Effects of Diacylglycerols on Conformation of Phosphatidylcholine Headgroups in Phosphatidylcholine/Phosphatidylserine Bilayers

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ABSTRACT The effects of five diacylglycerols (DAGs), diolein, 1-stearoyl,2-arachidonoyl-*sn*-glycerol, dioctanoylglycerol, 1-oleoyl,2-*sn*-acetylglycerol, and dipalmitin (DP), on the structure of lipid bilayers composed of mixtures of phosphatidylcholine and phosphatidylserine (4:1 mol/mol) were examined by ^2H nuclear magnetic resonance (NMR). Dipalmitoylphosphatidylcholine deuterated at the α- and β-positions of the choline moiety was used to probe the surface region of the membranes. Addition of each DAG except DP caused a continuous decrease in the β-deuteron quadrupole splittings and a concomitant increase in the α-deuteron splittings indicating that DAGs induce a conformational change in the phosphatidylcholine headgroup. Additional evidence of conformational change was found at high DAG concentrations ($\geq$20 mol%) where the α-deuteron peaks became doublets indicating that the two α-deuterons were not equivalent. The changes induced by DP were consistent with the lateral phase separation of the bilayers into gel-like and fluid-like domains with the phosphatidylcholine headgroups in the latter phase being virtually unaffected by DP. The DAG-induced changes in α-deuteron splittings were found to correlate with DAG-enhanced protein kinase C (PK-C) activity, suggesting that the DAG-induced conformational changes of the phosphatidylcholine headgroups are either directly or indirectly related to a mechanism of PK-C activation. ^2H NMR relaxation measurements showed significant increase of the spin-lattice relaxation times for the region of the phosphatidylcholine headgroups, induced by all DAGs except DP. However, this effect of DAGs did not correlate with the DAG-induced activation of PK-C.

INTRODUCTION

Protein kinase C (PK-C) is represented by a family of isoforms found in most animal cells and tissues and is particularly concentrated in the nervous system. The enzyme family performs a pivotal function in the intracellular signaling network (Nakamura and Nishizuka, 1994) and is considered to play a key role in numerous cellular processes including exocytosis, extracellular signal transduction, and cell growth and differentiation and probably performs an important function in synaptic transmission (Nishizuka, 1986). This regulatory enzyme is activated in vivo by the class of endogenous lipid second messengers, 1,2-*sn*-diacylglycerols (DAGs), which are produced as a result of a stimulus-induced activation of phospholipases (Berridge, 1987; Nakamura and Nishizuka, 1994), which in turn is initiated by numerous extracellular biological agents such as hormones, neurotransmitters, and growth factors.

Most current models of PK-C activation involve association of the inactive, cytosolic form of the enzyme with lipid membranes (Nelsestuen and Bazzi, 1991; Bell and Burns, 1991; Zidovetzki and Lester, 1992) where it binds to acidic lipids, primarily phosphatidylserine (PS), in a Ca^{2+}- and DAG-dependent manner (Castagna et al., 1982; Nishizuka, 1986). The efficiency of this process would be expected to depend on physicochemical properties (e.g., phase, fluidity, acyl side chain order, and domain formation) of the lipid membranes. Indeed, lipid bilayer structure has been implicated as an important factor in both protein-lipid interactions and activity of membrane proteins and membrane-active enzymes, particularly the phospholipases (Wilschut et al., 1978; Menashe et al., 1981; Jain and Jahagirdar, 1985; Romero et al., 1987; Gheriani-Gruszka et al., 1988; Jain and Berg, 1989; Roberts and Dennis, 1989; Sen et al., 1991; Zidovetzki et al., 1992).

It has been suggested (Epand, 1985; Das and Rand, 1986; Bolen and Sando, 1992; Zidovetzki and Lester, 1992; Senisterra and Epand, 1993; Slater et al., 1994) that DAG-induced physical perturbations in the lipid membranes may contribute to increased PK-C activity. DAGs induce structural changes in lipid membranes such as alterations of membrane curvature, modification of surface charge (Ohki et al., 1982), promotion of nonbilayer lipid phases (Dawson et al., 1984; Epand, 1985; Das and Rand, 1986; Epand and Bottega, 1988; Siegel et al., 1989; De Boeck and Zidovetzki, 1989; Ortiz et al., 1992; López-García et al., 1994a), and altered phospholipid headgroup spacing within the bilayer (Das and Rand, 1986; Bolen and Sando, 1992; De Boeck and Zidovetzki, 1992; Slater et al., 1994). These effects were interpreted as a destabilization of the bilayers and were suggested to play a role in DAG-induced activation of PK-C and phospholipases. DAG-induced nonbilayer phases have been shown to modulate the activity of the intracellular and secretory phospholipases A_2 and C (Dawson et al., 1984; Buckley, 1985; Sen et al., 1991; Zidovetzki et al., 1992).

Received for publication 10 March 1995 and in final form 6 June 1995.

