester 12-0-tetradecanoyl-phorbol-13-acetate. The phorbol ester was found to bring about a threefold increase in the microviscosity of the lipids in heavy rough membranes. Spin probe I (2,2,6,6-tetrahydro-4-capryloyl-oxypiperidine-1-oxyl), localized in the surface layer of the membrane lipids, gave results which indicate an increased number of thermoinduced structural transitions in the smooth membranes in the treated cells due to the transitions occurring at relatively low temperature and a decreased number of such transitions in the heavy rough fraction especially at high temperature. For 5,6-benzo-2,2,4,4-tetramethyl-1,2,3,4-tetrahydro-γ-carboline-oxyl, probe II, mainly distributed in the annular lipids, a decrease in the number of low temperature transitions in the smooth fraction was observed, while an increase occurred in the heavy rough one. The results obtained are discussed in terms of the effect of phorbol esters as promoters of tumor progression.

Key words: rough membranes, smooth membranes, structural transitions, transformation, spin probes

Phorbol esters are known to possess the ability of modulating the activity of important regulatory cellular systems such as phosphatidylinositol turnover [1–3] and production of cyclic nucleotides [4,5]. They also result in the transformation of normal cells [6,7] and potentiate the malignancy of already transformed ones [8,9]. Their effect is primarily directed at membranes whose composition and physico-chemical properties undergo marked changes upon contact with these compounds [10–14].

Due to the fact that membranes from tumor cells differ greatly from those in normal cells in such properties as intensity of lipid peroxidation [15] and their microviscosity [16], it was considered important to gain insight into the changes involved in the viscosity properties of the membranes after cells were treated with phorbol esters.

Three subfractions of endoplasmic reticulum (ER) membranes (S, smooth; LR, light rough; HR, heavy rough) have been earlier reported in the following transformed cell lines: L-929, MPC-11, Krebs II ascites, and HeLa cells [17–20], while in normal cells (C3H/10T$^{1/2}$) and tissues such as mouse liver, kidney, and heart, only the S and LR fractions have been observed [17,18]. The HR fraction, however, was observed in C3H/T10$^{1/2}$ cells after a 24 h period of incubation with the tumor promoter TPA [18]. The HR fraction, which appears to be specific for transformed cells, has been shown to differ from the LR fraction in a variety of parameters [for review, see reference 21]. The early features of TPA action on Krebs II ascites cells consisted of a stimulation in phosphatidylcholine synthesis in both S and LR membranes, but not in HR membranes [10], while incubation of cells with the non-tumor promoting phorbol ester 4α-phorbol 12, 13-didecanoate (4αPDD) had no effect on the endoplasmic reticulum subfractions (unpublished data). In a recent study Record et al. [22] found that acetyltransferase was the only marker enzyme to coincide with ^{3}H-uridine labeled RNA in a gradient fraction equivalent to HR membranes.

The purpose of this work was, therefore, to study the microviscosity and thermoinduced

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structural transitions in the membrane lipids of S and HR membranes isolated from Krebs II ascites cells, either untreated or treated with 12-0-tetradecanoyl-phorbol-13-acetate (TPA). In this study we used two stable iminoxyl radicals which localize mainly in the lipid and annular lipid layers of membranes, respectively, and are characterized by possessing low and high hydrophobicity factors. The probes chosen: 2,2,6,6-tetrahydro-4-capryloyl-oxypiperidine-1-oxyl (probe I) and 5,6-benzo-2,2,4,4-tetramethyl-1,2,3,4-tetrahydro-γ-carboline-3-oxyl (probe II) were convenient for the designed experiments since they localize in surface areas of membrane lipid layers [23–25]. These are directly affected by phorbol esters which do not penetrate deeply into the lipid bilayer [26].

MATERIALS AND METHODS

Krebs II ascites cells, a transformed cell line, were propogated in Balb/c mice, and then maintained in culture (19). TPA in acetone (at a concentration of $1 \cdot 10^{-3}$ M) was added to the culture medium at least 24 h before use and an equal volume of acetone was added to a similar volume of medium to be used for control cells. Cells were harvested from mice, washed, and then resuspended directly in the culture medium containing TPA at a final concentration of $1.7 \cdot 10^{-7}$ M or acetone [10,27]. Incubation was performed in roller suspension culture for 24 h.

