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Direct Evidence of Incorporation of 12–O–[120–$^2H_1$] Tetradecanoylphorbol–13–acetate into Artificial Membranes as Determined by Deuterium Magnetic Resonance*

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12-O-Tetradecanoylphorbol-13-acetate (TPA) is one of the most potent tumor promoters in skin carcinogenesis. As in the case of endogenous hormones or xenobiotic drugs, the first step in initiating a biological effect is believed to be the specific binding of TPA to cell receptors. Cell receptors for phorbol esters were identified in the membrane fraction of several types of cells and isolated receptor protein was recently analyzed. It was shown that this receptor protein requires phospholipid as well as cations for binding. The importance of phospholipid in the receptorsites of epidermis was also confirmed by Esumi and Fujiki. Pretreatment of the membrane fraction with phospholipase A_2 or C reduced the specific binding of TPA by 50%, while pretreatment with proteinase K reduced the binding by 27%. In addition, Deleers and Malaisse reported that the specific binding of phorbol esters in cell membranes is almost completely duplicated in artificial liposome membranes. These lines of experimental evidence suggest that the insertion of phorbol esters into the phospholipid domain of membranes does occur but this is difficult to prove by experiments with radioactive compounds.

Here, we attempt to elucidate the manner of interaction of TPA with cell membrane by analyzing the ^2H-NMR spectra of [20-$^2H_1$]TPA and the ^{31}P–NMR spectra in the system of dimyristoylphosphatidylcholine (DMPC) multibilayers.

TPA, phorbol 12,13-diacetate (PDA) and 20-oxo-20-deoxyphorbol 12-myristrate 13-acetate (PMAAL) were purchased from Consolidated Midland Corporation, New York, USA. [20-$^2H_1$]TPA was prepared by reduction of PMAAL at 0℃ in abs. MeOH by NaB^2H_4, followed by chromatographic purification on thin-layer plates of silica gel. Lamellar multibilayer dispersion, including TPA or PDA, was prepared by dissolving a stock solution (in acetone) of TPA or PDA into a chloroform solution of DMPC, followed by evaporation of these solvents completely *in vacuo* and hydration by distilled or ^2H-depleted water.

The ^2H- and ^{31}P–NMR spectra were recorded with a Bruker CXP-300 spectrometer at 46.06 and 121.5 MHz, respectively (7.0 T field strength). The high power ^2H-NMR

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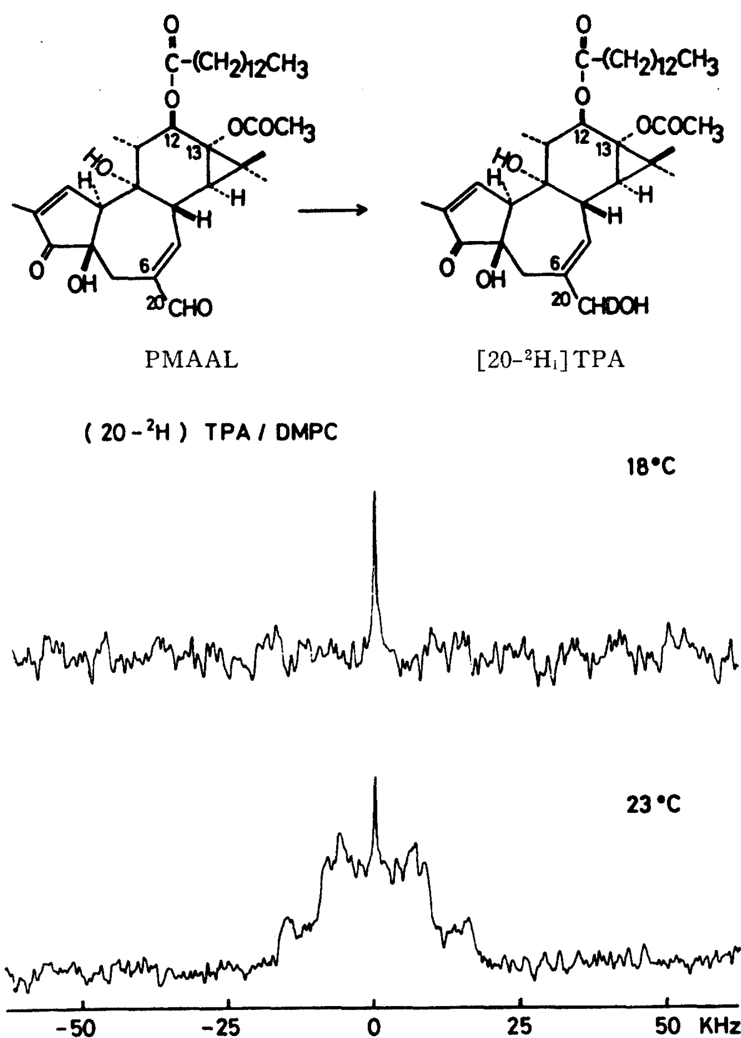