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0006-3495/95/09/965/09 $2.00

Variations in the hydrophobic portion of the activating phospholipids affect the lipid-dependent activation of PK-C and the action of DAG. Bolen and Sando (1992) showed that, in the absence of other unsaturated phospholipids, dioleoylphosphatidylserine (DOPS), but not dimyristoylphosphatidylserine (DMPS), could activate PK-C. This indicates that the presence of the acidic PS headgroup with all of the cofactors is not sufficient for PK-C activation, which also requires the presence of unsaturated acyl chains on either the PS or phosphatidylchoine (PC) lipid component. Both the degree of unsaturation and the length of the DAG fatty acid chain affect its capacity to activate PK-C (Kishimoto et al., 1980; Cabot and Jaken, 1984; Lapetina et al., 1985; Bonser et al., 1988; Snoek et al., 1988; Goldberg et al., 1994). These studies indicate that DAGs generate physical perturbations in the bilayer structure that may be sensed by PK-C and modulate its activation. Additionally, many agents that affect lipid membrane structure also modulate PK-C activity, supporting the view that physical properties of the membrane play a role in the PK-C activation process (Epand, 1987; Epand and Lester, 1990). However, not every type of lipid bilayer defect is associated with increased PK-C activity. It has been shown that the formation of membrane defects at the boundary between gel and liquid crystalline domains found at temperatures near the gel-to-liquid crystalline phase transition temperature in palmitoyloleoylphosphatidylserine vesicles (Senisterra and Epand, 1993) or those induced by dipalmitin (DP) in PC/PS bilayer mixtures (Goldberg et al., 1994) are not sufficient to promote PK-C activity.

Previously we have conducted ^{2}H nuclear magnetic resonance (NMR) studies of the hydrophobic region of the bilayer to characterize perturbations induced in PC/PS bilayers generated by a series of saturated and unsaturated DAGs and related those effects to the degree to which each of the DAGs induced PK-C activation (Goldberg et al., 1994). We have now correlated the DAG-induced activation of PK-C with the effects of DAGs on the polar headgroup region of the lipid bilayers. We used dipalmitoylphosphatidylcholine (DPPC) deuterated at the α- and β-positions of the choline headgroup as a ^{2}H NMR probe. ^{2}H NMR of headgroup-deuterated lipids has been extensively used to study a variety of factors affecting conformation of the lipid headgroups, such as the effects of polypeptides and proteins (Roux et al., 1989; Morrow et al., 1993; Hayer-Hartl et al., 1993), membrane potential (Leenhouts et al., 1993), size of lipid vesicles (Marassi et al., 1993a), inhomogeneity of the lateral distribution of charged membrane components (Marassi et al., 1993b), ethanol (Barry and Gawrisch, 1994), and many other charged and zwitterionic molecules (see Discussion). The use of the molecular voltmeter (Scherer and Seelig, 1989) and choline tilt (Macdonald et al., 1991) models allowed us to estimate the change, induced by DAGs, in the tilt of the choline headgroup relative to the bilayer surface.

MATERIALS AND METHODS

1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine-1,1,2,2-d4 (DPPC-d_4), diperdeuteriopalmitoylphosphatidylcholine (DPPC-d_{62}), dipalmitoylphosphatidylserine (DPPS), diperdeuteriopalmitoylphosphatidylserine (DPPS-d_{62}), and PC extracts from bovine liver were purchased from Avanti Polar Lipids (Alabaster, AL). 1,2-*sn*-Dioctanoylglycerol (diC$_8$), 1-stearoyl,2-arachidonoyl-*sn*-glycerol (SAG), and 1-oleoyl,2-acetyl-*sn*-glycerol (OAG) were obtained from Serdary Research Laboratories (London, Ontario, Canada). DP and diolein (DO) were purchased from Sigma Chemical Co. (St. Louis, MO).

Multilamellar lipid dispersions were prepared by mixing phospholipids disolved in chloroform and adding hexane- or chloroform-dissolved DAGs for phospholipid/DAG mixtures. The solvent was then evaporated with a stream of dry nitrogen and the sample was placed under a vacuum (<1 mtorr) for at least 8 h. The resulting thin lipid film was then hydrated with 25 mM Tris (2-amino-2-(hydroxymethyl)-propane-1,3-diol) (pH 7.4) buffer solution with 1 mM EDTA, prepared in ^{2}H-depleted H_2O (Sigma). The samples were always fully hydrated and were typically 1:10 (w/w) in lipid/water. As magnetic orientation of lipid bilayers is often observed at high magnetic field strength, spectra were examined for characteristics of orientation, such as low intensity in the low frequency shoulder of ^{31}P NMR spectra (Brumm et al., 1992; Qiu et al., 1993) or unusually high intensity in the wings of ^{2}H NMR spectra of DPPC-d_{62} or DPPS-d_{62} (Brumm et al., 1992). We consistently observed orientation only with OAG-containing samples. The samples with OAG were therefore made at reduced hydration (1:3 to 1:1, w/w in lipid/water), which completely abolished the orientation (Qiu et al., 1993). We made other samples, including controls, at the lower hydration levels and obtained results identical with those obtained at high hydration. A uniform lipid suspension was obtained by five freeze-thaw cycles (Westman et al., 1982; Mayer et al., 1985). The composition of the phospholipids was bovine liver PC/DPPC-d_4/DPPS = 3:1:1 (mol/mol/mol) giving the molar ratio of PC:PS as 4:1. For the relaxation measurements of the lipid acyl side chains, DPPC-d_{62} or DPPS-d_{62} were substituted for DPPC-d_4 or DPPS, respectively.