Cells were disrupted by nitrogen cavitation [28]. The HR and S ER membranes were separated by centrifugation on discontinuous sucrose gradients as described elsewhere [10,19, 28].

The protein concentration was measured by the method of Lowry et al. [29]. The microviscosity of the isolated membrane fractions was estimated from the rotational correlation time (τ_c) for probes I and II [30]. The probes, dissolved in alcohol (3.10^{-3} M), were added to the membrane suspensions containing ca. 3 mg protein/ml to a final concentration of 10^{-5}M. The ESR spectra were recorded with a Brucker 2000 D radiospectrometer (FRG) in a temperature range between 10° and 50°C with a thermostatting accuracy of ± 0.1°C. The τ_c value was determined using a formula for fast-rotating radicals [25,30].

RESULTS AND DISCUSSION

The effect of TPA on the τ_c temperature-dependent values in the range between 10° and 50°C was studied in S and HR subfractions of ER membranes isolated from Krebs II ascites cells [10,17,19]. In L-929 cells these two fractions of ER were shown to differ to a great extent both in microviscosity and thermoinduced structural transitions (results to be published).

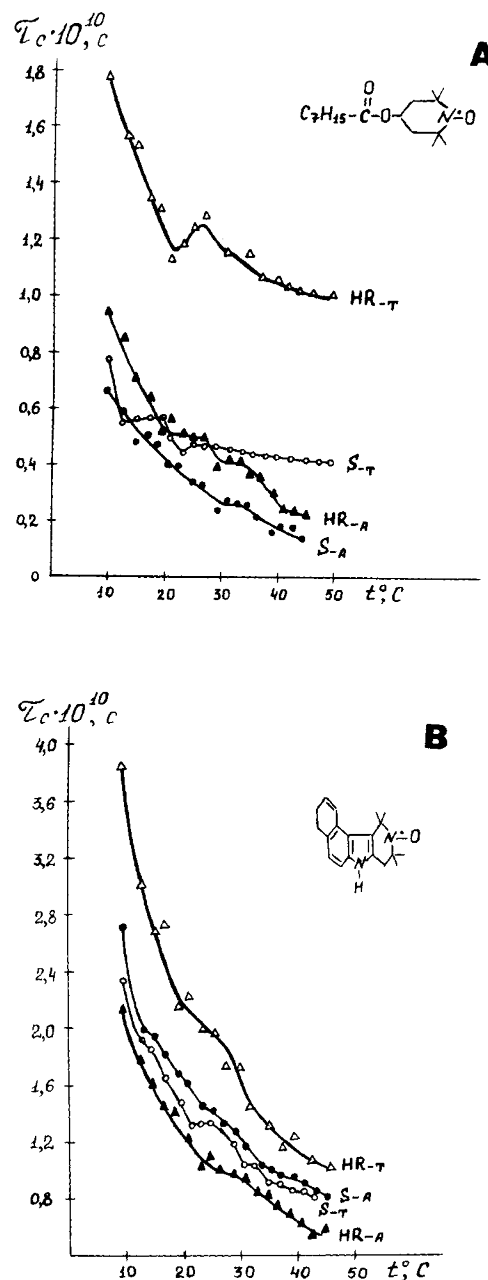

Fig. 1. Changes in rotational correlation time (τ_c) at various temperatures measured in smooth (S) and heavy rough (HR) endoplasmic reticulum membranes isolated from untreated (S_A and HR_A) and TPA treated (S_T and HR_T) Krebs II ascites cells. **A:** Probe I; **B:** Probe II. Each point represents the value obtained from three estimations (deviation ± 5%). See Materials and Methods for experimental details.

Figure 1A illustrates the temperature dependence of the τ_c values for probe I for S and HR membranes from Krebs II ascites cells. It can be seen that in the control group the HR fraction has higher τ_c values than for the S membranes, i.e., the former membranes are more viscous. After treating the cells with TPA, however, an increase in rotational correlation time was observed in both membrane fractions, the degree of increase being highest for HR membranes. The temperature dependency of microviscosity was less evident in both ER subfractions from TPA-treated cells, especially between 20° and 50°C.