Fig. 1. 46.06 MHz ^{2}H-NMR spectra of [20-$^{2}H_{1}$]TPA (12.3 mol%) in the presence of DMPC multibilayers (0.211mM). 90° pulse 8 μs; $\tau_1 = \tau_2 = 20\ \mu$s; recycle time 0.3s. Number of transients: bottom, 97176; top, 72834.

spectra were recorded by the quadrupole echo method, while the high resolution ^{31}P-NMR spectra were recorded by the pulse sequence of the gated ^{1}H-decoupling to prevent sample heating. Figure 1 shows the high power ^{2}H-NMR spectra of [20-$^{2}H_{1}$]TPA (12.3 mol%) in DMPC liposomes (0.211 mM in ^{2}H-depleted water) at temperatures below and above the liquid crystalline-gel phase transition (21℃). In the liquid crystalline phase (23℃, bottom trace), several pairs of the doublet peaks (30.7, 16.7 and 12.3 kHz) occur, but they are considerably suppressed in the gel phase lipid (18℃, top trace). The sharp central line derives from the residual HDO signal, which could not be removed during preparation of the liposomes. The absolute dependency of the ^{2}H-NMR spectrum on the phase transition clearly indicated that TPA molecules are incorporated within the

bilayer of DMPC.

The observed ^2H quadrupole splittings (D_q) are much smaller than that expected from the static value corresponding to solid molecules (172 kHz). Obviously, such reduction of the D_q is associated with the presence of internal rotation or anisotropic molecular motion as a whole. The most probable internal motion is the three-fold rotation of the hydroxymethyl group at 20-position. Nevertheless, the expected quadrupole splitting from this model is about 40 kHz which is still larger than the observed values. Therefore, it is obvious that further averaging of the quadrupole interaction occurs at certain axes at the C_{12}-O and C-C linkages at the ester bonds. The apparent 3 pairs of the D_q could be ascribed to the presence of 3 apecies whose motional states are different. Alternatively, such splittings could be explained by the asymmetric electric field gradient which was created by partial molecular motion. In any case, data are consistent with the assumption that the acyl chain of TPA is incorporated in the bilater, while the bulky tigliane portion is outside the bilayer. ^{31}P-NMR spectroscopy was applied to monitor the TPA-induced perturbation of liposome organization. ^{31}P Chemical shift anisotropy, the width of the power pattern of DMPC multibilayers, was decreased by 7.4% and 1.9% by incorporation of 33% TPA and PDA, respectively. In the presence of 16% phorbol esters, the decrease of the chemical shift anisotropy was 3.8% for TPA but negligible for PTA. The contrasting effect of PDA could be explained on the basis that this molecule with shorter aliphatic chain was not effectively incorporated into lipid bilayers.

It is noteworthy that similar ^2H quadrupole splittings and reduced ^{31}P chemical shift anisotropy were observed for selectively deuterated bile acids in phospholipid bilayers. Bile acids are moderate tumor promoters in colon carcinogenesis and they are unique in that no specific receptor for binding exists in colon tissues. The interaction with phospholipid membrane could be a common feature of several class of tumor promoters and the effect would be efficiently amplified in the presence of specific receptors.

The precent approach was also successfully applied to other potent tumor promoters such as teleocidins.