^{2}H NMR spectra were acquired at 11.74 T (corresponding to 500.13 MHz ^{1}H and 76.78 MHz ^{2}H frequencies) on a General Electric GN500 spectrometer using a high power, wide-line probe (Doty Scientific, Columbia, SC) and the standard quadrupole echo sequence (Davis et al., 1976). The spectral width was 0.5 MHz and refocusing time was 64 μs with a 90° pulse of 3.5 μs and typically 16,000 scans per spectrum. The relaxation delay was 200 ms for most measurements and 3 s for the relaxation experiments where longer T_{1z} (peak 13 of the chain-perdeuterated lipids) were determined. Spectra were de-Paked according to Sternin et al., (1983) using a computer analysis program, modified for the IBM personal computer, kindly supplied by Dr. S. Wassall. De-Paked spectra were compared with the original spectra to ensure that the features of the spectra were maintained through the de-Paking process. Spin-lattice relaxation (T_{1z}) times were measured by inversion recovery sequence (180° - τ - 90° - Δ - 90° - Δ). T_{1z} values for DPPC-d_{62} and DPPS-d_{62} spectra were determined using de-Paked spectra.

Choline tilt model

Use of the choline tilt model, as derived by Macdonald et al. (1991), provides a rough estimate of the change in tilt of the P$^+$-N$^+$ vector relative to the bilayer normal from changes in the quadrupole splittings of the headgroup-deuterated PC. Straightforward rearrangement of the equations in Macdonald et al., (1991) gives the following equation:

$$\sin\theta = \sin\theta_N \frac{[1/3 + (\Delta\nu_\alpha/\Delta\nu_Q) \times (2/3S_f)]^{1/2}}{[1/3 + (\Delta\nu_{\alpha N}/\Delta\nu_Q) \times (2/3S_f)]^{1/2}} \quad (1)$$

where θ is the angle formed between the P$^+$-N$^+$ vector and the bilayer normal, θ_N and $\Delta\nu_{\alpha N}$ are the P$^+$-N$^+$ vector angle and the quadrupole splitting, respectively, at a standard condition where both θ_N and $\Delta\nu_{\alpha N}$ are known (see below), $\Delta\nu_Q$ is the static quadrupolar coupling constant (170

kHz, Burnett and Muller, 1971) for a deuteron of a C-D bond, S_f is the order parameter, assumed to be 0.25 (Macdonald et al., 1991), and $\Delta\nu_\alpha$ is the measured quadrupole splitting.

The standard condition for θ_N and $\Delta\nu_{\alpha N}$ can be found in the literature and correspond to pure PC bilayers at low hydration. X-ray structural analysis data using dioleoylphosphatidylcholine (Wiener and White, 1992) showed that the angle between the P^--N^+ vector and the bilayer normal is $68 \pm 4°$ at 5.36 ± 0.08 water molecules per lipid. The corresponding $\Delta\nu_{\alpha N}$ was reported by Ulrich and Watts (1994) as 8.8 kHz. Thus, the use of Eq. 1 generates an estimate for θ from $\Delta\nu_\alpha$ under the conditions of the present work, such as addition of PS and different DAGs.

RESULTS

The ^2H NMR spectra of DPPC-d_4 in PC/PS mixtures in the absence or presence of 25 mol% DAGs are shown in Fig. 1. A ^2H NMR spectrum of fully hydrated lipid bilayers in the liquid crystalline phase is a superposition of the powder patterns arising from the deuterons at the α- and β-carbon positions of the choline headgroup. In the DPPC-d_4 spectra shown, the outer symmetric pair of peaks corresponds to the deuterons located at the α-carbon of the choline moiety and the inner pair corresponds to the deuterons at the β-carbon (Scherer and Seelig, 1987). The peak-to-peak quadrupole splittings ($\Delta\nu$) are determined by measuring the distance between symmetric peaks. Changes in the value of the splitting for a particular deuteron reflect changes in the average angle of the C-D bond with relation to the axis of rotation and in the average angular fluctuation of the C-D bond with respect to the average C-D bond angle (Seelig, 1977). In the case of the headgroup of a lipid molecule in a bilayer, the axis of rotation corresponds to the bilayer normal (McLaughlin et al., 1975).

The spectra in Fig. 1 exhibit the bilayer profile typical for the ^2H NMR spectrum of a randomly oriented dispersion of bilayers in the liquid crystalline phase. Addition of DP (Fig. 1 *F*) produces a prominent broad component superimposed on the narrower peaks, indicative of coexistent gel-like and liquid crystalline domains in the bilayer, consistent with our previous studies that have shown that DP causes a lateral phase separation of DP-enriched gel-like domains from the bulk phospholipids (De Boeck and Zidovetzki, 1989; Goldberg et al., 1994). The quadrupole splittings of the liquid crystalline component of the DP-containing samples are nearly identical with the control, indicating that DP is sequestered out of the liquid crystalline phase. Decrease of the line-widths of the β-peaks in the DAG-containing (except DP) lipids, which was more apparent in the de-Paked spectra (Fig. 2) is probably due to an increase of the spin-spin relaxation times (T_{2e}).