Table I presents microviscosity values measured at 37°C, i.e., culture incubation temperature, and also at 25°C, the temperature at which microviscosities are most frequently reported [16]. It is evident from the table that at 37°C, HR membranes from TPA treated cells show an elevated τ_c value for probe I by a factor of 3, whereas for the S fraction a twofold increase was observed. At 25°C the difference between microviscosities obtained for membranes from treated and untreated cells was less distinct.

Figure 1B shows the temperature dependency of τ_c for probe II in the ER membrane fractions. In control cells the microviscosity of the HR fraction is lower than that in S membranes. After treating cells with TPA these fractions behave differently: the S fraction shows a small, statistically insignificant drop in microviscosity at temperatures over 30°C, whereas the HR fraction displays a twofold increase in this parameter both at 25° and 37°C (see Table I). Thus in cells treated with the phorbol ester, a noticeable increase was observed in the membrane lipid microviscosity of HR membranes, i.e., a membrane fraction which is apparently specific to transformed cells. At the physiological temperature this increase may be as high as 3. The results indicate that in Krebs II cells the differences in microviscosity between S and HR subfractions are greatest in surface membrane lipid layers (probe I).

In order to detect thermoinduced structural transitions in ER membranes, the results are shown in Arrhenius-type plots (Figs. 2,3) [23,31,32]. Figure 2A indicates that in the control S fraction probe I has two transitions, at 31° and 35°C. After TPA treatment, however five temperature transitions were observed: at 15°, 19°, 23°, 29°, and 31°C, each of which is below the physiological. In the control HR fraction (Fig. 2B) there are four transitions, at 19°, 27°, 37°, and 41°C, i.e., below, equal to, or above the physiological value, whereas in treated cells three transitions were detected (at 19°, 27°, and 29°C). In the case of HR membranes it thus appears that TPA treatment leads to the disappearance of thermoinduced transitions at temperatures equal to and above the physiological. The results shown in Figures 2 and 3 indicate that the changes observed in viscosity at the membrane surface were of a greater magnitude than those occurring in the apolar core. Such changes may perhaps reflect earlier observations where marked differences were observed in the rates of incorporation of ^{3}H-choline into phospholipids in S and HR membranes in Krebs II ascites cells during a 24 h incubation with TPA [10].

The results demonstrate that TPA exerts a number of different effects on the microviscosity of S and HR membranes. First, there is an increase in the number of thermoinduced transitions for probe I in the S fraction, especially at relatively low-temperature, and a decrease in the number of such transitions in the HR fraction due to the appearance of a higher number of high-temperature ones. As a result, the "portrait" of rearrangements for probe I in the S and HR membranes is more similar than that observed in control cells.

TABLE I. Rotational Correlation Times ($\tau_c \cdot 10^{10}$ s) Estimated at 25° and 37° for Probes I and II in the Membrane Lipids of S and HR Fractions Isolated From Untreated and TPA-Treated Krebs II Ascites Cells*

		Untreated		TPA-treated	
Fraction	Temperature, °C	Probe I	Probe II	Probe I	Probe II
S	25	0.35 ± 0.02	1.45 ± 0.02	0.53 ± 0.02	1.26 ± 0.05
	37	0.21 ± 0.03	0.98 ± 0.02	0.44 ± 0.02	0.96 ± 0.04
HR	25	0.50 ± 0.04	1.02 ± 0.02	1.25 ± 0.05	2.00 ± 0.05
	37	0.36 ± 0.03	0.65 ± 0.02	1.08 ± 0.03	1.20 ± 0.02

*The results are from three estimations (± S.D.). See Materials and Methods for experimental details.