It has been shown, in PC or PC/PS bilayers, that the time-averaged environments for the two α-deuterons are similar. Without proton decoupling, the two signals overlap (Brown and Seelig, 1978; Akutsu and Seelig, 1981), although under decoupling conditions a small motional inequivalence (~300 Hz) has been reported (Gally et al., 1975; Akutsu and Seelig, 1981). Addition of DAGs resulted in the appearance of an additional pair of symmetric peaks close to the position of the α-deuterons, which were easily discernible without proton decoupling in the de-Paked spectra in all cases (Fig. 2) except DP where such detail could not be resolved (not shown). Increasing DAG concentration increased the frequency difference between the two $\Delta\nu_\alpha$ values, without altering the relative intensities, which were

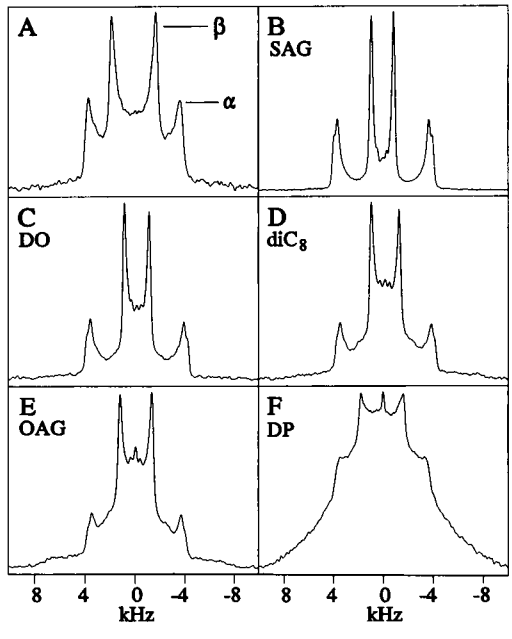

FIGURE 1 ^2H NMR spectra of DPPC-d_4 in PC/PS mixtures in the absence (*A*) or presence (*B–F*) of 25 mol% DAG at 37°C. The peaks are labeled in *A*.

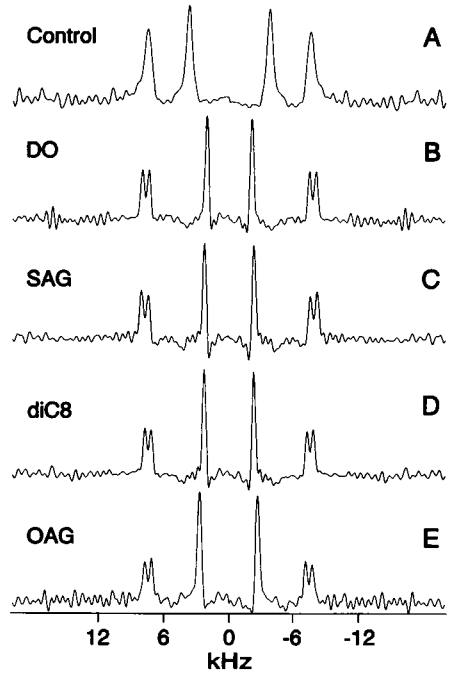

FIGURE 2 De-Paked ^2H NMR spectra of DPPC-d_4 in PC/PS mixtures in the absence (*A*) or presence (*B–E*) of 25 mol% DAG at 37°C.

always close to 1:1 and one-half the intensity of the β-deuteron signal (Fig. 3), indicating that the new peaks are due to the DAG-induced inequivalence of the α-deuterons, rather than DAG-induced lipid domains with different microenvironments. We have also observed no indication of multiple domains induced by these DAGs in our previous study (Goldberg et al., 1994). Inequivalence of the quadrupole splittings of the headgroup α-CD_2 deuterons was previously observed in PC (Gally et al., 1975), phosphatidylethanolamine and phosphatidylglycerol (Wohlgemuth et al., 1980), and PS (Browning and Seelig, 1980). The DAG-induced inequivalence may be due either to the rate of interconversion of two isoforms decreasing to a value slow enough to be observable on the ^2H NMR time scale or, alternatively, to the presence of DAG inducing additional conformational states in the PC headgroup (Sixl and Watts, 1982); however, the exact nature of the isoforms is not clear. Browning (1981) suggested that the observed increase of α-deuteron inequivalence by cholesterol may arise from the more parallel orientation of the headgroup relative to the bilayer surface, increasing steric interactions with the membrane surface under the headgroup and leading to steric crowding and loss of molecular freedom. As DAGs also tilt the headgroup toward the bilayer surface (see below), a similar argument may apply in the present case. Inequivalence of the α-deuterons was also observed in PC/PS bilayers upon addition of melittin (Dempsey et al., 1989) or in DPPC bilayers with cholesterol (Brown and Seelig, 1978) but not in the presence of either positively or negatively charged amphiphiles (Seelig et al., 1987), suggesting that DAGs may induce conformational changes in the PC headgroup in addition to changing the P^--N^+ dipole tilt (see below).