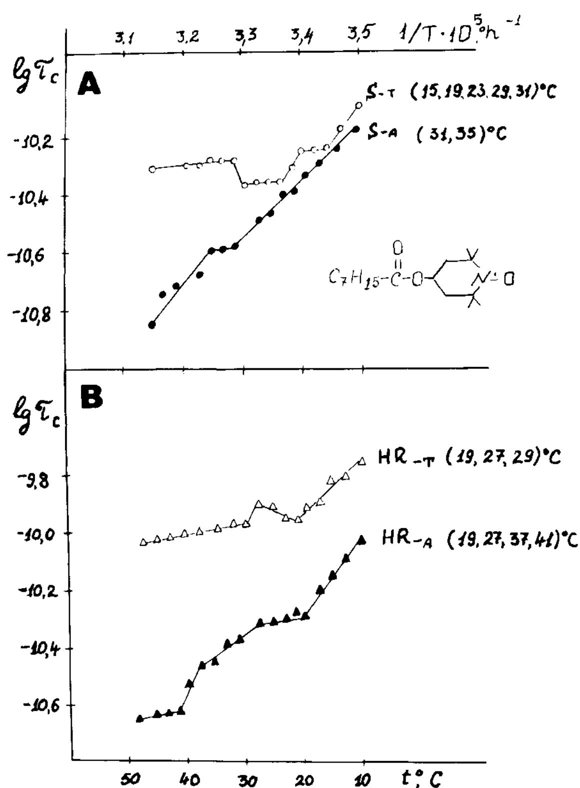

Fig. 2. Arrhenius-type plots of the τ_c values obtained with probe I for (A) smooth (S) and (B) heavy rough (HR) endoplasmic reticulum membranes isolated from untreated (S_A, HR_A) and TPA treated (S_T, HR_T) Krebs II ascites cells. Each point represents the value obtained from three estimations (deviation ± 5%). See Materials and Methods for experimental details.

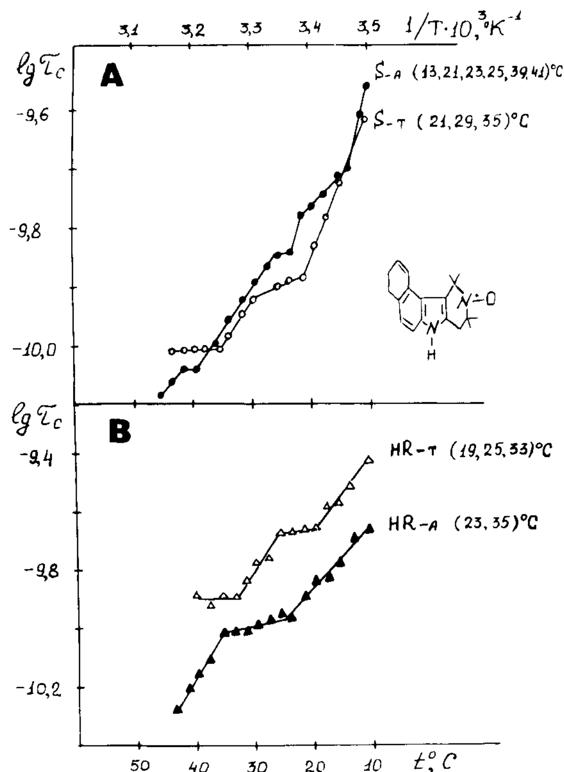

Fig. 3. Arrhenius-type plots of the τ_c values obtained with probe II for (A) smooth (S) and (B) heavy rough (HR) endoplasmic reticulum membranes isolated from untreated (S_A, HR_A) and TPA treated (S_T, HR_T) Krebs II ascites cells. Each point represents the value obtained from three estimations (deviation ± 5%). See Materials and Methods for experimental details.

Figure 3 depicts data for transitions resulting from the use of probe II. The S fraction in the control group has six transitions, at 13°, 21°, 23°, 25°, 39°, and 41°C (Fig. 3A). After TPA treatment the number dropped to three, with the disappearance of the lowest value and the two values above physiological temperature. In control cells the HR fraction showed two transitions, at 23° and 35°C (Fig. 3B). In TPA-treated cells, however, a further low-temperature transition occurred (at 19°C), while the other two were somewhat shifted along the temperature scale (to 25° and 33°C).

Thus, for probe II the effect of the phorbol ester on the ER fractions is different in that the number of thermoinduced structural transitions shows a significant drop in the S fraction and a similar rise in the HR one. As this takes place, the trend of rearrangement of the transition temperature interval in these fractions is also different: the HR fraction shows the appearance of a relatively low-temperature transition (at 19°C), whereas in S membranes the low temperature transition disappears (at 13°C).