Addition of each DAG caused a counterdirectional change in the Δν values: increase of $\Delta\nu_\alpha$ and concomitant decrease in $\Delta\nu_\beta$. Such a counterdirectional change in the α- and β-splittings demonstrates that the α- and β-carbon segments are moving in a coordinated manner, whereas the direction of this change indicates that the positive end of the P^--N^+ dipole of the choline headgroup is moving closer toward the membrane surface with the addition of DAGs (Seelig et al., 1987; Scherer and Seelig, 1989). Plotting β-splittings versus the corresponding α values for SAG, DO, and diC_8 yields straight lines (Fig. 4). The changes in quadrupole splittings upon addition of OAG were too small for a significant correlation to be obtained. Qualitatively similar observations were reported upon addition of charged amphiphiles (Seelig et al., 1987; Scherer and Seelig 1989) and changes in PS concentration (Roux et al., 1989). We have also observed that the addition of 20% (mol/mol) DPPS to PC increases $\Delta\nu_\alpha$ by 1.25 kHz and decreases $\Delta\nu_\beta$ by 1.27 kHz, which is similar to the effect observed upon addition of 17% DMPS to dimyristoylphosphatidylcholine (DMPC) membranes reported by Roux et al. (1989). Our results of the DPPS-induced change in $\Delta\nu_\alpha$ and $\Delta\nu_\beta$ of DPPC-d_4 are also comparable with values determined by Dempsey et al. (1989) where $\Delta\nu_\alpha$ was observed to increase by 1.4 kHz and $\Delta\nu_\beta$ decreased by 1.6 kHz. The slight differences are likely to be due to our use of bovine liver PC

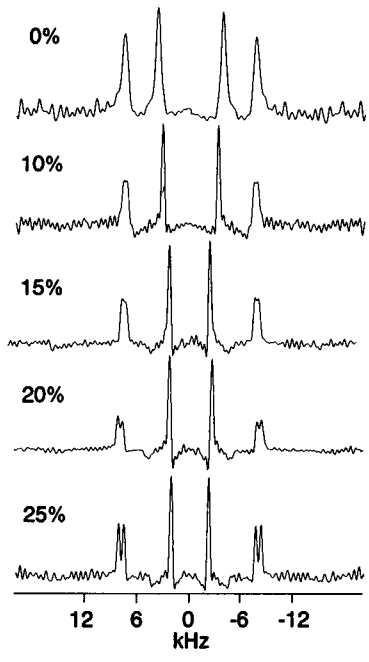

FIGURE 3 De-Paked ^2H NMR spectra of DPPC-d_4 in PC/PS mixtures with 0–25 mol% SAG.

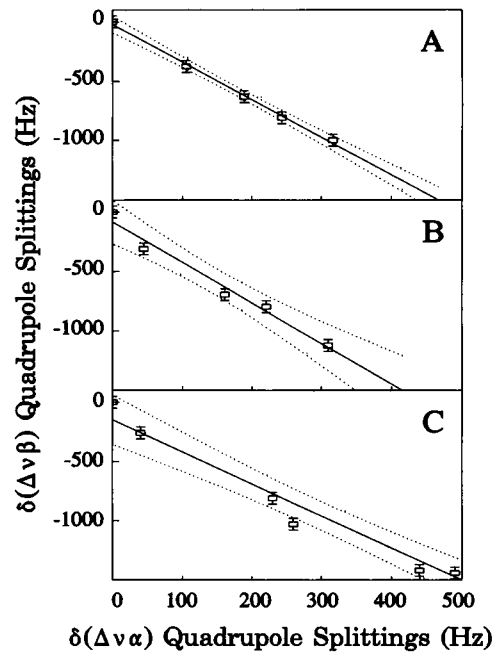

FIGURE 4 Plots of DAG-induced changes in β-peak quadrupole splittings versus changes in α-peak quadrupole splittings for DPPC-d_4 in PC/PS mixtures with 0–25 mol% DAG. The solid lines represent the linear regression of the data and the dotted lines indicate the 95% confidence interval for the regression fit. The slope for (*A*) SAG is −3.2 ± 0.2, (*B*) DO is −3.4 ± 0.4, and (*C*) diC_8 is −2.7 ± 0.3.

instead of purely synthetic PC and the labeled PC molecule bearing palmitoyl rather than myristoyl fatty acyl chains.

The addition of up to 25 mol% of DAGs decreases the membrane surface charge by diluting the PS fraction from 20 to 15% of the total lipid and would be expected to decrease (increase) $\Delta\nu_\alpha$ ($\Delta\nu_\beta$) by 0.31 (0.32) kHz, which is opposite to what is observed upon addition of DAGs. Thus the DAG-induced changes of quadrupole splittings cannot be explained by the dilution of the PS component. Furthermore, it should be noted that upon addition of DAGs the dilution of PS, reduces the observed change in $\Delta\nu_\alpha$ by approximately 30% in the case of SAG and by 45% for diC_8.