From the "portrait" of thermoinduced structural transition for S and HR membranes in control and TPA treated cells, it can be suggested that probes I and II are sensitive to variation in experimental conditions. Thus, in the S fraction, with the less hydrophobic probe (probe I), the effect of TPA results largely in the appearance of a variety of low-temperature transitions, whereas in the case of the more hydrophobic probe II such transitions disappear both in the low- and high-temperature regions. In the HR fraction the trend for the two probes is the same: for probe I a shift towards the lower temperature and for probe II appearance of a low-temperature transition.

Under the effect of the phorbol ester, the "portrait" of thermoinduced structural transitions in the S fraction undergoes more significant changes than in the case of HR membranes. This conclusion is additionally supported

by the fact that in the S fraction from TPA treated cells, two transitions appear common to the two probes (at 21–23° and 29°C), whereas in the control cells no common rearrangements take place. In the control HR fraction only one transition common to the two probes was observed at physiological temperature (35–37°C), whereas in treated cells the presence of two transitions was seen even at lower temperatures (19° and 25–27°C). The presence of a common *structural* transition in the lipid bilayer (probe I) and in the annular layers of the membrane lipids (probe II) only at physiological temperature has been demonstrated to provide conditions which promote the activity of membrane-bound enzyme complexes [32,33]. It can be therefore suggested that incubation of cells with TPA creates a less favourable environment in HR membranes, i.e., in the fraction specific with regard to the transformed cell.

It is also evident that in TPA-treated cells the ER fractions are more similar than those in the control. The same was observed both regarding the temperature scale of each of the probes and the appearance of common rearrangements detected by probes I and II. The first transition occurs at 19° and 21–23°C in the HR and S fractions, respectively, and the second one at 25–27°C and 29°C, respectively. This probably indicates the formation of a more cooperative ER structure upon treatment of cells with TPA.

Returning to the microviscosity of ER membranes, it should be pointed out that TPA, while leveling out differences between the two fractions with respect to the thermoinduced structural transitions, separates them on a membrane "rigidity" scale. Thus, in fractions from control cells where a high microviscosity is additionally characterized by the presence of a large number of transitions (probes I and II for the HR and S fractions, respectively), the same "rule" does not apply in TPA treated Krebs II cells. Under these conditions a marked increase in the viscosity in the HR fraction does not involve the appearance of a greater number of structural rearrangements.

Due to the fact that phorbol esters advance the *transformed cell* towards tumor progression [8,9], the enhanced viscosity of HR membrane lipids occurring with a simultaneous approach toward similarity in the thermoinduced structural transition patterns in the HR and S lipids can be probably interpreted as a further change in the ascites cell ER and a strengthening of its *tumor forming characteristics.* An increased rigidity in the microsomal membranes from ascites cells has been earlier reported using fluorescent and spin probes [34].

An alteration in the lipid composition of a membrane is considered to be a contributing factor in the ability of a membrane to undergo a modification in morphology such as formation of the hexagonal phase [35]. It is thus possible that the observed enhancement in microviscosity of membrane lipids in the HR fraction caused by the phorbol ester may have resulted in a modulation of the activity of membrane associated enzyme systems. It can be mentioned that a well-defined membrane structure is known to be essential for the functioning of such important enzymes as adenylate cyclase and membrane-bound protein kinase C [36,37], the latter being activated by phorbol esters and representing one of the major receptors for these esters. It is likely that the conformational dynamics of protein kinase C are modulated by the lipid environment [37].

In a previous paper it was suggested that phorbol esters may modify the mechanism by which polysomes are attached to the rough ER [21]. Such an effect may be manifested through the production of an enhanced rigidity of lipids in HR membranes, an ER subfraction found specifically in transformed cells [17–20]. Thus TPA, by causing changes in membrane lipid composition and subsequently altering membrane microviscosity, may have promoted a change in the manner of association between polysomes and a species of rough ER membranes, resulting in the formation of the HR subfraction. Polysomes in this fraction have been shown to be loosely associated with the membrane [38,39], in contrast to the situation in LR membranes [the rough ER component found in normal cells and tissues: reference 17,18], where tight association prevails [38]. Future work will be directed at gaining a better understanding of whether or not phorbol esters, by modifying membrane lipid composition and microviscosity, result in subtle changes in the protein synthetic apparatus associated with ER membranes.

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