The capacity of the DAGs to affect the $\Delta\nu_\alpha$ values for DPPC-d_4 was found to correlate with their efficiency as PK-C activators under similar conditions for both 15 and 25 mol% (Fig. 5), indicating that the DAG-induced changes in PC headgroup conformation and orientation are directly or indirectly related to DAG-induced PK-C activation. Similar analyses were performed on the relationship between PK-C activity and the quadrupolar splittings of the β-peak as well as the splittings for various segments of the hydrocarbon chain region as determined through ^2H NMR by using DPPC-d_{62} instead of DPPC-d_4 in the samples. The correlations were significant for the β-splittings of DPPC-d_4 and peak 2 of DPPC-d_{62} spectra, which corresponds approximately to the nine fatty acyl carbon segments nearest the headgroup. In both cases, the significance of the correlation was substantially lower than with the α-splittings.

NMR relaxation measurements were also performed for samples containing different DAGs at 25 mol%. T_{1z} measurements were taken on the headgroup deuterons and on the hydrocarbon chains for locations at the top and terminal regions (peaks 2 and 13, respectively; Table 1). In the cases of acyl chain-deuterated lipids, similar T_{1z} values were observed with DPPC-d_{62} or DPPS-d_{62} in the presence or absence of DAGs (Table 1). With the exception of a small change induced by DO near the top of the acyl chains, the addition of DAGs did not significantly change the T_{1z} values of either DPPC-d_{62} or DPPS-d_{62} in the acyl chain regions near the bilayer surface (Table 1, peak 2), nor in the bilayer interior (Table 1, peak 13). The lack of DAG-induced effects on T_{1z} values of the lipid acyl chains demonstrates that the addition of these molecules does not alter the dynamic properties of the membranes below the phospholipid glycerol backbone. A different picture emerges when considering the headgroup region. The T_{1z} values in the absence of DAGs are similar to those reported by Scherer and Seelig (1987). The presence of all DAGs other than DP caused both α- and β-deuteron T_{1z} values to increase nearly twofold (Table 1), reflecting an increased motional freedom of the choline headgroups. This is probably due to the creation of free volume in the surface region by the addition of DAGs that have virtually no headgroup. T_{1z} values for both positions increase similarly, whereas the corresponding quadrupole splittings change counterdirectionally. This rules out the T_{1z} increase as a major contributor to this effect, although a small decrease of both $\Delta\nu_\alpha$ and $\Delta\nu_\beta$ as a result of increased motion cannot be excluded. A more quantitative analysis of the relaxation data would require investigating the temperature dependece of T_{1z}. However, the complex phase behavior of the PC/PS/DAG system, described in our previous work (Goldberg et al., 1994) does not allow for the determination of the temperature dependence of T_{1z} to be observed without concurrent temperature-dependent phase changes.

Although all PK-C-activating DAGs increase T_{1z} values, the magnitudes of these increases did not correlate with the corresponding activation of PK-C.

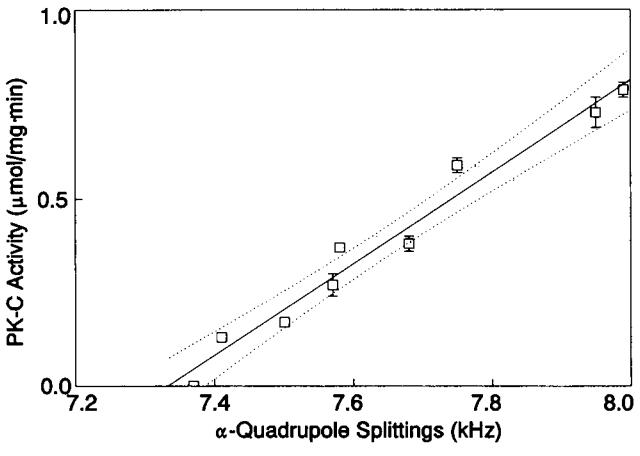

FIGURE 5 Correlation of PK-C activity with α-peak quadrupole splittings in the absence and presence of 15 and 25 mol% DAG. The correlation coefficient r is 0.9843, $r^2 = 0.9688$, and the p value is less than 0.0001. The solid lines represent the linear regression of the data and the dotted lines indicate the 95% confidence interval. The data for PK-C activity are taken from Goldberg et al., (1994).

DISCUSSION

This work was initiated to extend our previous studies that used acyl chain-deuterated lipid probes to investigate the relationship between the physical properties of lipid bilayers and the efficiency of DAG induction of PK-C activity (Goldberg et al., 1994). We have previously shown that with high DAG content nearly maximal PK-C activity can be attained without Ca^{2+} and that PK-C activity can be modulated by the incorporation of DAG into the bilayer in a concentration- and species-dependent manner (Goldberg et al., 1994). Furthermore, different species of DAG produce distinct physicochemical effects on the lipid mixtures such as tendencies to form nonbilayer phases, which were qualitatively correlated with PK-C activation, or the coexistence of liquid crystalline and gel phases, which were shown to be insufficient to activate PK-C.

Structural fluctuations in the lipid membrane bilayer are often implicated as a factor in the activity of the membrane-associated enzymes, notably phospholipases (Gheriani-Gruszka et al., 1988; Sen et al., 1991) and, more recently,

TABLE 1 Effects of 25 mol% DAGs on spin-lattice relaxation times (T_{1z}, ms) of different bilayer regions

	Control	SAG	DO	diC_8	OAG
DPPC-d_{62} peak 2*	38 ± 3	41 ± 3	48 ± 4	38 ± 2	38 ± 2
DPPC-d_{62} peak 13‡	396 ± 30	459 ± 53	476 ± 74	495 ± 35	353 ± 22
DPPS-d_{62} peak 2*	37 ± 3	40 ± 3	46 ± 3	41 ± 3	44 ± 4
DPPS-d_{62} peak 13‡	400 ± 51	390 ± 24	456 ± 68	378 ± 10	461 ± 67
DPPC-d_4 α-peak	19 ± 1	37 ± 2	34 ± 1	33 ± 2	35 ± 1
DPPC-d_4 β-peak	20 ± 1	41 ± 2	39.3 ± 0.4	37 ± 1	40 ± 1

* Corresponds approximately to the nine acyl side chain carbon segments adjacent to the head group.
‡ Corresponds to carbon segment 16 of the acyl side chain.

PK-C (Epand and Lester, 1990; Bolen and Sando, 1992; Senisterra and Epand, 1993). Indeed, our relaxation measurements demonstrate that all PK-C-activating DAGs significantly increase ^2H NMR spin-lattice relaxation times of the deuterated choline moiety, suggesting that DAGs induce an increase in the rate of the molecular motions in the headgroup region. The fact that no correlation was found between the magnitudes of the effects of DAGs on the relaxation times and their effects on PK-C activity suggests that these dynamic effects may be necessary, but not sufficient, factors in the mechanism of DAG-induced PK-C activation.

In the present work we show that DAGs also induce structural changes in the region of the choline headgroup in a concentration-dependent manner that can be quantitatively correlated with the efficiency of DAGs in activating PK-C. We observed a counterdirectional change in the α- and β-quadrupole splitting values with increasing DAG concentration. A change in the average order parameters of the PC headgroup would be expected to shift both α- and β-splittings in the same direction and thus cannot account for the counterdirectional change (Scherer and Seelig, 1989). A similar change in PC headgroup quadrupole splittings has been reported by others investigating effects on membrane surface charge density of various charge carriers including metal ions (Brown and Seelig, 1977; Akutsu and Seelig, 1981; Altenbach and Seelig, 1984; Macdonald and Seelig, 1987), hydrophobic ions (Altenbach and Seelig, 1985), the membrane-bound anion SCN^- (Macdonald and Seelig, 1988), charged amphiphiles (Seelig et al., 1987; Scherer and Seelig, 1989), the zwitterionic lipophilic molecule phloretin (Bechinger and Seelig, 1991), charged phospholipids (Sixl and Watts, 1983; Scherer and Seelig, 1987), and peptides (Dempsey and Watts, 1987; Dempsey et al., 1989; Kuchinka and Seelig, 1989; Roux et al., 1989; Beschiaschvili and Seelig, 1990a,b). These results have been used to develop a molecular voltmeter model (Scherer and Seelig, 1989) in which membrane-bound ions assert a Coulombic force on the P^--N^+ dipole of the choline moiety, repelling or attracting the positive nitrogen with respect to the membrane surface, which can cause changes in the conformation of the headgroup and alterations of average C-D bond angles with the molecular axis. This model has been developed further (Macdonald et al., 1991) into a choline tilt model using the assumptions that, as the N^+ is raised or lowered, the segmental order for the α- and β-carbons remains the same, the headgroup maintains its relative conformation, and the P^--N^+ vector changes only in angular orientation relative to the bilayer normal (see Konstant et al., 1994 for a recent critique of this model).

Addition of several different types of molecules or ions as well as changes in hydration all generate a counterdirectional change in the quadrupole splittings, giving characteristic $\delta(\Delta\nu_\beta)/\delta(\Delta\nu_\alpha)$ ratios. Charged amphiphiles (Scherer and Seelig, 1989) including DPPS (Goldberg and Zidovetzki, unpublished results), DMPS, and charged peptides (Roux et al., 1989) exhibit $\delta(\Delta\nu_\beta)/\delta(\Delta\nu_\alpha)$ ratios between −0.5 and −1.0, whereas the zwitterionic molecule phloretin has a value of approximately −2.7. In the case of DAGs, the ratios ranged from −2.7 to −3.4 (see the legend to Fig. 4), consistent with the absence of charge of these molecules. The differences in $\delta(\Delta\nu_\beta)/\delta(\Delta\nu_\alpha)$ values may be due to the relative positioning of the electrochemical groups in the bilayer, because in the case of the charged peptides and amphiphiles, the charges are assumed to be positioned on the surface of the membrane, whereas the dipoles of lipophilic molecules would more likely be positioned within or somewhat below the surface of the membrane. It has been previously shown that the quadrupole splittings of the PC headgroups do not respond directly to the net membrane surface charge, but rather the driving force for the changes in headgroup splittings is related to the intermolecular interactions within the local environment of neighboring lipids (Marassi and Macdonald, 1992).

Use of the choline tilt model provides an estimation of the change in the tilt of the choline headgroup caused by DAGs. The calculations using Eq. 1 indicate that the greatest DAG-induced change in α-splittings due to 25 mol% SAG corresponds only to a 1.7 ± 0.3° lowering of the P^--N^+ vector, with less effective DAGs such as diC_8 generating a deflection of only 1.0 ± 0.2°.

It has been suggested recently that conformation and movements of the phospholipid headgroups may present different motifs to an approaching protein molecule and therefore affect protein-membrane association (Stouch et al., 1994). However, we consider it unlikely that a maximal 1.7 ± 0.3° change in the choline headgroup orientation effected by DAGs and the corresponding small change in thickness of the hydrophilic surface region of the bilayer would have a distinct effect on PK-C activity. Alternatively,

both the observed changes in $\Delta\nu$ values and the PK-C activity may be affected by another factor that would be the primary cause for both phenomena. One such factor may be suggested from the observation that addition of phloretin to 1-palmitoyl,2-oleoylphosphatidylcholine bilayers was found to dramatically reduce the adsorption of ^2H_2O on the membrane surface (Bechinger and Seelig, 1991). It was proposed that the phloretin hydroxyl groups may be involved in hydrogen bonds with the lipid headgroup of 1-palmitoyl,2-oleoylphosphatidylcholine and displace water, whereas in the case of DAGs, it has been shown that DP reduces the degree of hydration at the fatty acyl carbonyl group of DPPC in DPPC/DP bilayers (López-García et al., 1994b). A study of the melting behavior of DMPC/dimyristoyl (DM) mixtures showed that a DMPC/DM complex may be stabilized by specific interactions, such as hydrogen bonding between the DM-OH group and the ester carbonyls of DMPC (Heimburg et al., 1991), which could dehydrate PC headgroups by displacing bound water. This raises the possibility that DAG-induced bilayer dehydration can both change the conformation of the PC headgroup and modulate the activity of membrane-associated PK-C. Indeed, DAG-induced changes in $\Delta\nu$ values observed by us resemble the changes observed by Ulrich and Watts (1994) upon dehydration of PC bilayers. By comparing the changes in $\Delta\nu_\alpha$ values with the data reported by Ulrich and Watts (1994) we can estimate that addition of SAG decreases the amount of water associated with PC from full hydration of ~22 water molecules per PC (Ulrich et al., 1990; Ulrich and Watts, 1994) to ~11. As all DAG headgroups are chemically identical, the differences in membrane surface effects generated by each type of DAG would depend primarily upon a465fig positioning and orientation of the headgroup, which in turn would be a function of the acyl chain composition. Such bilayer dehydration could, in principle, favorably change the association of PK-C molecules with the lipids, leading to enzyme activation. It has also been previously shown that PK-C activity is increased by synthetic PC analogues with hydrophobic groups near the headgroups (Charp et al., 1988), which may be due to the promotion of nonbilayer lipid phases by such substitutions (Lewis et al., 1994) and/or a decrease in the association with water molecules. Relatively small numbers of water molecules associated with phosphatidylethanolamine headgroups was suggested to be the cause for the tendency of this lipid to form inverted hexagonal phases (Yeagle and Sen, 1986). It is interesting to note that addition of phosphatidylethanolamine was shown to increase PK-C activity (Kaibuchi et al., 1981; Bazzi et al., 1992). We are currently testing the number of lipid-associated water molecules as a function of the presence of DAGs using ^2H_2O as a probe as described by Volke et al. (1994).

In the present study, we have identified a physicochemical parameter of the lipid bilayer membranes that is closely correlated with DAG-induced PK-C activity, namely the quadrupole splittings of the α-deuterons of the PC headgroup. It is important to consider that the ^2H NMR experiments in which changes in PC headgroup conformation were observed were performed in the absence of both PK-C and the histone III-S substrate, both of which were present in the assays for PK-C activity. Thus, under the conditions of the PK-C assays, those being low concentrations of PK-C and substrate in relation to lipids (Goldberg et al., 1994), predicting the capacity of lipid bilayer structure to affect PK-C activity can be reduced to considering only the lipid components. The situation may be different at high histone/lipid ratios (>1:2 w/w); our preliminary results show that, at the high ratios often employed in PK-C activity assays, histone begins to have substantial effects on lipid bilayer structure (Goldberg et al., 1995).

We thank R. Heyman and J. Huang for assistance in sample preparation for the NMR experiments.

This research was supported in part by a grant from the O. J. Holmok Cancer Fund. The NMR work was performed with a GN500 spectrometer, funded by National Science Foundation grant DMB840491 and National Institutes of Health grant BRSG2507.

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